REPLICATION OF HERPES SIMPLEX VIRUS DNA: STUDY OF AN ORIGIN BINDING PROTEIN

by

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TABLE OF CONTENTS

CHAPTER 3: RESULTS

SECTION 3A: Origin binding activity in cells infected with wt HSV-1

CHAPTER 4: DISCUSSION

SUMMARY

The herpes simplex virus type 1 (HSV-1) genome contains two distinct origins of viral DNA replication of related sequence. One (ori_L) lies close to the centre of the long unique region whilst two copies of the other (ori_S) are present within the inverted repeat regions flanking the short unique region. Previous experiments identified DNA fragments of 100 bp or less which specify a functional ori_S and include a 45 bp near perfect palindrome with a central AT-rich region. Moreover, DNase I footprint assays demonstrated the presence of a specific binding site for an HSV-1 encoded polypeptide which overlapped the end of the palindromic sequence. By analogy with other well-characterised origin binding proteins, the HSV-1 encoded origin binding protein was thought likely to play an important role in initiation of HSV DNA synthesis.

The work presented in this thesis describes the identification of the HSV-1 gene encoding this origin binding activity and investigates the role of its interaction with ori_S.

A sensitive gel retardation assay was set up to allow the identification of the virus induced origin binding activity. Incubation of nuclear extract from cells infected with wt HSV-1 with a radio-labelled ori_S fragment resulted in the formation of a major specific retarded complex. Experiments with synthetic oligonucleotides demonstrated the presence of two specific binding sites within the origin region, one of which (site I) corresponded to the previously described site whilst the other (site II) was located on the opposite side of the palindromic sequence.

While these experiments were in progress, Challberg and colleagues identified a set of seven HSV-1 genes which were necessary and sufficient for HSV DNA synthesis. These genes encoded the viral DNA polymerase (UL30), a single-strand-specific DNA binding protein (UL29), a double-stranded DNA binding protein (UL42) and four less well understood functions (UL5, UL8, UL9 and UL52). It was considered very likely that one of these latter four genes would encode the protein which binds specifically to ori_S.

Several approaches were used in attempts to identify the gene encoding origin binding activity. These included analysis of origin binding activity induced by ts mutants with defects in DNA synthesis at the non-permissive temperature and transfection of tissue culture cells with fragments encoding individual replication genes. Neither of these approaches however was successful.

HSV-1 tsK recombinants which express individual replication genes UL5, UL8, UL9 or UL52 at the non-permissive temperature were available in the laboratory. When these were tested, only the tsK/UL9 recombinant virus expressed origin binding activity at the non-permissive temperature. The protein-DNA complex obtained with extracts from cells infected with the tsK/UL9 recombinant virus exhibited a smeared binding pattern of lower mobility than previously seen with extracts from cells infected with wt HSV-1. Treatment of the tsK/UL9 extract with protease produced a smaller complex of similar mobility to that seen with wt HSV-1 extracts. In addition, antibody reactive with the UL9 protein further retarded the major complex observed with wt HSV-1 extracts, demonstrating that this complex contained a portion of the UL9 polypeptide.

Taken together, the above results suggested that a specific domain of the UL9 protein was probably responsible for sequence-specific binding. Fragments of the UL9 gene were therefore expressed as fusion proteins in Escherichia coli and the C-terminal 317 amino acids were found to bind specifically to HSV ori_S, indicating that sequence-specific recognition and binding activities resided within the C-terminal 1/3 of the protein. This result was independently confirmed by expressing the C-terminal 1/3 of the UL9 gene in an in vitro transcription and translation system.

Of the two UL9 binding sites within ori_S, one (site I) contains an 11 bp sequence which is also present in HSV-2 ori_S, HSV ori_L and varicella-zoster virus ori_S, and the other (site II) includes a related sequence element which differs in two positions from the corresponding region of site I. A third 11 bp sequence (motif III) which lies adjacent to site I and differs from the site I element at

only a single position was also recognised. Each of the three 11 bp sequences was deleted from within functional copies of HSV-1 ori_S and the effect on origin activity and binding of the UL9 protein examined. Gel retardation assays confirmed the importance of the 11 bp elements deleted from site I and II for UL9 binding. In transient replication assays, copies of ori_S lacking site I or site II elements exhibited undetectable or residual activity respectively. Hence, the 11 bp element in site I is essential for ori_S activity whilst the element in site II is required for fully efficient origin function.

The UL9 protein did not bind to motif III, even in the absence of site I sequences, although deleting motif III from ori_S caused a small reduction in activity. A single base change which converted the sequence of the 11 bp element within site I to that of motif III was sufficient to abolish both the interaction of the UL9 gene product at this locus and the replicative ability of ori_S. These results establish that the interaction of the UL9 protein with binding site I is essential for origin activity.

The implications of these results regarding the possible role of the UL9 protein and its recognition sites in the initiation of HSV DNA synthesis are discussed.

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ABBREVIATIONS

Abbreviations for media and solutions are given in Chapter 2A: Materials, and those for the HSV genome given in the legend to Figs. 1.1 and 1.5.

A	adenine
ATP	adenosine-5'-triphoshate
BHK	baby hamster kidney 21, clone 13 cells
bp	base pairs
C	cytosine
cDNA	complementary deoxyribonucleic acid
CAV	cell associated virus
Ci	Curie (s)
cm	centimetre
cpm	counts per minute
CRV	cell released virus
C-terminal	carboxy terminal
DBP or *dbp*	DNA binding protein
DNA	deoxyribonucleic acid
ds	double-stranded
dATP	2'-deoxyadenosine 5'-triphosphate
cCDP	2'-deoxycytidine 5'-diphosphate
dCMP	2'-deoxycytidine 5'-monophosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dNTP	2'-deoxyribonucleoside 5'-triphosphate
dUMP	2'-deoxyuridine 5'-monophosphate
dUTP	2'-deoxyuridine 5'-triphosphate
dUTPase	2'-deoxyuridine 5'-triphosphate nuclease
DMSO	dimethylsulphoxide
DNase I	deoxyribonuclease I
DTT	dithiothreitol
E	early (gene)
EBNA-1	Epstein-Barr nuclear antigen 1
EBV	Epstein-Barr virus
E.coli	*Escherichia coli*
EDTA	sodium ethylenediamine tetra-acetic acid
EM	electron microscopy
G	guanine
g	grams

GMEM	Glasgow modification of Eagle's medium
h	hour(s)
HCMV	human cytomegalovirus
HeBS	hepes buffered saline
HHV6	human herpes virus 6
HSV	herpes simplex virus
IE	immediate early (gene)
IEC	immediate early complex (on TAATGARAT elements)
IgG	immunoglobulin G
k	kilo
kbp	kilobase pairs
kD	kilodaltons
l	litre
L	long
LTR	long terminal repeat
M	molar
mDBP	major DNA binding protein
mg	milligrams
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
moi	multiplicity of infection
mol	moles
M_r	molecular weight
mRNA	messenger ribonucleic acid
n	nano
ng	nanograms
nm	nanometres
NPT	non-permissive temperature
N-terminal	amino terminal
OBP	origin binding protein
OD	optical density
ORF	open reading frame
ori	origin of replication
oriLyt	lytic origin of replication
oriP	origin of plasmid replication
ori_S	short region origin of replication
ori_L	long region origin of replication
^{32}P	phosphorus-32 radioisotope

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming units
pi	post infection
PK	protein kinase
pmol	picomole
PMSF	phenylmethylsulphonyl fluoride
pol	polymerase
PT	permissive temperature
R	purine moiety
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RR	ribonucleotide reductase
RT	room temperature
SDS	sodium dodecyl sulphate
sec	second(s)
ss	single-stranded
Staph A	*Staphylococcus aureus*
SV40	simian virus 40
syn	syncytial plaque morphology locus (syn^+ = non-syncytial, syn^- = syncytial
T	thymine
T-ag	T antigen
TK	thymidine kinase
Tris	tris(hydroxymethyl)aminomethane
TS	thymidylate synthetase
ts	temperature sensitive
TTP	2'-deoxythymidine 5'-triphosphate
UV	ultraviolet
V	volts
v	volume
Vmw	apparent molecular weight of virus-induced protein
vol	volume(s)
VP5	virion protein 5 (major capsid protein)
VZV	varicella zoster virus
ug	micrograms
ul	microlitres
w	weight
W	watts

wt	wild type
Y	pyrimidine moiety

AMINO ACID SYMBOLS

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

CHAPTER 1: INTRODUCTION

The research presented in this thesis concerns the interaction of a herpes simplex virus (HSV) type 1 gene product with a viral origin of DNA replication, and includes the identification of this protein and an investigation of its possible role in initiation of HSV DNA synthesis. The aim of this introduction therefore is to provide a background to the HSV lytic cycle and review our current knowledge of HSV DNA replication. Other aspects of the biology of herpesviruses, not directly related to this area, have, of necessity, been omitted or are dealt with only briefly.

SECTION 1A: The Herpesviruses

1. Description and classification

The family Herpesviridae contains a diverse array of large DNA containing viruses capable of infecting a wide variety of higher eukaryotes from reptiles to birds, fish to man (Roizman et al., 1981). Viruses are assigned to the family on the basis of distinct morphological features and all contain a double-stranded linear DNA genome in an enveloped virion particle, 150-200 nm in diameter. More specifically, the virus particle is composed of four distinct elements,namely the core, capsid, tegument and envelope (Roizman and Batterson, 1985).

The core of the virion contains the viral DNA (Epstein, 1962). The calculated dry mass of capsids (diameter <31 nm) indicates that some protein is present within the core in addition to the genomic DNA (Schrag et al., 1989). The HSV nucleocapsid also contains spermine which probably neutralises the charge of the DNA, facilitating dense packing of the genome (Roizman and Furlong, 1974).

Surrounding the virion core is an icosahedral capsid characteristically 100-110 nm in diameter and

composed of 162 capsomeres, 12 pentameric and 150 hexameric, arranged in 5:3:2 axial symmetry. Hexameric capsomeres are typically 12.5nm in diameter, 14nm in length and contain a 4nm wide axial hollow (Wildy et al., 1960).

Roizman and Furlong (1974) introduced the term 'tegument' to describe the layer of amorphous material between the capsid and the lipid envelope (Morgan et al., 1959). The tegument has no distinctive features in thin sections but does appear fibrous on negative staining (Morgan et al., 1959, Schwartz and Roizman, 1969). Fong et al. (1973) demonstrated differences in the thickness of the tegument depending on the location of the virion in the infected cell; nuclear enveloped particles on average being smaller then those found in cytoplasmic vacuoles. Other evidence suggests that the size of the tegument layer is determined by the virus strain (McCombs et al., 1971).

The outermost structure of the virion is the envelope which has a trilaminar appearance with surface spikes approximately 8nm long projecting from its outer surface (Wildy and Watson, 1963). The envelope itself is derived from nuclear and plasma membranes (Asher et al., 1969, Nii et al., 1968).

Apart from common morphological characteristics, a significant aspect of the biology of the Herpesviridae is their ability to establish and maintain a latent infection in their host. For the purposes of classification, the family has historically been divided into three sub-families, Alpha, Beta and Gammaherpesvirinae, based on biological properties such as host range, duration of reproductive cycle, cytopathology and characteristics of latent infection (Roizman et al., 1981).

Alphaherpesviruses

Members of this subfamily have a wide experimental host range in vivo and in vitro. The reproductive cycle is short and infection of cell cultures results in the destruction of the susceptible cell. Latent infections are primarily established in the ganglia. Examples are herpes simplex virus types 1 and 2 (HSV-1, HSV-2; human herpesviruses 1 and 2) and varicella-zoster virus (VZV; human herpesvirus 3).

Betaherpesviruses

Characteristic of this subfamily is the narrow *in vivo* host range with infection often restricted to the species or genus of the host. The reproductive cycle is relatively long with infected cells frequently becoming enlarged (cytomegalia). Latent infections are established in the secretory glands, lymphoreticular cells, kidneys and other tissues. Human cytomegalovirus (HCMV; human herpesvirus 5) is a member of this group.

Gammaherpesviruses

The host range of this group is often limited to the same family or order as the natural host. Viruses in this group are specific for either B or T lymphocytes with latent infection being frequently established in lymphoid tissue. The length of the reproductive cycle and cytopathology are variable. Epstein-Barr virus (EBV; human herpesvirus 4) belongs to this group.

It is now becoming increasingly evident that classification of the herpesviruses on the basis of biological properties may not accurately reflect their phylogenetic relationships. Limited sequence analysis of the recently identified human lymphotropic virus, HHV-6 (Salahuddin *et al.*, 1986) has shown that it is more closely related to the betaherpesvirus subfamily (Lawrence *et al.*, 1990) whereas its host range more closely resembles that of the gammaherpesviruses.

2. Epidemiology of human herpesviruses

There are six known herpesviruses that cause infection in man: HSV-1, HSV-2, VZV, EBV, HCMV and HHV-6.

HSV has a wide range of effects in infected individuals from totally asymptomatic to sporadic life-threatening diseases in a very small number of children and adults. HSV-1 is normally expressed as cold sore lesions of the lips and mouth (herpes labialis) and the eyes (herpes keratoconjunctivitis) whereas HSV-2 infections normally result in genitial lesions (herpes genitalis;

Whitley, 1985). Both infections are passed from person to person by direct contact. Following primary infection HSV can establish a latent infection in the neurons of sensory ganglia and brain tissue (Baringer and Swoveland, 1973; Fraser et al., 1981). Factors such as stress, fatigue and exposure to sunlight have been affiliated with the reactivation of latent virus and the recurrence of HSV-1 lesions. HSV has also been associated with skin infections (herpes herpeticum), a broad spectrum of neonatal infections from skin vesicles to liver and lung infections and sporadic fatal encephalitis (Whitley, 1985).

Varicella-zoster virus (VZV) causes both varicella (chicken-pox) and herpes zoster (shingles). Varicella is normally a childhood disease spread by the respiratory route and direct contact, resulting in a rash of skin lesions leading to pustules. VZV can establish a latent infection in the dorsal root ganglia and spinal cord (Bastian et al., 1974). The hypothesis of Hope-Simpson in 1965, that herpes zoster infection is due to the reactivation of a latent varicella infection, is now generally accepted to be true, based on various clinical and virological observations. The herpes zoster manifests itself in a painful rash with dermatomal distribution. The incidence and severity of herpes zoster increases with age as well as in immunocompromised individuals (Kennedy, 1987).

Primary infection with Epstein-Barr virus (EBV) usually takes the form of benign disease in children but if initial infection is delayed until adolescence or early adulthood, infectious mononucleosis normally results. EBV can produce a latent infection in B lymphocytes and epithelial cells. Unlike HSV, EBV has been directly associated with human cancers. Infection with EBV has been recognised as a contributory factor in the development of African Burkitt's lymphoma and the virus has also been linked to the high incidence of nasopharyngeal carcinoma in South East Asia (Epstein and Achong, 1986).

Human cytomegalovirus (HCMV) infections are normally asymptomatic with only an elevated antibody titre as evidence of infection. Latent HCMV infection has been detected in a number of cells which are implicated in the transmission of virus i.e. blood, kidney and sperm and also

in cells with a host-defence function such as macrophages (Huang et al., 1978). HCMV can also cause infectious mononucleosis but congenital HCMV infections are clinically the most significant due to the high percentage of cases resulting in mental retardation (Hamilton, 1982).

In 1986, Salahuddin et al. described a new human herpesvirus termed human B lymphotropic virus (now named human herpesvirus 6) which had been isolated from patients with AIDS or lymphoproliferative diseases. More recently, the virus has been etiologically implicated with exanthem subitum in children (Downing et al., 1987; Tedder et al., 1987; Yamanishi et al., 1988).

3. Structures of the human herpesvirus genomes

The human herpesviruses contain large double-stranded linear DNA genomes ranging in molecular weight from 80 x 10^6 (VZV) to approximately 150 x 10^6 (HCMV). The structures of the DNA genomes of the six human herpesviruses are shown in Fig. 1.1.

The HSV-1 genome has now been completely sequenced (McGeoch et al., 1985; McGeoch et al., 1986; Perry and McGeoch, 1988; McGeoch et al., 1988), contains approximately 152 kbp and has a molecular weight of 100 x 10^6 (Becker et al., 1968; Kieff et al., 1971). The DNA consists of two covalently joined segments, termed the long (L) and short (S) segments, and each contains unique DNA sequence (U_L and U_S). Each unique segment is flanked by a pair of oppositely orientated distinct repeat elements; the terminal and internal copies flanking U_L and U_S named TR_L and IR_L, TR_S and IR_S respectively (Sheldrick and Berthelot, 1974). The inverted repeats of L (R_L) are approximately 9200 bp long while those of S (R_S) are approximately 6600 bp long (McGeoch et al., 1986). A 400 bp sequence called the 'a' sequence (Davison and Wilkie, 1981) is present as a direct repeat at the termini of the genome and also in the inverted orientation at the joint between the L and S segments (Grafstrom et al., 1974; Sheldrick and Berthelot, 1974; Wadsworth et al., 1975, 1976; Fig. 1.2). The ends of the terminal 'a' sequences each have one overhanging residue

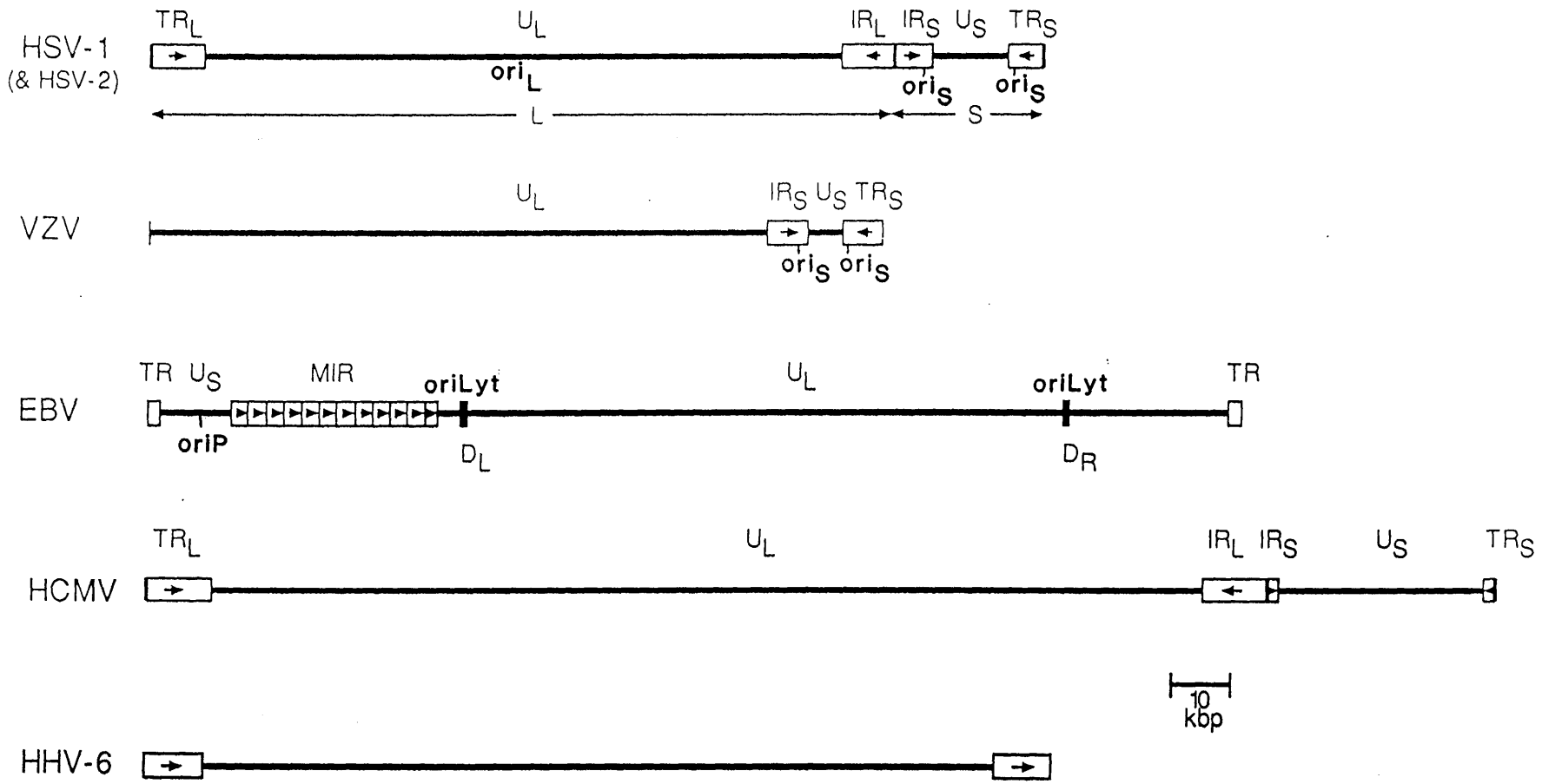

HSV-1
(& HSV-2)
TRL
UL
IRL
IRS
US
TRS
oriL
oriS
oriS
L
S
VZV
UL
IRS
US
TRS
oriS
oriS
EBV
TR
US
MIR
oriLyt
UL
oriLyt
TR
oriP
DL
DR
HCMV
TRL
UL
IRL
IRS
US
TRS
10
kbp
HHV-6

Figure 1.1 Structures of the genomes of the human herpesviruses

The genomes are presented as linear molecules. The bold lines represent unique DNA sequences and the boxes represent repeat sequences with arrows indicative of the relative sequence orientations.

Abbreviations

U_L	- long unique
U_S	- short unique
IR_L/TR_L	- internal/terminal long repeat
IR_S/TR_S	- internal/terminal short repeat
MIR	- major internal repeat
D_L	- left direct repeat
D_R	- right direct repeat
ori	- origin of DNA replication

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

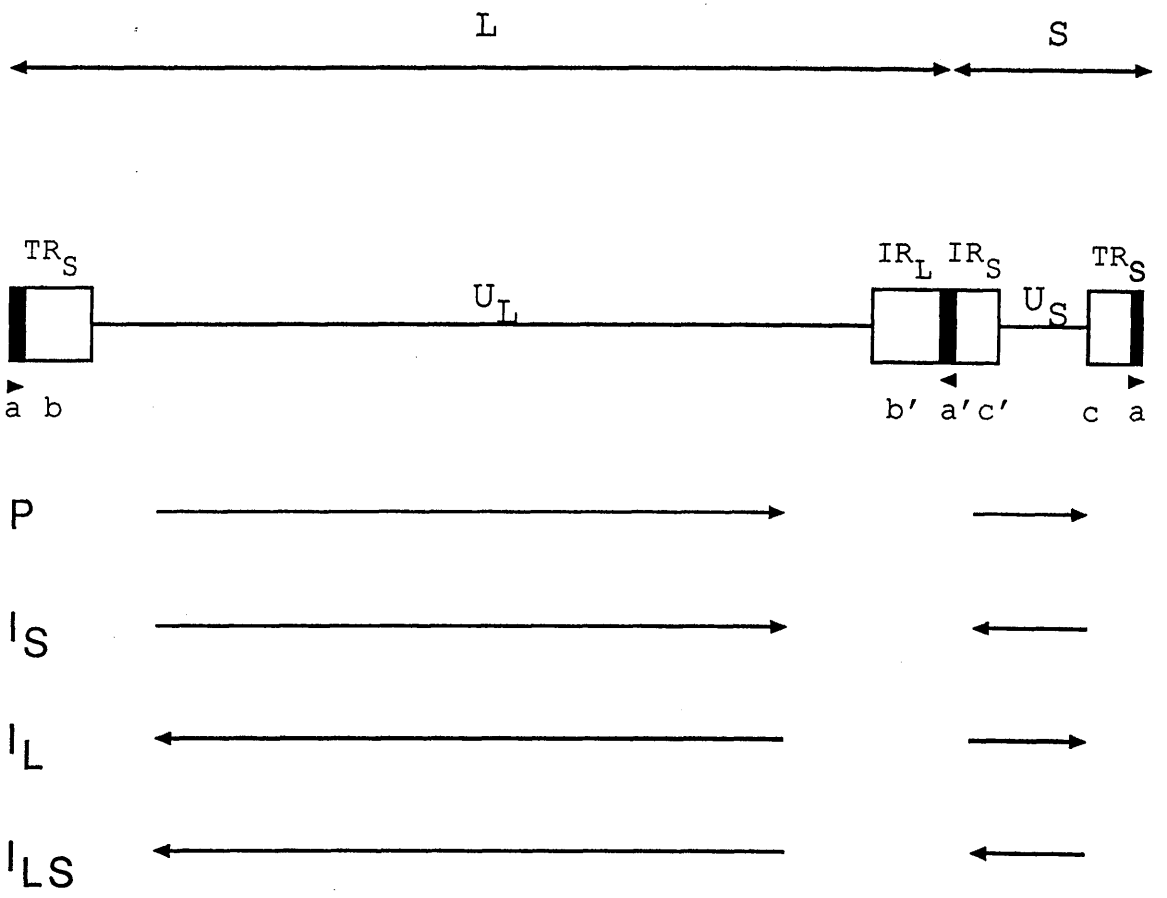

L
S
TR_S
U_L
IR_L
IR_S
U_S
TR_S
a b
b' a' c'
c a
P
I_S
I_L
I_LS

Figure 1.2 Schematic representation of the HSV genome

The genome consists of a long (L) and short (S) region flanked by inverted repeats (TR_L/IR_L, TR_S/IR_S). The 'a' sequence is present as a direct repeat at the genome termini and in inverted orientation at the L-S junction. Inversion of the L and S segments occurs between inverted copies of the 'a' sequence to generate four isomeric forms of viral DNA found in equimolar amounts. These are designated P (prototype), I_S (inversion of the short segment), I_L (inversion of the long segment) and I_{LS} (inversion of the long and short segments).

with a free 3' hydroxyl group (Mocarski and Roizman, 1982). In some virion DNA preparations the 'a' sequence is present in multiple copies at the L-S joint and the L terminus only (Wagner and Summers, 1978).

HSV-1 DNA preparations have been shown to contain equal amounts of four sequence orientation isomers arising from the L and S segments inverting relative to each other about the joint regions. These isomers are designated P (prototype), I_S (inversion of S), I_L (inversion of L) and I_{SL} (inversion of S and L) (Fig. 1.2; Hayward et al., 1975; Delius and Clements, 1976; Wilkie and Cortini, 1976; Roizman, 1979)

Through sequence analysis of HSV DNA, families of multiple copies of short direct repeat sequences have been identified and these are located mainly in the repeat regions, R_L and R_S. These sequences display a high G+C content and copy numbers are variable between individual plaque isolates (Davison and Wilkie, 1981; McGeoch et al., 1985; Rixon et al., 1984).

To date, nearly 33 kbp of HSV-2 DNA sequence has been published (Davison and Wilkie, 1981; McLauchlan and Clements, 1983; Swain and Galloway, 1983,1986; Whitton and Clements, 1984a,b; Swain et al., 1985; Lockshon and Galloway, 1986; Draper et al., 1986; McGeoch et al., 1987; Stuve et al., 1987; Worrad and Caradonna. 1988). HSV-1 and HSV-2 are closely related; they show many serological cross-reactions and DNA hybridisation studies have shown that the genome sequences are closely co-linear (Davison and Wilkie, 1983a). Coding sequences of corresponding genes show 70-80% nucleotide homology (McGeoch et al., 1987).

Complete sequencing of the 125 kbp VZV genome was carried out by Davison and Scott (1986). The overall structure of VZV is similar to HSV in that there are two covalently linked segments L and S flanked by inverted terminal repeats (Fig. 1.1). The repeats flanking U_L are only 88 bp long compared with the 9200 bp repeats of HSV (Dumas et al., 1981; Ecker and Hyman, 1982; Straus et al., 1982; Davison, 1984). VZV DNA preparations do contain four sequence orientation isomers but the two isomers representing one orientation of U_L constitute 80% of the total arrangements (Davison, 1984).

EBV strain B95-8 was the first herpesvirus sequence to be published (Baer et al., 1984). The genome consists of approximately 172 kbp, but as this strain of EBV was shown to contain a deletion of 13.6 kbp, a truer estimate of the genome length is 186 kbp (Raab-Traub et al., 1980). The overall structure of EBV differs greatly from HSV (Fig. 1.1). The termini of the genome contain several directly repeated copies of a 540 bp sequence. Another set of large direct repeats, each of 3072 bp are found internally towards one end of the genome. This major internal repeat separates a short unique region (U_S) from a larger long unique region (U_L). Towards either end of U_L are found two identical 1 kbp regions named D_L and D_R, both lying in the same orientation. Next to these identical repeats are found families of tandem repeat sequences which are related rather than identical (Raab-Traub et al., 1980; Laux et al., 1985).

The DNA of HCMV has a molecular weight of 147×10^6 (Geelen et al., 1978) and has the largest genome of the human herpesviruses containing approximately 230 kbp. Structurally the genome is similar to that of HSV whereby two unique segments L and S are bounded by inverted repeat sequences (Kilpatrick and Huang, 1977; Westrate et al., 1980; Fig. 1.1) and the DNA contains four equimolar populations differing in the relative orientation of the unique sequences (Kilpatrick et al., 1976). HCMV also possesses an equivalent of the HSV 'a' sequence at the ends of the terminal repeats and at the L-S joint region (Spaete and Mocarski, 1985). The 230 kbp sequence of the DNA of HCMV strain AD169 is now known (Chee et al., 1990).

Preliminary analysis of HHV-6 DNA has revealed a base composition of 41% G+C and a genome size of approximately 160 kbp. The DNA is thought to consist of a single unique sequence flanked by a set of large direct repeats (McGeoch, 1989). Recent sequence analysis of 21 kbp of the HHV-6 genome indicates that the virus is more closely related to HCMV than to the other human herpesviruses (Lawrence et al., 1990).

4. Genetic content of HSV-1

The acquisition of the complete sequence of the HSV-1 genome has allowed the assignment of open reading frames (ORFs), corresponding to proposed HSV-1 genes. It now appears that most of the HSV-1 sequence is capable of encoding proteins, with 89% of U_L and 97% of U_S occupied by ORFs (Rixon and McGeoch, 1985; McGeoch et al., 1985; McGeoch et al., 1986; Perry and McGeoch, 1988; McGeoch et al., 1988). The U_L region contains 56 genes, U_S contains 12 genes and one gene is contained in each repeat element yielding a total of 72 genes which encode 70 distinct polypeptides (McGeoch, 1989; see Fig.1.3). Of the 70 polypeptides there are presently 26 genes whose functions remain unknown. Genes whose functions are known have roles in transcriptional control, DNA replication, virion structure and assembly. Most of the genes located in U_S are not essential for growth of virus in tissue culture (Umene, 1986; Weber et al., 1987; Longnecker and Roizman, 1987) whereas presently nine genes in U_L have been identified as nonessential.

The present list of genes encoded by HSV-1 may not be complete. Published reports suggest the possible presence of other genes. These include a transcribed open reading frame containing the short region origin of viral DNA replication, ori_S (Hubenthal-Voss et al., 1987), and a gene upstream of immediate-early (IE) gene 1 in R_L (Ackerman et al., 1986a; Chou and Roizman, 1986). However in both cases the data is not yet conclusive. The region downstream of, and partly overlapping, the IE-1 gene has been shown to specify transcripts found in latently infected neuronal cells. Whether this transcript actually encodes a protein is presently uncertain (Stevens et al., 1987; Wagner et al., 1988).

The overall base composition of the genome is 68.3% G+C residues although in some regions , such as the IE-1 and IE-3 coding sequences, G+C content is as high as 75.4% and 81.5% respectively (Perry et al., 1986; McGeoch et al., 1986). The prevalence of G+C residues has two main consequences; a low number of nonsense codons in untranslated reading frames and a high content of amino acids encoded by GC-rich codons in HSV-1 proteins, namely, alanine, proline, glycine and arginine.

SECTION 1B: Herpes simplex virus lytic cycle

1. Initial stages of infection

An HSV infection begins with the initial binding of virus particles to the host cell membrane, subsequent penetration of the cell by fusion of the viral envelope with the plasma membrane and release of the viral DNA from the capsid into the cell nucleus. HSV appears to bind initially to heparin sulphate on the cell surface, as agents that can block this interaction effectively block HSV adsorption and infection (WuDunn and Spear, 1989). Following attachment of the virion to the cell surface, the viral envelope fuses with the plasma membrane (Morgan et al., 1968) and the viral capsid is released into the cytoplasm (Fuller and Spear, 1985). Adsorption is thought to be mediated via attachment of the viral surface glycoproteins to specific receptors on the cell surface, although these receptors have yet to be identified. Four glycoproteins gB, gC, gD and gE have been implicated in this event (Para et al., 1982; Johnson et al., 1984; Fuller and Spear, 1985). Experiments with ts mutants have identified two genes (gB and UL25) which play important roles in the penetration process (Sarmiento et al, 1979; Addison et al., 1984; Cai et al., 1988).

On entering the cell, the capsid is transported to the nuclear pore where viral DNA is released into the nucleus (Knipe et al., 1981; Batterson and Roizman, 1983). Again, this activity may require a virion function as studies on an HSV-1 mutant, tsB7 (UL36), revealed an accumulation of viral capsids at nuclear pores at NPT (Batterson et al.,1983).

2. Effect on host cell macromolecular synthesis

Infection of cultured cells with HSV results in the rapid inhibition of host cell polypeptide synthesis (Roizman et al., 1965) and cellular DNA synthesis, disaggregation of

polyribosomes and a reduction in the level of host mRNA (Nishioka and Silverstein, 1977; Fenwick, 1984; Schek and Bachenheimer, 1985). These processes have been divided into two distinct phases: 'early` shutoff, which involves the rapid degradation of host polyribosomes and reduction in host mRNAs and is mediated by a virion component (Nishioka and Silverstein, 1977) and a delayed 'late` shutoff which involves enhanced degradation of host mRNA and requires the expression of viral genes (Nishioka and Silverstein, 1978; Isom et al., 1983; Fenwick, 1984). The mechanism of host shut-off may differ between HSV-1 and HSV-2 as some strains of HSV-2 appear to induce a more rapid inhibition of host protein synthesis (Pereira et al., 1977; Schek and Bachenheimer, 1985). HSV-2 strain G seems to encode a very efficient virion associated host shut-off (vhs) function which also destabilises viral IE mRNAs in the absence of de novo viral protein synthesis (Fenwick and Owen, 1988).

The virion-associated primary inhibition of host protein synthesis is non-essential for virus replication as viable mutants (vhs mutants) have been isolated which fail to degrade pre-exiting mRNAs in the absence of viral gene expression but are not deficient in the delayed 'late` shut-off activity (Read and Frenkel, 1983; Kwong and Frenkel, 1987; Strom and Frenkel, 1987). The vhs factor appears to be responsible for degradation of both host and viral mRNA. Both host and viral mRNA are significantly more stable in cells infected with vhs mutants and the failure of these mutants to shut-off IE polypeptide expression indicates the importance of the shut-off for rapid regulation of viral gene expression (Kwong and Frenkel, 1987). The vhs mutation has been mapped to within the UL41 open reading frame (Kwong et al., 1988). Based on studies with the vhs mutant , it has been proposed that the wild type (wt) vhs protein interacts with a cellular factor which controls the half-life of cellular mRNA, resulting in the destabilisation of both host and viral mRNA (Kwong and Frenkel, 1989).

In contrast to the inhibition of host protein synthesis described above, certain cellular genes, notably stress and heat shock proteins, are induced during HSV infection (LaThangue et al., 1984; Patel et al., 1986; Kemp

et al., 1986).

3. Transcription and regulatory features of HSV-1

Transcription of HSV-1 genes takes place in the nucleus of infected cells using the cellular RNA polymerase II (Alwine, 1974; Preston and Newton, 1976) in conjunction with cellular and viral trans-activating factors. The HSV mRNAs are similar to cellular mRNAs in that they possess 5' and 3' non-coding sequences and a 3' poly(A) tract (Silverstein et al., 1976).

Within the HSV-1 genome, genes are transcribed in both orientations and each gene has its own promoter. Hence there is no long range ordering of genes where many genes are served by a small number of promoters as is the case for adenoviruses (Fig. 1.3; McGeoch et al., 1988a; McGeoch, 1989). However, the occurence of 3' co-terminal families of transcripts is common. For example in U_S, 11 of the 13 mRNAs (encoding 12 proteins) are arranged in four nested families with unique 5' ends and common 3' ends (Rixon and McGeoch, 1984, 1985; McGeoch et al., 1986). Other features of transcriptional organisation include transcripts with overlapping reading frames such as US10 and 11 (Rixon and McGeoch, 1984), overlapping 5' ends on opposite strands (Wilkie et al.,1980) and spliced transcripts, which although not common, notably encode the IE proteins Vmw110 (Perry et al., 1986), Vmw68 and Vmw12 (Watson et al., 1981; Rixon and Clements, 1982) and also the UL15 polypeptide (Costa et al., 1985; McGeoch et al., 1988a)

HSV transcription is divided into three temporally distinct phases : immediate-early (IE), early (E) and late (L). The first viral genes to be transcribed from the parental genome are designated IE or alpha (α) genes. The IE mRNAs are produced in the absence of viral protein synthesis (Kozak and Roizman, 1974; Clements et al., 1977; Jones and Roizman, 1979) and functional IE gene products are required for subsequent expression of early or beta (β) genes from unreplicated genomes. The IE and E gene products are in turn required for efficient expression of the late or gamma (γ) genes (Honess and Roizman, 1974, 1975; Preston,

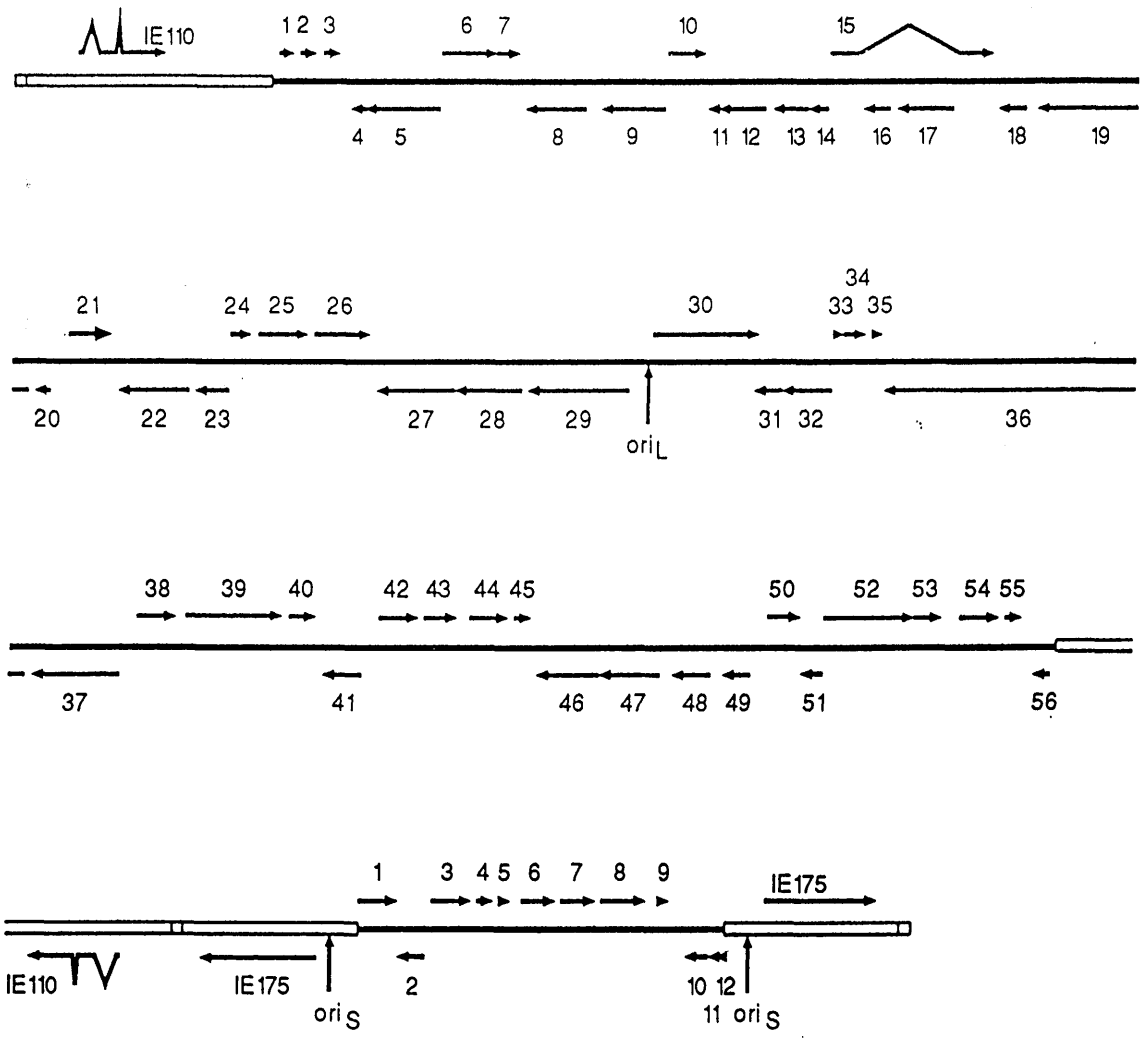

IE110
1 2 3
6 7
10
15
4 5
8
9
11 12
13 14
16 17
18
19
21
24 25 26
30
34
33 35
20
22
23
27
28
29
oriL
31 32
36
38
39
40
42 43
44 45
50
52
53
54 55
37
41
46
47
48
49
51
56
1
3
4 5
6
7
8
9
IE175
IE110
IE175
oriS
2
10 12
11 oriS

Figure 1.3 The location of genes in the HSV-1 genome
(reproduced from McGeoch, 1989)

The HSV-1 genome is shown on four successive lines (40 kbp per line) with unique regions represented by solid lines and major repeat sequences as open boxes. The sizes and orientation of proposed functional open reading frames are shown by arrows. In the top four lines, genes UL1-UL56 are numbered 1-56 and in the bottom line genes US1-US12, as 1-12. The location of introns in the coding regions of gene UL15 and the two copies (TR_L and IR_L) of the IE-1 gene are indicated. Reports concerning the functions of the HSV-1 genes are referenced in McGeoch (1989) and in relevant sections of the thesis.

1979a,b; Everett, 1984a,b; O'Hare and Hayward, 1988a). The late genes have been further divided into two subclasses : early-late (γ_1) and 'true' lates (γ_2) with the distinction being that true late gene expression is barely detectable in the absence of viral DNA replication (Jones and Roizman, 1979; Holland et al., 1980; Hall et al., 1982; Godowski and Knipe, 1985; Johnson et al., 1986).

The expression of HSV genes is controlled primarily at the level of transcription initiation and certain HSV gene products play important regulatory roles in this process. The following sections describe the control of HSV gene transcription in more detail.

4. Transcription and regulation of immediate-early genes

There are five IE genes of HSV-1 named IE-1, IE-2 (UL54), IE-3, IE-4 (US1) and IE-5 (US12). These genes encode polypeptides Vmw110, 63, 175, 68 and 12 respectively. The IE mRNAs are synthesised in the absence of viral protein synthesis and in vivo labelled IE mRNAs have been used to locate the position of the IE genes on the HSV-1 genome (Watson et al., 1979; see Fig. 1.3). The genes for IE-4 and IE-5 have identical promoters located in the repeat sequences of U_S while their coding sequences differ due to their location in the U_S region itself. Both genes are spliced with exon sequences lying in the repeat regions (Watson et al., 1981; Rixon and Clements, 1982). The IE-1 gene is also spliced and is located within the TR_L/IR_L regions whereas IE-3 gene is unspliced and lies wholly within IR_S/TR_S; both genes are therefore diploid (Perry et al., 1986; Rixon et al., 1982). The unspliced mRNA specified by IE-2 lies within the U_L region and is therefore the only IE gene not associated with the major repeat regions (Whitton et al., 1983).

The IE upstream promoter regions have been sequenced (Mackem and Roizman, 1980, 1981; Murchie and McGeoch, 1982; Whitton et al., 1983; Whitton and Clements, 1984a) and are now known to contain a number of cis-acting elements which are recognised by both viral and cellular

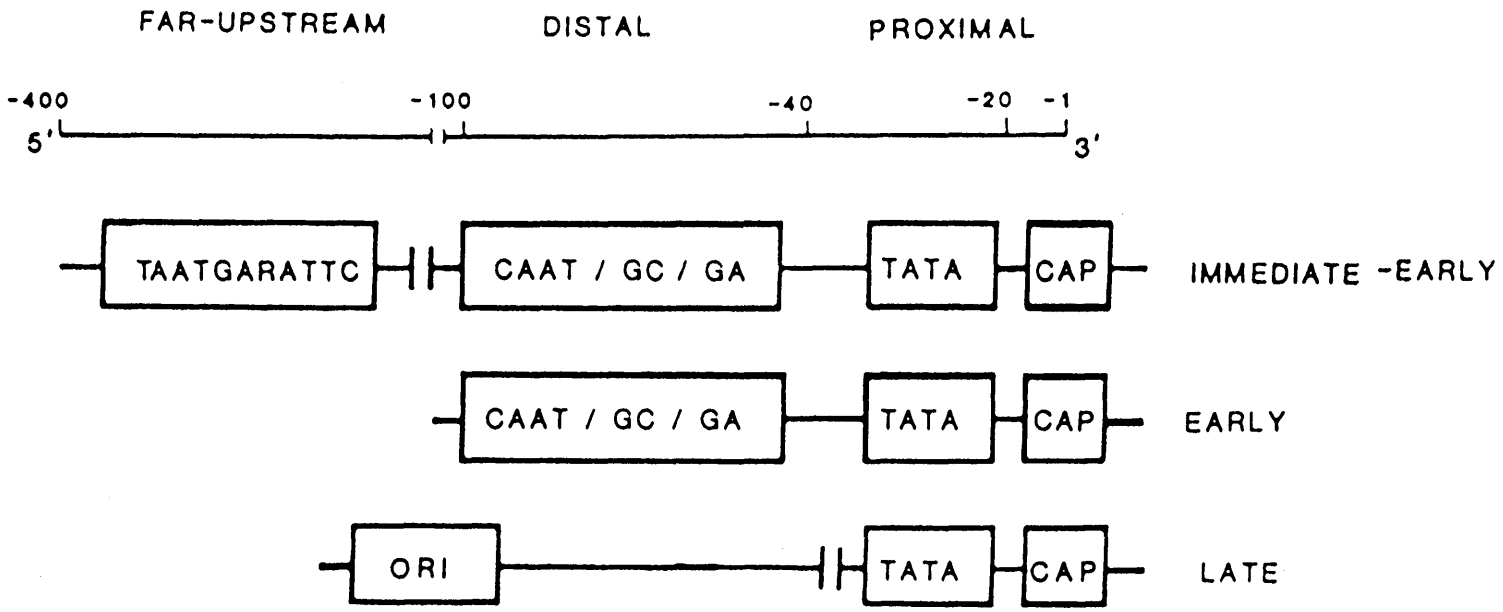

FAR-UPSTREAM
DISTAL
PROXIMAL
-400
-100
-40
-20
-1
5'
3'
TAATGARATTC
CAAT / GC / GA
TATA
CAP
IMMEDIATE -EARLY
CAAT / GC / GA
TATA
CAP
EARLY
ORI
TATA
CAP
LATE

Figure 1.4 Cis-acting signals for the regulation of HSV transcription (reproduced from Johnson and Everett, 1986a)

IE promoter regions contain a cap site, a TATA box, distal promoter elements, including CAAT and GA- and GC-rich motifs and a far upstream element required for activation by the virion component Vmw65, which includes the consensus sequence, TAATGARAT. E promoters include only the distal and proximal promoter regions. Late promoters consist of only a TATA box and cap site, although an active origin of DNA replication (ORI) is also required in *cis* for efficient gene expression.

trans-activating factors. Deletion analysis of the promoter and upstream regions of IE genes has identified essentially two distinct domains; a promoter region and a regulatory domain (Mackem and Roizman, 1982a,b,c; Cordingley et al., 1983, Preston et al., 1984; Fig 1.4).

The promoter region lies proximal to the mRNA initiation site and allows expression of downstream coding sequences but not regulation as an IE gene (Mackem and Roizman, 1982a,c; Cordingley et al., 1983). Not surprisingly, a TATA box, which is known to play an important role in the precise initiation and efficiency of transcription of eukaryotic genes, is located 20 to 30 bp upstream of the start site (Benoist et al., 1980; Benoist and Chambon, 1981).

The far upstream regulatory region can itself be divided into two distinct domains; one element which allows the downstream coding sequences to be regulated as an IE gene and an enhancer-like sequence which allows high level expression (Post et al., 1981; Mackem and Roizman, 1982a,c; Cordingley et al., 1983; Lang et al., 1984; Preston and Tannahill, 1984).

Evidence that IE gene expression was positively regulated by a virion component came from observations that expression from IE upstream regions could be stimulated by infection with HSV ts mutants which could penetrate cells but did not express IE genes or by infection with UV light-irradiated virus (Post et al., 1981; Mackem and Roizman, 1982a; Batterson and Roizman, 1983; Batterson et al., 1983; Cordingley et al.,1983; Preston and Tannahill, 1984). The UL48 gene encoding the virion polypeptide responsible for IE stimulation was identified, sequenced and shown to encode a phosphorylated protein of apparent M_R of 65,000 (Dalrymple et al., 1985; Pellet et al., 1985; Campbell et al., 1984). The virion stimulatory polypeptide, Vmw65 (or VP16) is a major structural component of the virion tegument (Marsden et al., 1976; Hall et al., 1982). Recently, an HSV-1 Vmw65 insertion mutant has been constructed in which the ability of Vmw65 to trans-activate IE gene expression was abolished. The accumulation of IE mRNAs 1,2 and 4 were reduced 2 to 5-fold but IE mRNA 3 was unaffected. The mutant has a high particle/PFU ratio and at

high moi, many of the particles are capable of trans -activation in the early stages of infection. Activation of IE transcription by Vmw65 is therefore important at low moi and in vivo, but at high moi, this function is not required (Ace et al., 1989).

The trans-inducing activity of Vmw65 is mediated essentially through interaction with cellular factors at an AT-rich element located in the IE upstream regulatory region which has the consensus sequence 5'-TAATGARAT-3' (R=purine; Mackem and Roizman, 1982a,c; Cordingley et al., 1983; Whitton and Clements, 1984a; Preston et al., 1984; Campbell et al., 1984; Kristie and Roizman, 1984; Gaffney et al., 1985; Bzik and Preston, 1986; Fig.1.4).

Although the action of Vmw65 is mediated through a sequence specific element, the protein itself is unable to bind directly to this element or indeed non-specifically to double-stranded (ds) DNA (Marsden et al., 1987). However, Vmw65 forms a complex (IEC) with at least two cellular proteins on the TAATGARAT element (McKnight et al., 1987; Preston et al., 1988; O'Hare and Goding, 1988; Gerster and Roeder, 1988). The formation of this complex correlates with stimulation of IE gene expression (O'Hare and Goding, 1988; Ace et al., 1988). Evidence that OCT-1, a ubiquitous cellular protein which binds to the octamer sequence, ATGCAAT, interacts with Vmw65 was given by Gerster and Roeder (1988) who showed that, in the presence of Vmw65, the addition of OCT-1 protein to OCT-1 depleted extracts stimulated the formation of IEC complex.

The amino-terminal 411 amino acids of Vmw65 are required for binding to cellular factors (McKee et al. submitted for publication) and the acidic carboxy-terminal domain contains the trans-inducing activity (Triezenberg et al., 1988).

Other regulatory elements in IE genes include multiple GC-rich sequences, located in the enhancer region of the upstream regulatory domain, which act as binding sites for the eukaryotic cellular transcription factor Sp1 (Jones and Tjian, 1985; Briggs et al., 1986) and GA-rich elements which may possibly enhance the TAAGARAT element (O'Hare and Hayward, 1987).

5. Properties and functions of IE polypeptides

The IE polypeptides have been the subject of intensive study due to early observations that in the absence of their polypeptide products there was no expression of E and late genes (Honess and Roizman, 1974; Swanstrom *et al.*, 1974; Clements *et al.*, 1977; Anderson *et al.*, 1980). The main features and characteristics of these proteins are described below:

Vmw175

The IE-3 gene product, Vmw175, accumulates in the nucleus of infected cells and is post-translationally modified by phosphorylation and also by poly-ADP ribosylation *in vitro*, such that three forms of the protein can be separated by differences in electrophoretic mobility (Pereira *et al.*, 1977; Preston and Notarianni, 1983). *Ts* mutants have been particularly useful in the study of the role of Vmw175. At NPT, a mutant, *ts*K, with a lesion in IE-3, overproduces the IE-1,2,4,5 products and an aberrant form of the IE-3 gene product which is found largely in the cytoplasm. Early or late genes are not expressed (with the exception of the large subunit of ribonucleotide reductase encoded by UL39). A functional Vmw175 is therefore required for the transcription of E and late classes of viral genes and also for the repression of IE gene expression (Preston, 1979a; Watson and Clements, 1980). Further evidence for the role of Vmw175 in coordinating the transition from IE to E gene expression has been obtained from transient transfection assays where transcription from early gene (e.g. glycoprotein D; thymidine kinase) promoters linked to the gene for chloramphenicol acetyltransferase (CAT) was monitored following co-transfection with plasmids containing a copy of IE-3 (Everett, 1984a, 1986; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986). Vmw175 was shown to *trans*-activate an E gene promoter but a greatly enhanced level of transcription was observed when a plasmid containing a copy of IE-1 was also present (DeLuca and Schaffer, 1985; Everett, 1986; O'Hare and Hayward, 1985a).

The role of Vmw175 in autoregulation and repression has been studied by assaying the level of expression from IE promoter-CAT constructs in cells containing plasmids expressing Vmw175. Vmw175 was shown to repress basal levels of transcription from IE promoters and to reduce transcription from IE promoters stimulated by Vmw65 (O'Hare and Hayward, 1985b) although at low doses of Vmw175, the IE-3 and IE-4/5 promoters were both stimulated (DeLuca and Schaffer, 1985, Gelman and Silverstein, 1986).

While most of the IE polypeptides bind DNA to some extent *in vitro* (Hay and Hay, 1980; Metzler and Wilcox, 1985) Vmw175 is the only IE protein known to interact specifically with a conserved binding sequence, ATCGTC, which is found in the promoter/regulatory region of the IE-1, IE-2 and IE-3 genes and of some early and late genes (Faber and Wilcox, 1988; Kristie and Roizman, 1986; Paterson and Everett, 1986). Whether this sequence is involved in *trans*-activation remains to be resolved as most early gene promoters lack sequences closely related to the consensus sequence. There is also no evidence for any *trans*-activation specific sequences in early promoters being required for the action of either Vmw175 (Everett, 1984a; Coen *et al*., 1986) or indeed any other viral or cellular encoded proteins (Michael *et al*., 1988. Kattar-Cooley and Wilcox, 1989).

Vmw110

The HSV IE-1 gene product is a phosphorylated protein found mainly in the nucleus of infected cells (Pereira *et al*., 1977, Hay and Hay, 1980; Ackerman *et al*., 1984). Studies on the role of Vmw110 have been limited due to the failure to isolate any *ts* mutants. However, variants of HSV-1 and 2 have been isolated that contain only one copy of the gene (Poffenberger *et al*., 1983; Harland and Brown, 1985) and more recently, mutant viruses have been constructed which contain large deletions in both copies of IE-1 (Stow and Stow, 1986; Sacks and Schaffer, 1987). At low multiplicities of infection (moi) the mutant *dl*1403 (Stow and Stow, 1986) grows poorly in tissue culture cells and has a 50 to 100-fold lower yield than *wt* HSV-1. At high moi the virus expresses similar amounts of viral proteins and

replicates as well as wt HSV, although virus stocks have significantly higher particle to pfu ratios than wt HSV-1. It appears that Vmw110 is not absolutely essential for virus growth in tissue culture but rather ensures that there is sufficient viral gene expression at low moi for a productive infection (Stow and Stow, 1986; Everett, 1989).

As in the case of Vmw175, the use of individually cloned plasmid copies of IE-1 has allowed the study of the effect of Vmw110 on HSV promoter activation in co-transfection experiments. The experiments demonstrated that Vmw110 is a strong trans-activator of IE, E and late classes of genes (Everett, 1984a; Quinlan and Knipe, 1985; Mavromara-Nazos et al., 1986) which can act synergistically with Vmw175 to increase activation from a number of viral promoters (DeLuca and Schaffer, 1985; Everett, 1986; O'Hare and Hayward, 1985a) as well as some cellular promoters (Everett, 1985).

A large number of in-frame insertion and deletion mutants within the coding region of IE-1 have been constructed, recombined into virus and used to define regions of the polypeptide sequence that are important for its function in transfection assays and virus infection (Everett, 1987b, 1988, 1989). Two regions most sensitive to mutation lie in the second exon (region 1) and at the carboxy terminus (region 5). Region 1 contains a potential zinc-finger DNA binding motif which is crucial for trans-activation of the E gene (gD) promoter in the absence of Vmw175 but is not required to the same extent when Vmw175 is supplied. The converse situation applies to region 5. It is possible that in transfection assays Vmw110 modulates transcription by two different mechanisms, one of which requires Vmw175. The Vmw110 synthesised in tsK infected cells, which express a mutated Vmw175, is not of itself sufficient for activation of E promoters suggesting that in vivo, transcriptional activation by Vmw110 proceeds by a mechanism which also requires Vmw175.

Vmw110 is the only HSV gene product required for reactivation of HSV-2 from in vitro latently infected cells. Interestingly, a deletion in region 1 prevents the reactivation event which suggests that events leading to reactivation are initiated by the activation of gene

expression by Vmw110 alone (Harris et al., 1989).

Vmw63

The IE-2 gene product is a phosphoprotein located in the nucleus of the infected cells (Pereira et al ., 1977; Hay and Hay, 1980; Wilcox et al., 1980). Mutant viruses containing ts lesions have been isolated and found to overproduce Vmw175 and Vmw63 at NPT. Synthesis of early proteins and DNA was not affected but production of many late proteins was restricted (Sacks et al., 1985). Further studies using deletion mutants which do not express Vmw63 demonstrated that these viruses overexpressed early genes, displayed reduced levels of late ($gamma_1$) polypeptides and were replication incompetent on non-complementing cell lines. Vmw63 therefore has an essential role in regulating early and late gene expression and possibly also in regulating IE gene expression (McCarthy et al., 1989).

The use of transient expression assays for analysis of the ability of Vmw63 to regulate HSV-1 genes has produced conflicting reports. While some studies have produced evidence for the trans-activation of an HSV early gene (gB) by Vmw63 alone (Rice and Knipe, 1988) it would appear that Vmw63 alone does not invariably exhibit such activity. However, in concert with Vmw175 and Vmw110, Vmw63 can further stimulate the expression of genes under late promoter control (Everett, 1986; Sekulovich et al., 1988; Rice and Knipe, 1988).

Vmw68

IE gene 4 encodes Vmw68, a nuclear phosphorylated protein (Pereira et al., 1977; Hay and Hay, 1980; Ackerman et al., 1984). A deletion mutant which lacks the carboxy terminal third of Vmw68 has been constructed (Post and Roizman, 1981; Sears et al., 1985) and shown to exhibit growth properties dependent on the cell line used. This implies that host factors in some cell lines can complement the function of Vmw68. In mutant infected resting cells, a reduction in viral growth has been attributed to a reduction in late ($gamma_2$) gene expression (Sears et al., 1988).

Vmw12

Unlike the other IE polypeptides, Vmw12, encoded by IE gene 5, is not phosphorylated and is located in the cytoplasm (Preston, 1979b; Marsden et al., 1982). IE-5 is not an essential gene for viral growth in tissue culture as viable deletion mutants lacking the entire IE-5 gene have been isolated (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

6. Regulation of early gene expression

Following the synthesis of IE gene products, expression of early genes can occur (Honess and Roizman, 1974; Clements et al., 1977). Early genes are sometimes sub-divided due to the variation in kinetics of gene expression amongst this group. Thus, the large sub-unit of ribonucleotide reductase (RR_1), has traditionally been classed as a $beta_1$ gene product (Roizman and Batterson, 1983) but is in fact expressed very early in infection and can be detected in the absence of activation by IE proteins (Roizman and Batterson, 1985; Wymer et al., 1989). Other early genes e.g. gB, are transcribed at early times but are not fully expressed until after the onset of DNA replication (Gibson and Spear,1983; Johnson and Everett, 1986a).

Early experiments to evaluate the promoter sequence requirements for the trans-activation of E gene promoters by viral gene products used deletion mutagenesis of E promoter sequences (of the TK and gD genes) linked to indicator genes which were assayed by microinjection into xenopus oocytes (McKnight et al., 1981; Jones et al., 1985). Later experiments by McKnight and Kingsbury (1982) used linker scanning mutations in which small sections of promoter sequence were replaced by synthetic linker DNA. By assaying these mutants in xenopus oocytes, and therefore in the absence of IE gene products, only promoter elements recognised by the cellular transcription machinery could be detected. Several functional elements were present and included a TATA box, upstream GA- and GC-rich (Sp1 binding site) regions and an inverted CCAAT motif present in the TK promoter (McKnight and Kingsbury, 1982; Everett, 1983,

1984b; Eisenberg et al., 1985; El Karah et al., 1985; see Fig. 1.4). When the TK linker-scanning mutants were introduced into the HSV-1 genome at the normal TK promoter location (Coen et al., 1986) the upstream elements in the promoter region were less crucial for gene expression while greater importance was placed on the requirement for a TATA box and cap site region. Analysis of deletion and insertion mutants within the gD promoter region, induced by HSV-1 infection, or activated by an SV40 enhancer linked upstream of the promoter, showed that mutations which resulted in a reduced response to viral trans-activation also incurred a reduction in cis-activation by the SV40 enhancer. It would therefore appear that there are no promoter elements upstream of the gD and TK gene that are specific for trans-activation by HSV products (Everett, 1984b). This implies that IE gene products activate E gene transcription by their interaction with, or alteration of, the activity of cellular transcription factors (Eisenberg et al., 1985; Coen et al., 1986)

7. Regulation of late gene expression

Late gene expression is first detected at 2-3 h post infection although maximal expression is not reached until 10-16 h post infection (Munk and Sauer, 1964; Roizman, 1969). Late gene expression requires HSV DNA replication for maximum activity as noted by analysis of the polypeptide profiles of DNA negative ts mutants or under conditions where DNA synthesis is inhibited (Honess and Roizman, 1974; Powell et al., 1975; Holland et al., 1980; Conley et al., 1981; Pederson et al., 1981).

Late genes have been divided into two groups; 'leaky late' (γ_1) whose expression is reduced but still detectable in the absence of DNA replication and 'true lates' (γ_2) whose expression is barely detectable (<1%) before DNA synthesis (Wagner, 1985; Roizman and Batterson, 1985). The prototype of the (γ_1) genes is UL19 encoding the major capsid protein, Vmw155. The promoter of this gene extends no more than 125 bp upstream of the RNA cap site and can be activated by IE gene products (Costa et al., 1985).

Detailed analysis of the promoter region of a(γ_2) gene has been carried out using transient transfection assays in which US11 promoter deletions were carried on a plasmid containing an HSV-1 origin of replication, ori_S, which enables replication of the plasmid DNA in the presence of infecting HSV-1 (Johnson and Everett, 1986a; Stow and McMonagle, 1983). Surprisingly, DNA promoter elements required for regulation of gene expression were contained within a 31 bp sequence upstream of the RNA cap site containing only a TATA box and the cap site region (Fig.1.4). When sequences upstream of the early gD promoter TATA box were deleted, the gene was regulated as a true late gene and required the activity of an origin of replication (Johnson and Everett, 1986b).

The requirement for viral DNA synthesis in late gene regulation is not yet understood. An actively replicating template will undoubtedly result in structural changes in the DNA which may provide a switch for L gene expression, or the replication machinery itself may play some role in situ.

8. Herpes simplex virus DNA replication

This section summarises the general features of HSV DNA replication. A more detailed account of the enzymology and cis-acting sequences required is presented in section 1D.

(a) in vivo studies

On the basis of velocity sedimentation characteristics of newly synthesised viral DNA, and EM and restriction endonuclease analysis of replicative intermediates, the replication of HSV can broadly be divided into three phases:

(i) Initial phase of replication

Upon entering the cell nucleus, the HSV genome is thought to circularise by direct ligation of the termini forming a joint fragment in which the two terminal 'a' sequences share a DR_1 element (section 1B part 10; Davison

and Wilkie, 1983; Poffenberger et al., 1983). Although circularisation was originally thought to occur by exposure of complementary cohesive termini digested by exonuclease (Roizman, 1979), it now appears more likely that it takes place by ligation of the termini via the complementary 3'-single base extensions on the L and S termini (Mocarski and Roizman, 1982a,b; Davison and Rixon, 1985). HSV genomes containing heterotypic 'a' sequences (Davison and Wilkie, 1983) and VZV, which lacks a terminal redundancy, are both assumed to carry out this process (Davison, 1984). Studies using an HSV-1 deletion mutant showed that the covalent head-to-tail linkage of infecting viral DNA reached maximum levels less than 30 min after exposing the cells to virus and took place in the absence of protein synthesis (Ben-Porat and Veach, 1980; Poffenberger and Roizman, 1985).

(ii) Early phase of replication

Newly synthesized herpesvirus DNA, pulse-labelled in vivo with ^{3}H-thymidine, has been analysed by velocity sedimentation in neutral sucrose gradients (Jacob and Roizman, 1977; Ben-Porat and Tokazewski, 1977). Viral DNA synthesised 2-3 h after infection sediments heterogenously at approximately twice the rate of unit-length viral DNA (Ben-Porat and Tokazewski, 1977). During the early phase, the genome continues to remain 'endless' (Jongeneel and Bachenheimer, 1981) and it has been suggested that viral genomes therefore undergo an initial template amplification stage as circular monomers (Ben-Porat and Tokazewski, 1977).

(iii) Late phase of replication

Herpesvirus DNA synthesised at late times (4-6 h after infection) sediments at approximately 100 times the rate of unit length viral DNA (Jacob and Roizman, 1977) and has been described as being in the form of "a large tangled mass" (Ben-Porat and Rixon, 1979). Restriction endonuclease analysis shows a decrease in the concentration of end fragments consistent with the hypothesis that the viral DNA accumulating in the cell consists of head-to-tail concatemers arising from the replication of the DNA by a rolling circle mechanism (Jacob et al., 1979).

It is also possible that the rapidly sedimenting

DNA synthesised late in infection is generated by a 'Cairns' type mechanism of replication where the daughter molecules remain topologically linked at the end of a round of replication (Jongeneel and Bachenheimer, 1981) or perhaps from homologous recombination between nascent DNA circles. This latter possibility cannot be ruled out as HSV is known to exhibit a high rate of recombination which, furthermore, appears to be dependent on the viral replication machinery (Schaffer et al., 1974, Smiley et al., 1980; Weber et al., 1988).

HSV DNA fragments upon denaturation with alkali (Kieff et al., 1971; Wilkie, 1973). Although HSV DNA does contain short stretches of ribonucleotides (Biswal et al., 1974), it is unlikely that these are entirely responsible for the fragmentation in alkali since similar amounts of fragmentation have been reported on formamide gradients (Roizman, 1979). It has been suggested that fragmentation is due to the presence of single-strand nicks or gaps in HSV DNA which appear to be randomly distributed on both strands. The functional significance of these nicks is presently unknown (Frenkel and Roizman, 1972; Wilkie, 1973; Wadsworth et al., 1976).

(b) in vitro studies

Another approach to the analysis of the mode of replication of HSV DNA has been the development of sub-cellular systems that support the synthesis of HSV DNA. These have included nuclear monolayers (Bell, 1974), isolated nuclei (Becker and Asher, 1975; Bolden et al., 1975), nucleoplasmic extracts (Pignatti et al., 1979; Muller et al., 1981) and permeabilised infected cells (Jongeneel and Bachenheimer, 1980). In general these systems have been unable to faithfully reproduce in vivo patterns of viral DNA synthesis and fail to synthesise unit-length DNA or support semi-conservative replication. However, permeabilised infected cells were capable of incorporating labelled triphosphates into rapidly sedimenting DNA (Jongeneel and Bachenheimer, 1980).

(c) inversion/recombination of the HSV genome

Following circularisation of the infecting viral genome, it is thought that limited synthesis of circular intermediates occurs and that inversion of the L and S segments may take place by recombination within the 'a' sequences. Whether inversion is merely a consequence of the genome structure or has some biological role is unknown. All four isomers are functionally equivalent in tissue culture since variants have been isolated which are frozen in the P, I_S and I_{LS} configurations (Preston et al., 1978; Poffenberger et al., 1983; Jenkins and Roizman, 1986). In addition, single plaque isolates of wt HSV-1 yield equimolar amounts of the four isomers; hence each isomer can be rapidly converted to the other three forms. Pairs of the four isomers can be generated without an inversion event through utilisation of different cleavage sites in the concatemeric or circular DNA e.g. P and I_{LS} can be generated by alternative cleavage of the same concatemeric molecule and similarily so can I_S and I_L. Although many studies indicate that the 'a' sequence is sufficient for inversion, it is clear that inversion events can also occur between inverted copies of other sequences present in an HSV genome (Pogue-Geile et al., 1985; Jenkins et al., 1985; Varmuza and Smiley, 1985; Weber et al., 1987). Experiments by Weber et al. (1988) have demonstrated that the DNA replication process is intrinsically recombinogenic and argues against a specific inversion mechanism operating on the 'a` sequence.

9. Herpes simplex virus structural proteins

Many early genes encode enzymes and proteins involved in HSV DNA replication and are dealt with in more detail in section 1C. The rest of this section deals with HSV-1 encoded structural proteins and events following the synthesis of HSV DNA.

There appear to be at least 30 different virion structural polypeptides, the majority of which are encoded by the HSV late genes. These proteins have been grouped into three main classes; glycoproteins, tegument proteins and capsid proteins. HSV encodes seven known glycoproteins;

gB (encoded by UL27), gC (UL44), gD (US6), (Spear, 1976), gE (US8) (Bauke and Spear, 1979), gG (US4) (Ackerman et al., 1986b; Richman, 1986), gH (UL22) (Marsden et al., 1978; Frame et al., 1986; McGeoch et al., 1987,1988a) and gI (US7) (Longnecker et al., 1987). In addition, an ORF in the U_S region, US5, specifies an amino acid sequence characteristic of a glycoprotein although the protein has yet to be identified. The glycoproteins gB, gD and gH are essential for growth in tissue culture (Little et al., 1981; Weller et al., 1983a; Buckmaster et al., 1984; Fuller and Spear 1985; Gompels and Minson, 1986) whereas gC, gE, gG, gI and US5 appear to be non-essential (Longnecker and Roizman, 1986; Longnecker et al., 1987; Harland and Brown, 1988).

The number of virus encoded proteins that constitute the tegument layer is uncertain. HSV-1 tegument polypeptides are normally recognised as proteins not belonging to the capsid or envelope that are released by solubilisation of the virus with non-ionic detergent (Lemaster and Roizman, 1980; Roizman and Furlong, 1974). The IE transcription activator, Vmw65 (UL48) is a major constituent of the tegument as is the UL36 gene product, Vmw273 (Batterson et al., 1983; McGeoch et al., 1988a). The UL47 gene is also now thought to be located in this layer (McLean et al. submitted for publication).

The HSV capsid is composed of at least 6 polypeptides including the major capsid protein of M_r 155,000 (ICP5, VP5, Vmw155) which is encoded by UL19 (Marsden et al., 1978; Morse et al., 1978; McGeoch et al., 1988a). The family of polypeptides encoded by UL26 of approximate M_r 40,000 (p40) are also associated with different forms of the HSV-1 capsid (Addison, 1986; Rixon et al., 1988). Recent studies by Rixon et al. (submitted for publication) have identified UL18 and UL38 as encoding two capsid proteins.

10. Packaging, encapsidation and assembly of virions

Herpes simplex virus infected cells contain large concatemeric DNA molecules arising from replication of the viral genome. For the assembly of mature virions

site-specific cleavage events take place to generate unit-length genomic molecules that are packaged into virus particles. Studies using ts mutants have demonstrated that the cleavage and packaging processes are tightly linked as mutants which fail to encapsidate replicated DNA are also unable to cleave it into unit lengths (Ladin et al., 1980; Preston et al., 1983; Addison et al., 1984; Deiss and Frenkel, 1986).

The presence of the 'a sequence at both genomic temini suggests that this sequence may be involved in the cleavage of the DNA. Insertion of an additional 'a' sequence into the viral genome results in the generation of novel termini corresponding to the position of the inserted 'a sequence (Smiley et al., 1981; Mocarski and Roizman, 1982; Varmuza and Smiley, 1985). Replicated plasmid DNA containing a functional viral origin of replication can only become packaged and propagated in virus stocks as defective genomes if the plasmid contains an 'a sequence (Stow et al., 1983, 1986; Deiss and Frenkel, 1986) indicating that cis-acting maturation and packaging signals reside in the 'a' sequence.

The structure of the 'a' sequence is shown in Fig 1.5). Short (17-21 bp) direct repeat sequences, DR_1, are located at each end of the 'a' sequence. However DR_1 elements located at the end of the L and S termini are incomplete. For example, the L terminus of HSV-1 strain 17 possesses 20.5 bp of a 21 bp DR1 sequence (i.e. 20 bp plus a single nucleotide 3' extension) and the remaining 0.5 bp is at the end of the S terminus. Hence in concatemeric DNA molecules, adjacent 'a' sequences share an intervening DR_1 element (Mocarski and Roizman, 1981). Adjacent to the DR_1 repeats are two unique regions called U_b and U_c according to their location nearest to the flanking b and c sequences respectively. Separating U_b and U_c are two additional repeat elements, DR_2, a 12 bp repeat unit present in 1 to 22 copies and DR_4, a 37 bp sequence present in 0 to 3 copies (Davison and Wilkie, 1981; Mocarski and Roizman, 1981). Although there is variation in the nucleotide composition of the 'a' sequence depending on the virus strain, two highly conserved sequences are located in U_b and Uc termed pac-1 and pac-2 respectively (see Fig. 1.5).

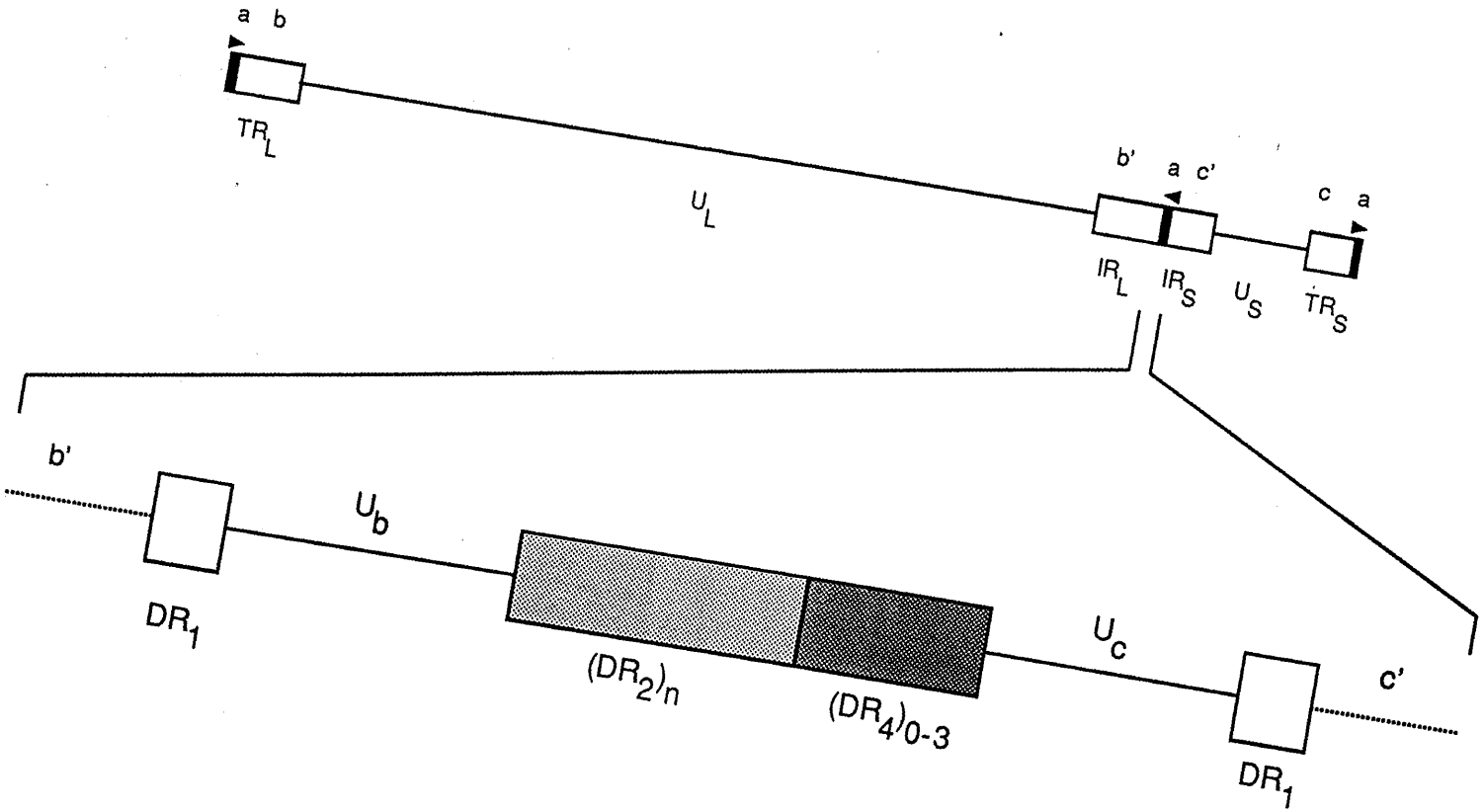

a b
TR_L
U_L
b' a c'
c a
IR_L
IR_S
U_S
TR_S
b'
U_b
DR_1
(DR_2)_n
(DR_4)_0-3
U_c
c'
DR_1

Figure 1.5 Structure of the HSV-1 'a' sequence

The viral genome is shown with an expanded region below showing the structure of the 'a' sequence in the orientation found in the L-S junction.

U_b - a unique sequence located toward the b' sequence
U_c - a unique sequence located toward the c' sequence
DR1 - a 17-21 bp element present as a direct repeat at the ends of the 'a' sequence
DR2 - a 12 bp repeat element present in 1 to 22 copies
DR4 - a 37 bp element present in 0-3 copies

The structure of the 'a' sequence at the L and S termini suggest that cleavage of concatemeric DNA occurs through the shared DR_1 element of two adjacent 'a' sequences. However, certain defective genomes capable of undergoing cleavage and packaging were shown to contain only 4 bp of the DR_1 element, suggesting that the recognition signal for cleavage is not identical to the cleavage site (Mocarski et al., 1985). Several studies using deletion analysis or generation of novel termini using sub-fragments of the 'a' sequence have demonstrated that sequences within U_b and U_c are required for cleavage and packaging.

Based on these findings, a simple model for cleavage and packaging of HSV DNA can be proposed. Circularisation of linear genomes containing an 'a' sequence at each end results in the generation of a novel joint fragment containing tandem 'a' sequences sharing a DR_1 element. When such molecules are replicated by a rolling circle mechanism the novel joint fragment is reiterated at intervals corresponding to a unit length genome in the concatemers. A putative cleavage/packaging protein complex recognises the packaging signal at this joint and produces a staggered cleavage through the shared DR_1. Packaging of the resulting molecule continues until the next paired 'a' sequence in the same orientation is encountered (i.e. one genome length away). Repetition of the cleavage event completes the packaging of one genome with an 'a' sequence at each terminus and enables that of the next to be initiated. More elaborate models have been proposed to explain the cleavage/packaging and amplification of the 'a' sequence in the case of artifically constructed defective molecules which contain a single 'a' sequence. Following replication and propagation, these molecules are found to contain an 'a' sequence at each terminus (Varmuza and Smiley, 1985; Deiss et al., 1986).

Some evidence for the above model has come from gel retardation analysis of protein-DNA interactions using probe DNA containing various elements from the 'a' sequence. Sequence-specific complexes were obtained using pac-2-DR_1 sequence but not with DNA containing either element alone or pac-1 and DR_1 (Chou and Roizman, 1989). Purification of proteins which formed complexes revealed a previously

reported DNase activity (Hoffman, 1981), probably the viral exonuclease encoded by UL12; the VP1 tegument protein (Lemaster and Roizman, 1980) encoded by UL36 and a third unidentified protein of apparent M_r 140,000. Although the action of the viral DNase is normally exonuclear and non-specific, its DNA binding and cleavage activity could be directed by its interaction with a cellular or viral protein. A host cell protein has also been identified as binding to the conserved pac-2 sequence in HCMV (Kemble and Mocarski, 1989).

Further studies using ts mutants tsl204 and tsl201 (Preston et al., 1983; Addison, 1986) which are defective in both cleavage and packaging events have suggested a role for the p40 proteins encoded by UL26 (Preston et al., 1984; McGeoch et al., 1988a). At NPT tsl201 is unable to process p40; the unprocessed proteins become associated with empty capsids which accumulate in the cell nucleus. Tsl233 (whose mutation maps to UL33) is similarily unable to package viral DNA at NPT and assembles partially cored capsids but is able to process p40 normally (Al-Kobaisi, 1990). The p40 protein is found in empty capsids but appears to be present only as a minor component of full capsids or mature virions. It has been suggested therefore that p40 is only briefly associated with capsids at an early stage in assembly and is lost during packaging of the viral DNA (Rixon et al., 1988).

Nucleocapsids are enveloped at the inner lamella of the nuclear membrane; only capsids containing an approximate genome length are enveloped and transported to the cytoplasm forming mature virions (Roizman and Furlong, 1974, Vlazny et al., 1982; Stow et al., 1986). Virions are proposed to leave the cell via the Golgi apparatus following a pathway similar to that taken by secreted soluble proteins (Johnson and Spear, 1983).

SECTION 1C: Cis- and trans-acting requirements for HSV DNA replication

1. Cis-acting signals required for HSV DNA replication

The existence of specific regions within the HSV genome which allow the initiation of DNA synthesis was first suggested by EM studies of replicating DNA molecules and analysis of the structures of defective genomes which arose during serial passage of HSV at high moi.

Friedmann et al. (1977) observed, using EM, DNA molecules that contained replicative loops or Y-shaped structures, indicative of replicative intermediates. The position of these structures suggested that the genome contained probably three specific origins of DNA replication.

Extension of these observations came from the study of defective virus genomes. These genomes do not contain the complete genetic information of the virus and as a consequence are only replicated when helper functions are supplied by non-defective genomes (Schroder et al., 1975/6). That defective genomes could be maintained in virus stocks indicated that they contained cis-acting sequences required for initiation of DNA synthesis and for cleavage and packaging of the viral DNA (Frenkel et al., 1975). Characterisation of defective genomes by restriction digestion and Southern blot analysis revealed that they consist of repeated identical copies of small segments of viral DNA (Wagner et al., 1974; Frenkel et al., 1976, 1980; Schroder et al., 1975/76; Graham et al., 1978; Locker and Frenkel, 1979; Kaerner et al., 1979, 1981; Denniston et al., 1981). On the basis of the origin of DNA sequences within the repeating units, defective genomes were divided into two classes: class I defective genomes, containing DNA sequences from the short segment of the genome including the 'a` sequence (Denniston et al., 1981; Frenkel et al., 1980, 1981; Vlazny and Frenkel, 1981) and class II defective genomes, containing sequences derived from the middle of the long unique region as well as the 'a' sequence (Schroder et al., 1975/76; Kaerner et al., 1979, 1981; Frenkel et al., 1980). This suggested that the HSV genome might contain two distinct origins of replication, one in L and the other in S. Vlazny and Frenkel (1981) provided direct evidence that repeat units of class I and II defective DNAs contain origins of replication by demonstrating that individual units generated by restriction enzyme cleavage could

replicate and regenerate tandemly repeated structures in the presence of helper virus.

To identify the precise sequences required for DNA replication, an assay was developed whereby plasmids containing inserts of viral DNA were transfected into tissue culture cells which were subsequently infected with wt HSV-1 to provide any necessary trans-acting helper functions. The presence of a viral origin of replication within the plasmid molecule allows replication of the linked vector sequences. Amplification of non-HSV sequence in the plasmid could be detected by hybridisation of extracted DNA with labelled parent vector DNA. In this manner, an origin of replication was located within a 995 bp fragment containing only sequences from within R_S (Stow, 1982). Furthermore, the products of plasmid replication were high molecular weight molecules containing tandem duplications of the plasmid DNA, indicative of a rolling circle mechanism of replication. This origin has been designated ori_S due to its location within the inverted repeats of the S component; therefore there are two copies of ori_S in the HSV genome.

The origin within class II defective genomes, designated ori_L, has similiarly been mapped and originates from near the middle of the U_L component of the HSV genome (Frenkel et al., 1980; Locker et al., 1982). Hence, in total, the HSV genome contains three origins of replication.

(a) the short region origin of replication

Using a systematic deletion analysis of ori_S-containing plasmids, Stow and McMonagle (1983) identified a 90 bp region within the above 995 bp fragment from the TR_S/IR_S region that contained the minimum signals necessary for ori_S activity (Fig. 1.6). The minimal sequence lies within an untranscribed region located between the 5' ends of two divergently transcribed IE genes. The ori_S sequence contains several distinguishing features including an almost perfect palindromic sequence 45 bp long containing 18 consecutive A and T residues at its centre. Intra-strand base pairing could potentially generate a cruciform structure. The removal of 23 bp from the centre of the palindrome followed by the insertion of an 8 bp

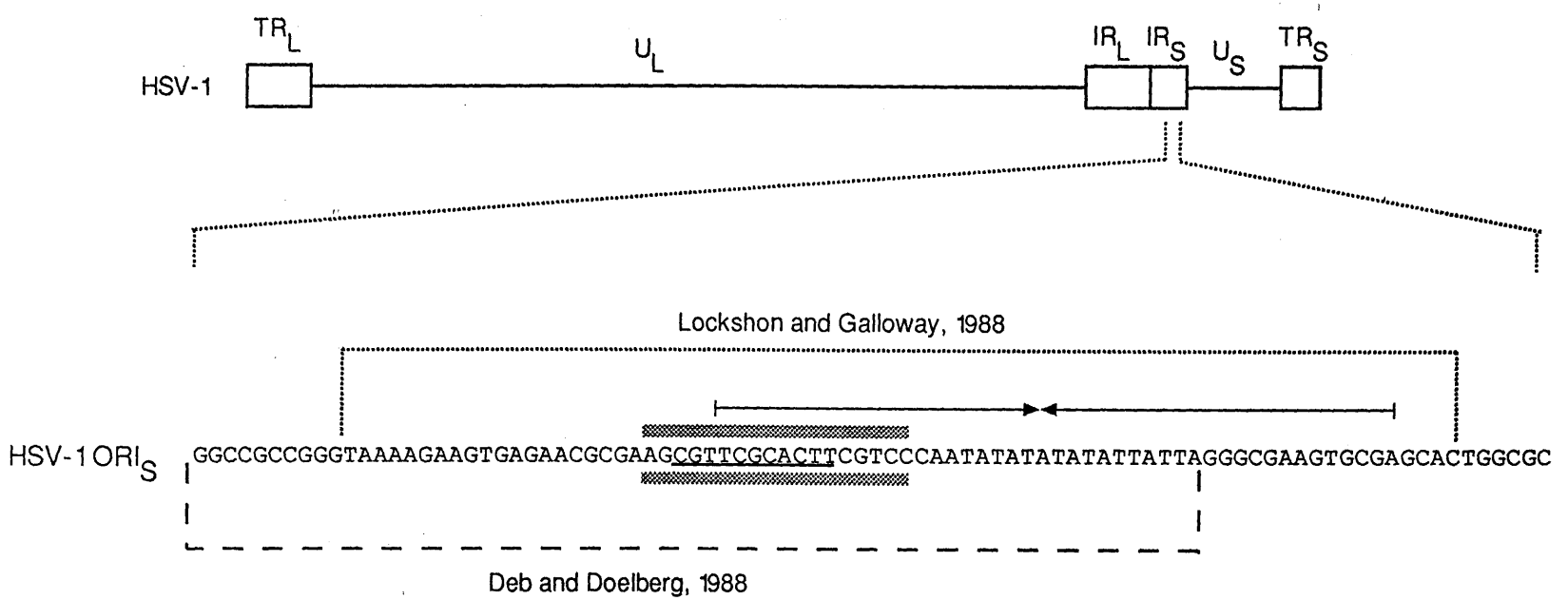

TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$
HSV-1
Lockshon and Galloway, 1988
HSV-1 ORI$_S$
GGCCGCCGGGTAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGCGC
Deb and Doelberg, 1988

Figure 1.6 DNA sequence of the 90 bp HSV-1 ori_S region as defined by Stow and McMonagle (1983)

The 45 bp palindrome is indicated by arrows and the 18 bp region protected from DNase I digestion by an origin binding protein is shown as a hatched box at the left hand end of the palindrome (Elias et al., 1986). This region includes an 11 bp sequence (underlined) which is conserved in HSV ori_L and VZV ori_S (Fig. 1.7 and 1.8). The limits of the minimal HSV-2 ori_S sequence as defined by Lockshon and Galloway (1988) are marked on the HSV-1 ori_S sequence, as are the limits of the minimal ori_S sequence as defined by Deb and Doelberg (1988).

HindIII linker, resulted in the loss of ori_S activity, demonstrating an essential role for sequences within the palindrome for initiation of DNA replication (Stow, 1985).

An 11 bp sequence located at the left hand end of the palindrome is conserved in HSV ori_L and VZV ori_S (Weller et al., 1985; Stow and Davison, 1986; see Fig. 1.9 section 1D). Interestingly, this conserved sequence lies within an 18 bp region covering the left hand end of the palindrome which is protected from DNase I digestion by an HSV encoded origin binding protein {Elias et al., 1986; see section 1C part 2(a)}.

More recent studies carrying out deletion analysis of ori_S-containing plasmids in order to define the limits of sequence required for origin activity have presented conflicting results. Using plasmids containing an HSV-2 ori_S sequence, which is highly homologous to HSV-1 ori_S (Whitton and Clements, 1984b), Lockshon and Galloway (1988) identified a 75 bp core origin sequence containing a palindromic region. The limits of this core origin on the corresponding HSV-1 sequence are shown in Fig. 1.6. Within this region, HSV-1 and HSV-2 are identical in 70 out of 75 residues. Deletion of the central AT-rich region of the palindrome abolished ori_S activity, as did replacement of AT bp with GC bp. When additional copies of an AT-dinucleotide were introduced progressively into the centre of the palindrome, ori_S activity oscillated with a periodicity of approximately 10 bp (one helical turn). In marked contrast to these observations, Deb and Doelberg (1988) have reported that a plasmid lacking the right arm of the palindrome was unimpaired in its replication (Fig. 1.6).

The reason for HSV-1 possessing two copies of ori_S is not known. Viable deletion mutants lacking one copy of ori_S have been isolated, but attempts to generate viable deletion mutants lacking both copies of ori_S have so far proved unsuccessful (Longnecker and Roizman, 1986; Smith et al., 1989). This suggests that at least one copy of ori_S may be essential for viral replication. In the case of HSV-2 strain HG52, the genome contains four copies of ori_S due to a tandem duplication of a 137 bp element in the TR_S and IR_S regions (Whitton and Clements, 1984b).

Despite the location of ori_S between divergently

transcribed genes, there is no direct evidence for the promoter or regulatory elements of these genes having any effect on ori_S activity or vice versa. In identifying the minimal ori_S functional region, sequences outwith the core origin sequence appeared to have some enhancing effect on origin activity but determination of the actual sequences involved has yet to be carried out (Stow, 1982; Stow and McMonagle, 1983; Lockshon and Galloway, 1988). A transcript in HSV-infected cells has been identified that extends through the ori_S sequence (Hubenthal-Voss *et al.*, 1987; Hubenthal-Voss and Roizman, 1988) although the 5' and 3' ends of the transcript are not located within sequences which affect ori_S function. The role of this transcript (if any) in regulation of DNA replication has yet to be determined.

(b) the long region origin of replication

Early attempts to analyse the HSV origin of replication in class II defective genomes were hampered due to the tendency of this region to suffer deletions when cloned into *E.coli* vectors (Spaete and Frenkel, 1982; Weller *et al.*, 1985). Plasmids containing such deletions could produce defective genomes when transfected into cells along with HSV-1 DNA, but only following restoration of the deleted region by recombination with the helper HSV-1 DNA (Spaete and Frenkel, 1982). This suggested that the deleted region contained the origin of replication, designated ori_L.

The first ori_L sequence to be determined was from a class II defective DNA of HSV-1 strain Angelotti. Gray and Kaerner (1984) sequenced a 296 bp fragment of virion DNA which was prone to deletions when cloned in *E.coli*. Subsequent studies by Weller *et al.* (1985) and Quinn and McGeoch (1985) reported the sequence of an undeleted ori_L from strains KOS and 17*syn*$^+$. Weller *et al.* (1985) successfully cloned the 425 bp deletion-prone region of the non-defective HSV-1 strain KOS into a yeast vector in an undeleted form. Using the plasmid amplification assay developed by Stow and McMonagle (1983) this sequence was shown to exhibit origin activity.

The ori_L sequence of HSV-1 lies between two

divergently transcribed genes encoding the major DNA binding protein (UL29) and the DNA polymerase (UL30), notably both components of the DNA replication machinery, suggesting a possible relationship between ori_L activity and transcriptional activity.

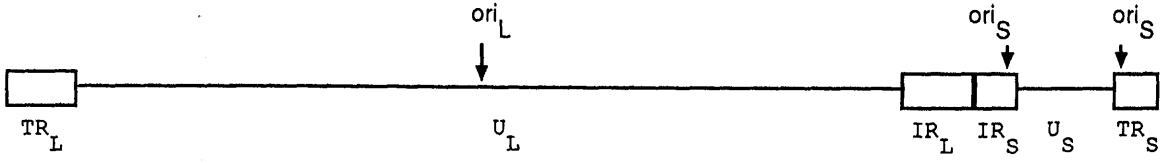
The ori_L region of KOS and 17*syn*$^+$ contains a 144 bp perfect palindrome, the probable reason for the cloning instability. As with ori_S, the ori_L sequence contains an AT-rich region at the centre of the palindrome (Fig. 1.7). The entire ori_S palindrome is highly homologous to the central region of the ori_L palindrome (85% identical residues) and sequence homology extends about 40 bp to the left of the ori_S palindrome. The ori_L of HSV-2 has been functionally localised to a 241 bp fragment from a region homologous to that containing HSV-1 ori_L. Sequencing of the HSV-2 ori_L has identified a 136 bp, almost perfect, palindrome which shows 88% identity to HSV-1 ori_L (Lockshon and Galloway, 1986). The original ori_L sequence determined by Gray and Kaerner (1984) consisted of two large tandemly repeated palindromic regions and is not therefore the simplest form of sequence associated with ori_L.

The limits of the minimal ori_L sequence required for origin activity have not been determined. By comparison with studies carried out with ori_S and from the striking sequence similarity between both origins, it is likely that the limits lie within the 72 bp arms of the palindrome. Due to the nature of the palindrome, the 11 bp conserved sequence, located at the left hand end of the ori_S palindrome, is present twice within the ori_L palindrome.

The isolation of a viable deletion mutant lacking 150 bp of ori_L demonstrates that this origin is dispensible for virus replication and moreover, the closely related alphaherpesvirus, VZV, which contains two copies of ori_S, does not appear to have an equivalent of ori_L (Davison and Scott, 1986; Stow and Davison, 1986; Polvino-Bodnar *et al.*, 1987).

2. Genetics of HSV DNA replication

Three complementary approaches have been used to


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

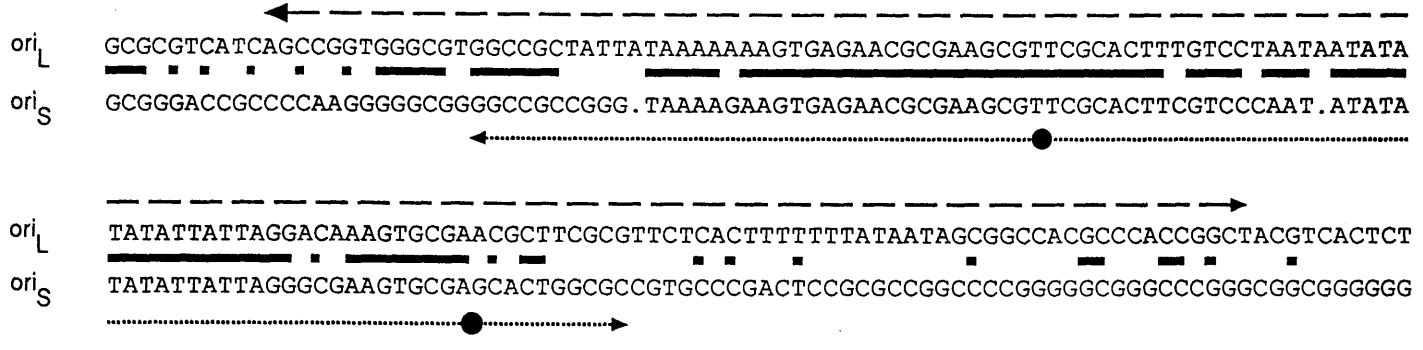

ori L
GCGCGTCATCAGCCGGTGGGCGTGGCCGCTATTATAAAAAAAGTGAGAACGCGAAGCGTTCGCACTTTGTCCTAATAATATA
ori S
GCGGGACCGCCCCAAGGGGGCGGGGCCGCCGGG.TAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAAT.ATATA
ori L
TATATTATTAGGACAAAGTGCGAACGCTTCGCGTTCTCACTTTTTTTTATAATAGCGGCCACGCCCACCGGCTACGTCACTCT
ori S
TATATTATTAGGGCGAAGTGCGAGCACTGGCGCCGTGCCCGACTCCGCGCCGGCCCCGGGGGCGGGCCCGGGCGGCGGGGGG

Figure 1.7 Comparsion of the DNA sequence of HSV-1 ori_S and HSV-1 ori_L.

The location of the HSV-1 ori_S and ori_L are marked on the schematic diagram of the HSV-1 genome and the sequence from these regions is shown below. Solid bars represent conserved bases. Arrowed dashed lines above the ori_L sequence represent the limits of the 144 bp perfect palindrome and the arrowed dotted lines below the ori_S sequence represent the limits of the minimal ori_S region as defined by Stow and McMonagle (1983). The limits of the ori_S palindrome are marked by dots.

identify the trans-acting viral gene products involved in the replication of HSV DNA.

Approximately 10 complementation groups of ts mutants have been characterised and designated defective for DNA synthesis at NPT. Some of these mutations map to genes encoding IE regulatory proteins (Preston, 1979; Dixon and Schaffer, 1980). Since the transcription of early genes depends on prior expression of one or more IE genes, IE polypeptides are likely to have an indirect role in DNA replication. However, complementation groups of mutants have been identified with lesions in each of the seven genes encoding products directly involved in DNA replication (see below).

The second approach has involved the biochemical analysis of cell-free extracts from infected cells for enzyme activities, which, by analogy with more extensively characterised replication systems, may have a role in DNA replication. The biochemical activities presently identified include a viral DNA polymerase (Keir and Gold, 1963; Hay et al., 1971), a single-stranded major DNA binding protein (Bayliss et al., 1975; Purifoy and Powell, 1976), an origin-specific DNA binding protein (Elias et al., 1986), helicase-primase activity (Crute et al., 1989), thymidine kinase (Kit and Dubbs, 1963), ribonucleotide reductase (Cohen, 1972), an exonuclease (Hay et al., 1971; Morrison and Keir, 1968), uracil-DNA glycosylase (Caradonna and Cheng, 1981), deoxyuridine triphosphate nucleotidyl hydrolase (Wohlrab et al., 1982) and protein kinase activities (Blue and Stobbs, 1981; Purves et al., 1987). Some DNA negative ts mutants have been shown to specify thermolabile forms of some of these enzymes e.g. DNA polymerase (Purifoy and Powell, 1981), single-stranded major DNA binding protein (Lee and Knipe,1983; Ruyechan et al., 1986), ribonucleotide reductase (Preston et al., 1984) and the exonuclease (Moss, 1986).

The complete set of viral genes required for DNA replication has now apparently been identified using a third approach based on the transient replication of transfected plasmid DNA containing an HSV origin of replication. When purified HSV DNA is transfected into tissue culture cells, viral genes are expressed which provide nec essary

trans-acting functions required to amplify an ori_S-containing plasmid concurrently transfected into the cells. Challberg (1986) constructed a set of five plasmids containing XbaI fragments, which between them contained most of the HSV-1 genome, and found that these plasmids behaved similarly to intact viral DNA in supporting amplification of an ori_S-containing plasmid.

Following systematic subcloning, deletion analysis and inactivation by restriction enzyme digestion, and in conjunction with the HSV-1 sequence data, a set of seven genes was identified, which were necessary and sufficient for plasmid amplification (Wu *et al.*, 1988). In addition, three other genes were identified which stimulated amplification, namely the IE proteins Vmw175, 110 and 63 and these were therefore proposed to have an indirect role in DNA replication. The identification of seven genes, essential for DNA synthesis (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) correlates well with the mapping data of the DNA negative *ts* mutants. *Ts* defects in these seven genes all lead to a DNA negative phenotype (Purifoy *et al.*, 1977; Dixon and Schaffer, 1980; Chartrand *et al.*, 1980; Purifoy and Powell, 1981; Conley *et al.*, 1981; Weller *et al.*, 1983; Littler *et al.*, 1983; Coen *et al.*, 1984; Weller *et al.*, 1987; Zhu and Weller, 1988; Marchetti *et al.*, 1988). The location of the genes are shown in Fig. 1.3 and the predicted molecular weights of the products are shown in Table 1.1. In addition, deletion and insertion mutants have been characterised which map to the UL8, UL9 and UL52 genes and verify their essential nature. These host range (hr) mutants only replicate in cell lines expressing the corresponding *wt* gene product (Carmichael *et al.*, 1988; Goldstein and Weller, 1988a; Carmichael and Weller, 1989).

Our knowledge of the functions of the products of these seven genes varies considerably. Three of the genes encode the previously well-characterised viral DNA polymerase (UL30), the single-stranded major DNA binding protein (mDBP; UL29) and the 65-kD double-stranded DNA binding protein (65-kD DBP) whereas the products of the other four genes were, until recently, unknown.

The high degree of correlation between genes required for DNA synthesis in the transient system and genes

Table 1.1 HSV-1 genes required for DNA replication (adapted from McGeoch, 1987)

HSV-1 gene (*)	Protein M_r	homologous VZV gene (1)	homologous EBV gene (2)	homologous HCMV gene (3)
UL5	99,000	55	BBLF4	UL105
UL8	80,000	52	?	?
UL9	94,000	51	?	?
UL29	128,000	29	BALF2	UL57
UL30	136,000	28	BALF5	UL54
UL42	51,000	16	?	?
UL52	115,000	6	BSLF1	UL70

(*) The HSV-1 genes identified from functional assays of Challberg, (1986) and Wu *et al*. (1988)

(1) Otained by comparison with the sequence of Davison and Scott, (1986)

(2) Obtained by comparison with the sequence of Baer *et al*. (1984)

(3) Obtained from Chee *et al*. (1990)

'?' indicates that no reading frame showing sequence homology could be found

identified by genetic studies to be essential for viral DNA replication would indicate that the major genetic requirements have now been identified. However, it remains possible that certain functions important for replication of intact viral genomes may not have been detected using the transient plasmid replication assay (e.g. circularisation, sealing of nicks). The remainder of this section will outline the information that is available on the functions of the genes whose products are essential or may play an indirect role in HSV DNA synthesis.

(a) genes essential for HSV DNA replication

UL30 - viral DNA polymerase

The induction of a novel DNA polymerase in extracts of HSV-infected cells was first described by Keir and Gold (1963). This activity is distinguishable from cellular DNA polymerases on the basis of biochemical characteristics such as sensitivity to inhibitors, salt concentration required for maximal activity and also by immunological criteria (Keir et al., 1966; Weissbach et al., 1973; Powell and Purifoy, 1977; O'Donnell et al., 1987a). Studies of HSV ts mutants and drug-resistant mutants have demonstrated that this enzyme is virally encoded. Several ts mutants with lesions in the polymerase gene (pol) have been characterised and fail to synthesis viral DNA at NPT (Purifoy et al., 1977; Dixon and Schaffer, 1980; Chartrand et al., 1980; Coen et al., 1984). The protein has been purified and has an apparent molecular weight around 140,000 (Powell and Purifoy, 1977; Knopf, 1979; O'Donnell et al., 1987a) which is in close agreement with the predicted molecular weight of 136,000 based on sequence analysis of the pol gene (Gibbs et al., 1985; Quinn and McGeoch, 1985). In common with other DNA polymerases, the HSV polymerase has an intrinsic 3'-5' exonuclease activity that would appear to provide a proofreading function to increase fidelity of replication (Knopf, 1979; Powell and Purifoy, 1977; O'Donnell et al., 1987a). Crute and Lehman (1989) have recently demonstrated that the pol gene product also contains an intrinsic ribonuclease H activity that specifically degrades RNA/DNA heteroduplexes or duplex DNA substrates in the 5'-3'

direction. This activity is analogous to the 5'-3' exonuclease associated with E.coli DNA polymerase I which is required for DNA repair and removal of RNA primers (Kornberg, 1980).

A purified preparation of the HSV DNA polymerase is catalytically active on simple primer-templates and is highly processive in the absence of accessory factors, although the addition of single-stranded DNA binding protein of E.coli greatly stimulates the rate of synthesis on primed single-stranded DNA templates (O'Donnell et al., 1987a). The polymerase has also been expressed in yeast (Haffey et al., 1988) and by in vitro transcription/translation (Dorsky and Crumpacker, 1988). In both cases, the enzyme is catalytically active and is therefore functional in the absence of any accessory factors. However, other virus encoded polypeptides may be required to increase the efficiency or modify the activity of the polymerase. The polymerase of HSV-2 has been shown to co-purify with a 55-kD polypeptide, known to be encoded by the UL42 gene and it is now generally considered that the holoenzyme is a heterodimer containing UL30 and UL42 subunits (Vaughan et al., 1984; Parris et al., 1988; see below).

The HSV DNA polymerase activity can be blocked by the action of several antiviral drugs, namely deoxyribonucleotide and pyrophosphate analogues . Drug-resistant mutants have been isolated (Hay and Subak-Sharpe, 1976; Crumpacker et al., 1980; Coen et al., 1982; Honess et al., 1984) which, through characterisation of their mutations have identified a region within the carboxy terminal portion of the enzyme involved in interactions with deoxyribonucleotide triphosphates. This region is highly conserved in other herpesvirus polymerases as well as some eukaryotic and prokaryotic enzymes (Gibbs et al., 1985; Larder et al., 1987; Gibbs et al., 1988; Wong et al., 1988).

UL29 - the major single-stranded DNA binding protein

Next to the polymerase, the best characterised protein required for HSV DNA replication is the major single-stranded DNA binding protein (mDBP; ICP8). This species was identified as an abundant HSV induced protein

with a molecular weight of around 130,000, which was present in HSV-infected cells but not virions (Honess and Roizman, 1973; Powell and Courtney, 1975). Ts mutants with amino acid substitutions in the gene for the DNA binding protein (dbp) fail to synthesise viral DNA at NPT indicating that the protein is essential for HSV DNA replication (Conley et al., 1981; Weller et al., 1983; Littler et al., 1983).

Mapping of the ts lesions and analysis of the properties of mutated polypeptides has allowed the identification of distinct functional domains within the polypeptide. Some mutants produce proteins that are defective in nuclear localisation while others are unable to bind DNA (Lee and Knipe, 1983; Leinbach et al., 1984; Quinlan et al., 1984; de Bruyn-Kops and Knipe, 1988; Gao et al., 1988; Gao and Knipe, 1989). The DNA binding domain appears to be located within the carboxy terminal region (Leinbach and Heath, 1988) although some of the amino acid residues between positions 346 and 450 also play some role in DNA binding, perhaps by stabilizing the single-stranded DNA (ssDNA) binding site in the carboxy terminus or by forming intermolecular contacts required for cooperative DNA binding (Gao et al., 1988).

Other dbp mutants have also been shown to display altered sensitivity to drugs that inhibit the viral DNA polymerase suggesting some interaction between the polymerase and the mDBP (Chiou et al., 1985). In addition, some dbp ts mutants over-express certain late genes at NPT suggesting a role for the protein in transcriptional regulation (Godowski and Knipe, 1985, 1986). Interestingly, the mDBP has moderate affinity for binding to polyriboadenylic acid suggesting that the protein may possibly influence gene expression at the post transcriptional level by binding to the poly(A) tail of mRNA transcripts (Ruyechan and Weir, 1984). As the mDBP protein binds tightly to single-stranded DNA cellulose columns (Bayliss et al., 1975; Purifoy and Powell, 1976; Powell et al., 1981; Knipe et al., 1982; Ruyechan, 1983) it has been postulated that the protein is analogous to the gene 32 protein of bacteriophage T4 and the single-stranded binding protein (SSB) of E.coli. These proteins bind to single-stranded DNA at the replication fork and stabilise

the strands for use as templates by the DNA polymerase. In support of this, mDBP binds more tightly to single-stranded than double-stranded DNA (Ruyechan, 1983; O'Donnell *et al.*, 1987b) and binding activity is cooperative and sequence-independent (Ruyechan, 1983). The mDBP has a small stimulatory effect on HSV polymerase activity (Ruyechan and Weir, 1984), and can reduce the melting temperature of duplex DNA *in vitro* (Powell *et al.*, 1981).

Using monoclonal antibodies against mDBP, the location of the protein throughout the viral replicative cycle has been examined (Showalter *et al.*, 1981; Knipe and Spang, 1982; Quinlan and Knipe, 1983; Quinlan *et al.*, 1984; de Bruyn-Kops and Knipe, 1988). The intracellular distribution of mDBP in infected cells is dependent on the status of viral DNA replication (Quinlan *et al.*, 1984). When replication occurs, the protein accumulates in large, globular replication compartments in association with viral DNA. When replication is blocked either chemically, or by the use of pol *ts* mutants, mDBP is found at numerous foci called pre-replicative sites in association with a nuclear protein framework. On lifting the replicative block, the protein redistributes itself back to replicative compartments (Quinlan *et al.*, 1984). Hence, the protein may possibly play some role in organising cellular and viral components into structures related to HSV DNA replication (de Bruyn-Kops and Knipe, 1988). Finally, Matsumoto *et al.* (1987) noted that the amino terminal 1/5 of mDBP shares some sequence similarity with rat proliferating cell nuclear antigen (PCNA). PCNA (also called cyclin or DNA polymerase delta auxiliary protein) is a highly conserved protein whose intranuclear distribution is regulated by cellular DNA replication and which is also required for stimulation of DNA polymerase delta (Bravo and MacDonald-Bravo, 1985).

UL42 - double-stranded DNA binding protein

The HSV-1 double-stranded DNA binding protein (dsDBP), of apparent molecular weight 65,000, is the counterpart of the HSV-2 55-kD polypeptide (described above) which co-purifies with the product of the polymerase gene (Vaughan *et al.*, 1984). This polypeptide is encoded by the UL42 gene (Parris *et al.*, 1988) and is therefore distinct

from the 65-kD virion trans-inducing factor (Marsden et al., 1987). Immunoaffinity purification of the UL42 protein has shown that there is a close association between it and the polymerase but the strong non-sequence specific DNA binding activity is an intrinsic property of the UL42 gene and is not due to its interaction with polymerase (Gallo et al., 1988).

The role of dsDBP in DNA synthesis is not completely resolved. As stated earlier, the protein is not required for HSV DNA polymerase activity. Gallo et al. (1989) have however demonstrated that purified dsDBP can specifically stimulate the activity of purified HSV DNA polymerase up to 10-fold. The ability of a monoclonal antibody to dsDBP to remove the stimulatory activity from a dsDBP preparation indicates this protein does act as a polymerase accessory factor although the mechanism for stimulation remains to be determined. Under appropriate assay conditions, the processivity of purified polymerase on ss primer-template molecules is enhanced by addition of purified UL42 product (M. Challberg, personal communication). It thus seems probable that the in vivo polymerase is a heterodimer consisting of the UL30 and UL42 subunits.

UL5, UL8, UL9 and UL52

The products of the four remaining DNA replication genes, UL5, UL8, UL9 and UL52 had (until 1988) not been identified amongst the proteins of infected cells suggesting that they were present at low abundance. Identification of these products depended upon their cloning into expression vectors (Olivo et al., 1988, 1989; Calder and Stow, maunscript submitted for publication).

By analogy with other replication systems, a number of possible functions for these proteins can be postulated, such as origin recognition, primer synthesis and degradation, unwinding of duplex DNA, polymerase accessory factors and topoisomerases. A recent report by Olivo et al. (1988) and the results presented in this thesis have identified the UL9 gene as encoding the origin binding activity initially detected by Elias et al. (1986). This work will be described and discussed in detail in sections 3

and 4. Analysis of the predicted amino acid sequence of the four proteins has suggested that UL5 and UL9 contain purine NTP-binding sites of the type found in many helicases (Hodgeman, 1988; McGeoch et al., 1988; Gorbalenya et al., 1989; Gorbalenya and Koonin, 1989).

Infection of cells with HSV has been shown to induce novel helicase and primase activities (Crute et al., 1988; Holmes et al., 1988; Crute et al., 1989). The primase activity detected by Holmes et al. (1988) now however appears to be an RNA polymerase activity released from Vero cell mitochondria upon infection by HSV-1 (Tsurumi and Lehman, 1990). The helicase activity (Crute et al., 1988) has been purified to homogeneity (Crute et al., 1989) and additionally contains a tightly associated DNA primase. Both activities are components of a three-subunit enzyme composed of the products of UL5, UL8 and UL52. The helicase can use either ATP or GTP as a cofactor in displacing an oligonucleotide annealed to M13 single-stranded DNA. Experiments with model substrates suggest that the polarity of unwinding is 5'-3' relative to the M13 DNA, thus, the enzyme may prime lagging strand synthesis as it unwinds DNA at the replication fork. As yet, the helicase and primase activities have not been assigned to individual gene products although recent studies in our laboratory have shown that helicase activity can be obtained with a combination of the UL5 and UL52 proteins (Calder and Stow, manuscript submitted for publication).

(b) genes indirectly involved in HSV DNA replication

Apart from the trans-activating roles of the IE gene products, several HSV encoded enzymes may also indirectly influence viral DNA replication by modifying nucleic acid metabolism. There are two HSV-specified enzymes which catalyse reactions in the biosynthesis of DNA precursors : thymidine kinase (TK) and ribonucleotide reductase (RR). TK, first described by Kit and Dubbs (1963), phosphorylates, in addition to thymidine, deoxycytidine (Jamieson and Subak-Sharpe, 1974) and thymidylate (Chen and Prusoff, 1978), and uses nucleoside triphosphates, usually ATP, as a phosphate donor. Thus, the

enzyme possesses both pyrimidine deoxyribonucleoside kinase and thymidylate kinase activity. However, exogenously added deoxycytidine fails to be incorporated into DNA suggesting that reduction of CDP, rather than phosphorylation of dCMP is the major route of dCDP and therefore dCTP synthesis (Jamieson and Subak-Sharpe, 1976). Studies of TK negative mutants have shown that TK (encoded by UL23) is not essential for virus growth in dividing cells but is required in serum-starved cells (Jamieson et al., 1974). TK is not essential for the establishment of a latent infection in mice, but may have some role in either reactivation or virus replication following reactivation (Efstathiou et al., 1989).

Ribonucleotide reductase catalyses the reduction of ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphates and is essential for the replication of DNA in prokaryotic and eukaryotic cells. HSV RR differs from its cellular counterpart in that it is insensitive to inhibition by TTP (Cohen, 1972; Dutia, 1983). The enzyme is composed of large, (RR_1), and small, (RR_2), subunits encoded by contiguous genes, UL39 and UL40 respectively (Preston et al., 1984; Bacchetti et al., 1984; Frame et al., 1985; McLauchlan and Clements, 1983). Interaction of the subunits is essential for enzyme activity. A synthetic oligopeptide corresponding to the carboxy terminus of RR_2 specifically inhibits the viral enzyme by interfering with the subunit interaction (Frame et al., 1985; Cohen et al., 1986; Dutia et al., 1986). Contrasting evidence has been presented for the requirement of HSV RR for growth in tissue culture cells. Goldstein and Weller (1988b) have shown, using an insertion mutant in RR_1, that the enzyme is dispensible for growth in actively dividing cells whereas a ts mutant, ts1207, which contains a lesion in RR_1, exhibited decreased virus yields at NPT (Preston et al., 1984). However, characterisation of another mutant, ts1222 has indicated that, as with TK, RR is essential for the replication of HSV in tissue culture cells under certain conditions. These conditions include non-dividing, serum-starved cells and actively dividing cells at 39.5°C (Preston et al., 1988). The dependence of these mutants on the state of the cell for their replication

probably reflects the requirement for cellular RR to compensate for the loss of the virus encoded enzyme.

HSV also encodes a deoxyuridine-5'-triphosphate nucleotidyl hydrolase, or dUTPase, which catalyses the conversion of dUTP to dUMP and pyrophosphate (Wohlrab and Francke, 1980; Preston and Fisher, 1984). The enzyme (encoded by UL50) more likely functions to minimise the incorporation of uracil into nascent DNA rather than provide dUMP for TTP synthesis. The gene is dispensible for growth in tissue culture (Fisher and Preston, 1986).

Caradonna and Cheng (1981) reported the induction of a uracil-DNA glycosylase activity in HSV-infected cells. It seems likely that the role of this enzyme is the removal of uracil residues created by deamination of cytosine in DNA rather than uracil residues directly incorporated into the DNA. This enzyme is encoded by UL2 and is dispensible for growth in tissue culture (Mullaney et al., 1989).

The HSV exonuclease was first described by Keir and Gold (1963) as an increased alkaline nuclease activity in infected cells. The enzyme, encoded by UL12, has 5' and 3' exonuclease activities and an endonuclease function (Morrison and Keir, 1968; Hoffman and Cheng, 1979; Hoffman, 1981). Studies with ts mutants have produced conflicting results with regard to the requirement of the exonuclease for virus growth and DNA synthesis (Moss et al., 1979; Francke and Garret, 1982; Moss, 1986). More definitive experiments using an insertion mutant in the UL12 gene, have confirmed that the alkaline nuclease is required for virus replication in tissue culture. This mutant is unimpaired in viral DNA synthesis but exhibits a defect in the production of infectious virus particles. This suggests a possible role for the exonuclease post DNA replication (Weller et al. unpublished data).

A novel HSV-induced topoisomerase activity has been detected in HSV-infected cells and virions (Biswal et al., 1983; Leary and Francke, 1984; Muller et al., 1985). Muller et al. (1985) suggested that the topoisomerase was of type I activity i.e. produces transient single-strand breaks in the DNA, and was a component of the viral envelope or tegument structure, whereas Biswal et al. (1983) have reported their activity as copurifying with the DNA polymerase. Whether

this enzyme is virus encoded remains to be determined.

Finally, a protein kinase (PK) activity is induced after HSV-1 infection of cultured cells (Blue and Stobbs, 1981) which could possibly be involved in the modification of replication proteins. The HSV US3 gene was first identified as a putative PK gene based on amino acid sequence similarity to known eukaryotic PKs (McGeoch and Davison, 1986). Biochemical and genetic studies have demonstrated that a virus-induced PK is encoded by US3 and that the activity is not essential for virus growth in tissue culture (Frame et al., 1987; Purves et al., 1987). Recently, a second protein kinase function has been suggested for UL13 (Smith and Smith, 1989). The substrates for these enzymes are not yet known.

SECTION 1D: DNA replication in other human herpesviruses

1. VZV DNA replication

Comparisons of the VZV DNA sequence with that of the closely related herpes simplex virus type 1 have significantly extended our knowledge of the proteins involved and possible mechanism adopted by VZV in replicating its DNA. The relative scarcity of information on the molecular biology of VZV is due primarily to the growth characteristics of the virus. VZV has a restricted host range in vitro and is highly cell-associated. It is difficult to obtain a cell-free virus of reasonable titre and few VZV mutants have been isolated. Despite the considerable difference in G+C content between VZV and HSV (46% and 68% respectively) molecular hybridisation and DNA sequence studies have shown that these two viruses exhibit similar gene arrangements and hence have allowed functions to be provisionally assigned to VZV genes products which are homologous to HSV-1 counterparts (Davison and Wilkie, 1983; McGeoch, 1984; Davison and McGeoch, 1986; Table 1.1).

The model for VZV DNA replication is basically identical to that proposed for HSV. The virus circularises after entry into the cell by ligation of the termini. VZV

does not possess a terminal direct repeat equivalent to the 'a' sequence of HSV-1 therefore circularisation of the viral genome on entering the cell is most likely to take place via direct ligation of the termini to produce a novel L-S joint identical in sequence to the normal L-S joint. Due to the low frequency of inversion of U_L compared to U_S, replicated viral DNA predominantly contains two isomers representing a single orientation of U_L.

From DNA sequence studies, an origin of replication was predicted in the TR_S/IR_S region (Davison and Scott, 1985) which contained a 46 bp palindrome similar in size and location to ori_S of HSV (Stow and McMonagle, 1983). A 259 bp fragment of VZV DNA containing the palindrome was subsequently shown to function as an origin of replication and deletions within the palindrome affected replicative ability (Stow and Davison, 1986).

A comparison of HSV-1 ori_S and VZV ori_S sequence is shown in Fig. 1.8. The palindromes are of similar length although the AT-rich region of VZV is approximately twice as long as HSV. To the left of the VZV palindrome is an 11 bp sequence identical to a sequence found in ori_S and ori_L of HSV-1 and HSV-2 and which is part of the 18 bp sequence protected from DNase I digestion by an HSV-1 induced origin-binding protein (Elias et al., 1986). Unlike HSV, in which the 11 bp sequence lies partly inside the palindrome, in VZV the sequence lies completely outwith this region. The observation by Stow and Davison (1986), that plasmids containing VZV ori_S were able to replicate in the presence of superinfecting HSV-1 with approximately 5% the efficiency of an HSV-1 ori_S-containing plasmid, is consistent with the conserved 11 bp region forming part of a sequence involved in origin recognition. Results and discussion presented in this thesis will expand upon this point.

Although VZV contains an origin of replication equivalent in position to that of HSV-1 ori_S, no origin is present in the region corresponding to HSV-1 ori_L. The cloned VZV KpnI s fragment which includes the intergenic region between the pol and dbp genes and which is inactive in replication assays was shown not to have undergone any deletion, thereby excluding the possibility that the viral genome contains a deletion prone origin (like HSV-1 ori_L) at

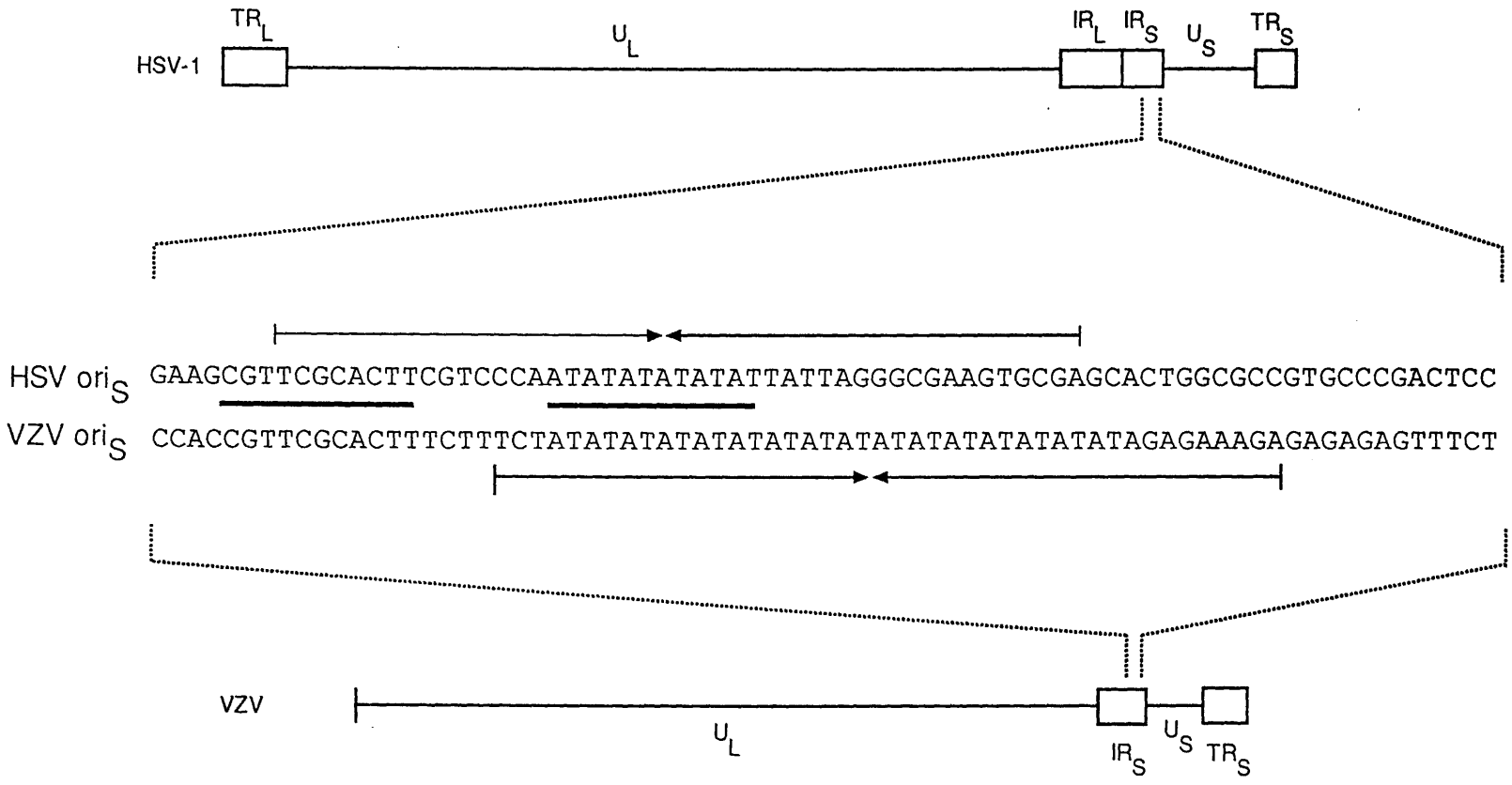

TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$
HSV-1
HSV ori$_S$ GAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGCGCCGTGCCCGACTCC
VZV ori$_S$ CCACCGTTCGCACTTTCTTTCTATATATATATATATATATATATATATATATATATAGAGAAAGAGAGAGAGTTTCT
VZV
U$_L$
IR$_S$
U$_S$
TR$_S$

Figure 1.8 Comparison of the DNA sequences within HSV ori_S and VZV ori_S

The sequences shown are: HSV-1 ori_S, nucleotides 568 to 642 of the sequence of Stow and McMonagle (1983) and VZV ori_S, nucleotides 5590 to 5662 of the sequence of Davison and Scott (1985).

The limits of the palindromes are shown by arrows and conserved regions are marked by solid bars.

this locus (Stow and Davison, 1986).

The ability of HSV to stimulate replication of a VZV ori_S-containing plasmid suggests similarities in the function of viral encoded polypeptides in initiation of DNA replication. VZV homologues of all seven HSV genes essential for DNA synthesis have been recognised (Table 1.1).

VZV also encodes a thymidylate synthetase (TS), not found in HSV, whose existence was predicted from DNA sequence analysis (Thompson et al., 1987). TS catalyses the reductive methylation of deoxyuridylate to thymidylate and is encoded by VZV gene 13. The function of TS was confirmed by expressing the polypeptide in an E.coli mutant from which the bacterial TS gene had been deleted. The only VZV replicative activities so far described are the polymerase, exonuclease and several DNA binding proteins.

2. EBV DNA replication

The 172kbp genome of the gammaherpesvirus, Epstein-Barr virus is maintained as an extrachromosomal multicopy (usually 1 to 10 copies per cell) supercoiled plasmid in tumour cells from Burkitt's lymphoma and nasopharyngeal carcinoma biopsies. These multiple copies arise soon after infection by amplification of the viral DNA relative to cell DNA (Sudgen et al., 1979). Latent infections, characterised by limited viral gene expression and faithful replication of the EBV episome once (and only once) per cell cycle, can be established in primary lymphocytes immortalised in vitro. Such transformed cell lines have been extensively used to identify EBV functions involved in DNA replication. In addition to the latent (maintenance) phase during which viral genome replication is tightly coupled to that of the cell, lytic replication of EBV can occur under certain conditions and is characterised by multiple rounds of DNA synthesis within the infected cell. Recent experiments have shown that separate origins of DNA replication are utilised during the latent and lytic phases.

To map the sequences required for maintenance,

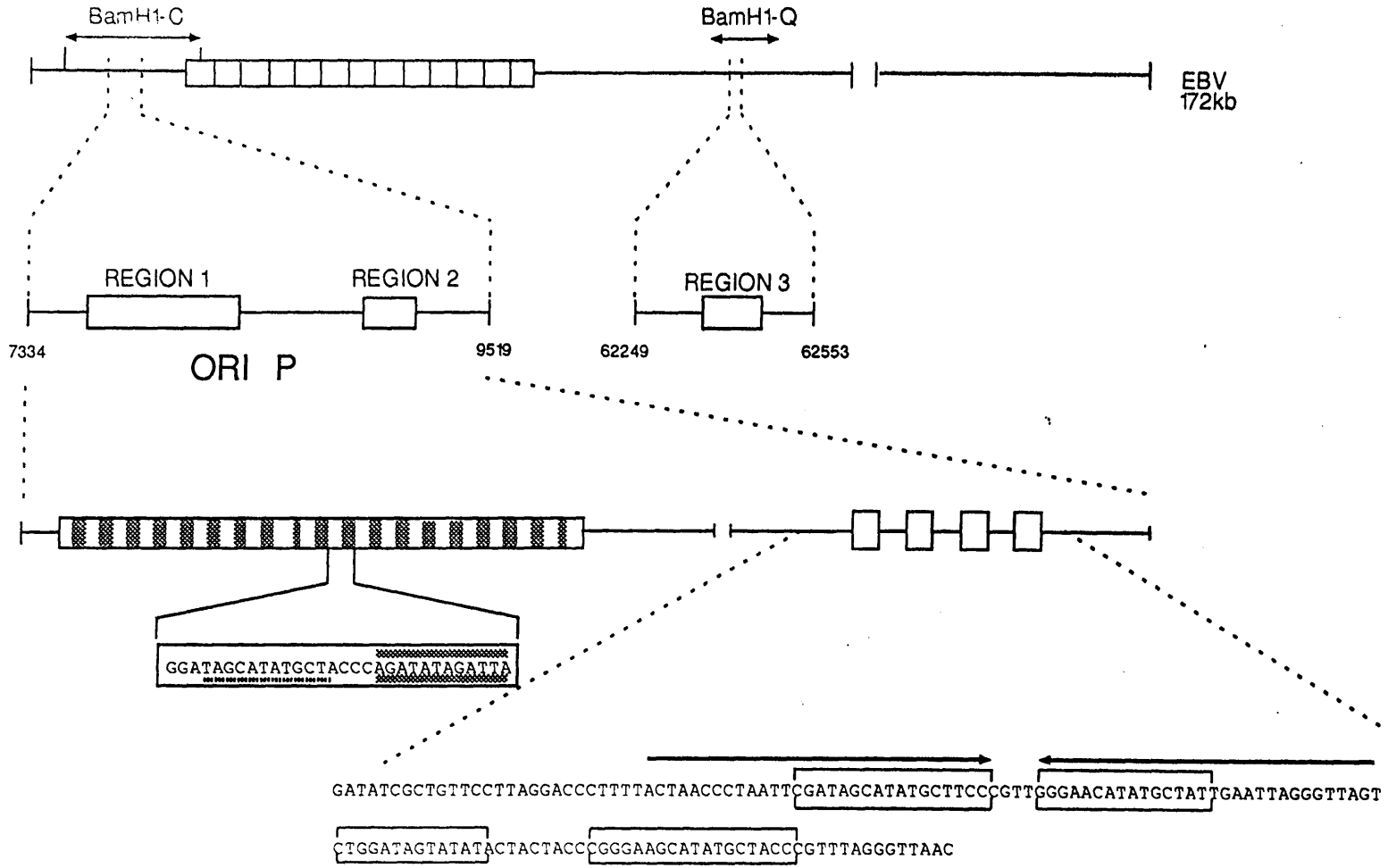

BamH1-C
BamH1-Q
EBV
172kb
REGION 1
REGION 2
REGION 3
7334
9519
62249
62553
ORI P
GGATAGCATATGCTACCCAGATATAGATTA
GATATCGCTGTTCCTTAGGACCCTTTTACTAACCCTAATTCGATAGCATATGCTTCCCGTTGGGAACATATGCTATTGAATTAGGGTTAGT
CTGGATAGTATATACTACTACCCGGGAAGCATATGCTACCCGTTTAGGGTTAAC

Figure 1.9 Structure of the EBV origin of replication, oriP, and location of EBNA-1 binding sites

A schematic diagram of the EBV genome is shown with an expanded region below showing oriP and three distinct loci (regions 1, 2 and 3) which contain EBNA-1 binding sites.

At the left of oriP (region 1) is a 20 member family of tandemly repeated sequences. This family consists of 17 imperfect copies and three truncated copies of a 30 bp sequence. The 30 bp EBNA-1 consensus sequence, shown below this region, contains a central 12 bp palindrome (underlined) followed by a 12 bp region containing a 4 bp direct repeat, AGAT. separated by two bp (shaded block).

At the right of oriP (region 2) are four truncated copies of the 30 bp sequence described above (located in marked boxes). Two of the blocks of repeats plus additional flanking sequences make up a 65 bp region of dyad symmetry (marked by arrows above the sequence). Region 3 contains two EBNA-1 binding sites which lie in the EBNA-1 coding region. The base pair coordinates are from Baer et al. (1984).

plasmids containing fragments of the EBV genome and a selectable marker were transfected into EBV transformed cells to determine the region of the genome which facilitates autonomous replication of the input plasmid. In this manner, Yates et al. (1984) identified a cis-acting 1.8 kbp element, oriP (origin of plasmid replication), within the U_S region of the genome which confers stable plasmid maintenance during latent infections and did not allow runaway replication under conditions where lytic EBV DNA synthesis was induced (Yates and Camiolo, 1988). Further analysis of this region identified two non-contiguous sequences which were essential for activity; a region of approximately 20 tandem, imperfect copies of a 30 bp sequence (region 1; Fig. 1.9) and a second sequence of 114 bp approximately 1 kbp downstream, which contains a 65 bp region of dyad symmetry composed of 4 related copies of the 30 bp sequence (Reisman et al., 1985; region 2 Fig. 1.9). Since oriP dependent replication required the presence of the EBV genome in tranfected cells, it was postulated that replication from oriP required trans-acting products encoded by the EBV genome (Yates et al., 1984).

To map the gene(s) encoding the trans-acting function(s), cells containing individual overlapping segments of the EBV genome were transfected with plasmids containing oriP and a selectable marker. The EBV BamHI-Z/SalI-P fragment encoding the 80-kD viral nuclear antigen, EBNA-1, was the sole requirement for plasmid maintenance; the DNA polymerase and other components of the replicative machinery are presumably supplied by the cell (Yates et al., 1985; Lupton and Levine, 1985). Not surprisingly, EBNA-1 is a member of a family of genes (EBNA-1 to 6) which are normally expressed during latent infection. No counterparts to these genes have been identified in HSV or VZV (Sample et al., 1986; Speck and Strominger, 1985; McGeoch, 1989).

Using filter binding and DNase I footprinting assays, Rawlins et al. (1985) demonstrated that the carboxy terminal domain of the EBNA-1 protein could specifically bind to three loci in the EBV genome. Two of these loci corresponded to essential elements within oriP. The third site within the BamHI-Q fragment (region 3; Fig. 1.9)

contained two tandem copies homologous to the tandem repeat units found in regions 1 and 2. The EBNA-1 protein bound to each of the 20 tandem repeats in regions 1 and 2 protecting a 25 bp region containing a 12 bp palindromic consensus sequence, TAGCATATGCTA (Kimball et al., 1989; Fig. 1.9). A later report demonstrated that tandem copies of the dyad symmetry region alone (region 2) could cooperatively activate replication suggesting that region 2 contains the actual minimal origin of replication in latently infected cells (Wysokenski and Yates, 1989). Indeed, experiments using a gel electrophoresis technique developed by Brewer and Fangman (1987) to study replicative intermediates, indicated that the site of initiation of DNA replication within oriP is at, or very near the dyad symmetry element in region 2 while the direct repeats in region 1 contain a replication fork barrier and termination site. Hence, replication from oriP appears to proceed in a predominantly unidirectional manner (Gahn and Schildkraut, 1989).

Studies using purified EBNA-1 and either monomeric or multimeric region 1 consensus binding sites have indicated that cooperative interactions between binding sites have little impact on EBNA-1 binding to region 1 (Kimball et al., 1989). Conflictingly, another report suggests that a minimum of seven EBNA-1 binding sites are required to activate oriP efficiently through a highly cooperative process (Wysokenski and Yates, 1989).

EBNA-1 has different affinities for binding to the three naturally occurring loci, with region 1 having the highest affinity and region 3 the lowest. The increased affinity of region 1 over region 2 is greater than would be predicted from the number of individual binding sites in each locus (Jones et al., 1989). Based on these findings, a 'sink and origin' model has been proposed in which the initiation of plasmid replication is controlled by the interaction of EBNA-1 with region 2 in oriP (Rawlins et al., 1985). The amount of EBNA-1 available for initiation of DNA replication might then be regulated by sequestration of protein at region 1 (the sink). Moreover, since overproduction of EBNA-1 could potentially result in increased initiation of plasmid DNA replication and loss of synchrony with cell division, it seems likely that EBNA-1

synthesis is itself strictly controlled. The EBNA-1 primary transcript passes through region 3 and it is possible that EBNA-1 binding to this site might negatively regulate its own transcription. Occupation of region 3 (the weakest binding region) would occur when EBNA-1 protein concentration increased beyond that necessary to saturate regions 1 and 2 (Jones et al., 1989).

In addition, it appears that EBNA-1 can positively regulate its own expression. Reisman and Sugden (1986) demonstrated that region 1 of oriP acts as an EBNA-1-dependent enhancer. Analysis of cDNA clones has shown that the EBNA-1 gene is transcribed as a highly spliced mRNA from promoters within the BamHI c fragment although the open reading frame encoding the gene lies approximately 90 kbp downstream in the BamHI k fragment (Summers et al., 1982; Hennessy and Kieff, 1983; Sample et al., 1986; Bodescot et al., 1987). Although the region 1 enhancer is approximately 3 kbp from the EBNA promoter, in the presence of EBNA-1 this promoter depends on region 1 in cis for efficient expression (Sudgen and Warren, 1989). Such negative and positive feedback controls of EBNA-1 expression would be expected to tightly regulate the protein present in the cells, and hence the amplification of episomal EBV genomes.

A significant advance has been made in the study of lytic phase replication of EBV DNA with the identification of a portion of the genome, termed oriLyt, that enables enhanced replication of plasmids in cells in which EBV is in the lytic phase (Hammerschmidt and Sudgen, 1988). The EBV lytic phase can be induced in some latently infected cell lines by chemical treatments (ZurHausen et al., 1978) or by introduction of a vector expressing the BZLF-1 gene of EBV, whose product is a transcriptional activator (Countryman and Miller, 1985; Takada et al., 1986). Fragments from throughout the EBV genome were co-transfected into latently infected cells along with the BZLF-1 gene and assayed for their capacity to allow replication of linked plasmid DNA sequences. Sequences containing the minimal oriLyt were mapped to within a set of directly repeating elements, D_L and D_R, which themselves contain multiple repeat sequences and are situated at the ends of the U_L region (see Fig.1.1; section 1.1). Most naturally occurring strains of EBV have

two copies of oriLyt although some strains (e.g. B95-8) can replicate in the lytic phase with only one copy. Unlike the lytic origins of HSV and VZV, oriLyt is a complex sequence consisting of two essential regions of approximately 300 bp separated by at least 260 bp of non-essential sequence. The second 300 bp region can be functionally substituted by a transcriptional promoter/enhancer.

OriLyt differs functionally in at least three ways from oriP. Firstly, replication from oriLyt yields a 100 to 1000 fold amplification of the plasmid content per cell whereas replication from oriP yields a stable number of plasmid molecules per cell in a dividing population. Secondly, the products of oriLyt replication are concatemeric DNA molecules whereas replication via oriP produces circular episomal molecules. Thirdly, the only viral gene product that oriP requires to function is EBNA-1; oriLyt requires at least one viral gene, the DNA polymerase, which is not expressed in latently infected cells (Leinbach et al., 1976). The EBV genome possesses clear counterparts to four of the HSV-1 DNA replication genes, namely UL5, UL29, UL30 and UL52 (Table 1.1; McGeoch, 1987) and it would be expected that these also play important roles in the lytic phase.

OriLyt does not contain an AT-rich sequence within a palindromic sequence analogous to the human alphaherpesviruses. Its complex structure and association with potential binding sites for transcription factors distinguish it from the origins of HSV-1 and HSV-2. Interestingly, there is no clear counterpart for the HSV UL9 origin binding protein in EBV. Despite probable differences in the initiation of DNA replication, both viruses, during the lytic phase appear to amplify plasmid containing origins in a similar manner as the products of replication in both cases consist of concatemeric DNA. No latent origin of replication has been identified in HSV. Based on the lack of detectable viral gene expression in HSV latently infected neuronal cells, and the fact that these cells are non-dividing, it would appear that an equivalent of oriP is not required for HSV to be maintained in the latent phase.

3. HCMV DNA replication

By DNA sequence comparisons of HCMV with HSV-1, homologues to four of the seven HSV-1 genes known to be essential for DNA replication have been identified (Chee et al., 1990). These are the DNA polymerase (Kouzarides et al., 1987), the major DBP, UL5 and UL52 (Table 1.1). HCMV origins of DNA replication have yet to be identified. HCMV, like HSV, possesses a terminal 'a' sequence. The HCMV 'a' sequence can provide the cleavage/packaging signals for defective HSV genomes (Spaete and Mocarski, 1985).

SECTION 1E: The role of sequence-specific DNA binding proteins in the initiation of DNA replication

The subject of this thesis concerns the identification and characterisation of an HSV origin-binding protein which, by analogy with other well-characterised prokaryotic and eukaryotic origin-binding proteins is believed to play a critical role in directing the formation of a replication complex at the HSV origins of DNA replication. Initiation of DNA synthesis has been investigated in many systems and several other examples in which initiation takes place at internal origins following interactions with sequence-specific DNA binding proteins are known. The following section briefly describes the role of sequence-specific DNA binding proteins in three of the best studied systems (E.coli, bacteriophage lambda, and simian virus 40 [SV40]) and attempts to identify some common features.

In prokaryotic and eukaryotic cells, the initiation of DNA replication is a tightly controlled event such that the chromosomes are replicated once per cell cycle. In contrast, many DNA viruses have evolved replicative mechanisms to escape such stringent controls and allow autonomous replication of the viral genome. This can be achieved by specifying a replicative machinery that is distinct from the host cell (e.g. adenoviruses), or by synthesising proteins that preferentially recognise and

direct replication of the viral DNA from a specific origin sequence using host-cell enzymes (e.g. SV40).

The development of origin-dependent *in vitro* DNA replication in several systems e.g. *E.coli* (Fuller *et al.*, 1981), bacteriophage lambda (Wold *et al.*, 1982) and SV40 (Li and Kelly, 1984) has demonstrated that, whether prokaryotic or eukaryotic, these systems have a common pathway for initiating DNA replication. This pathway can be envisaged as a three step process:

(1) a protein recognises and binds to a specific sequence within the origin of replication (initial complex)

(2) this complex leads to the melting of a small stretch of duplex DNA within the origin (open complex)

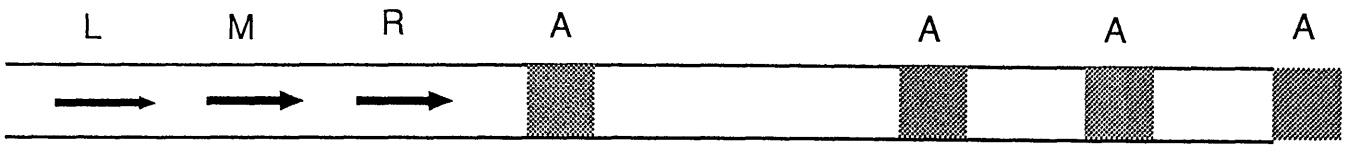
(3) a helicase activity extends the primary replication bubble (pre-priming complex) which marks the site at which the synthesis of daughter strands is initiated.

Examples of well-characterized proteins that are required to mediate steps 1 and 2 are the *E.coli* dnaA protein, the bacteriophage lambda O protein and SV40 T antigen.

1. *E.coli dnaA* initiation protein

DNA replication of the *E.coli* chromosome initiates at a unique site called oriC. The minimal oriC consists of a 245 bp DNA sequence which contains all the *cis*-acting information required for autonomous replication (Fig. 1.10(a); Oka *et al.*, 1980). Mutational analysis of this sequence has revealed two essential sequence elements; protein recognition elements and spacer elements that maintain a required distance between the protein recognition elements (Oka *et al.*, 1984). The boundary at the right end of oriC coincides with the end of the rightmost of four 9 bp elements (dnaA boxes) bound by the *E.coli* dnaA protein {Fig. 1.10(a)}. The leftmost 60 bp of oriC contains three tandem repeats of a 13 bp motif (L-left, M-middle, R-right) which are highly conserved among the Enterobacteriaceae.

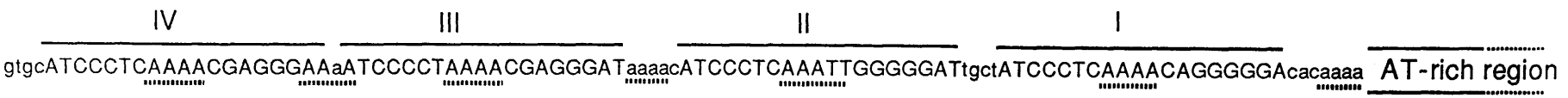
(a) E.coli minimal oriC



13 mer consensus sequence GATCTnTTnTTTT

dnaA box consensus sequence TTATC(C/A)A(C/A)A

b) bacteriophage lambda origin



(C) SV40 core origin

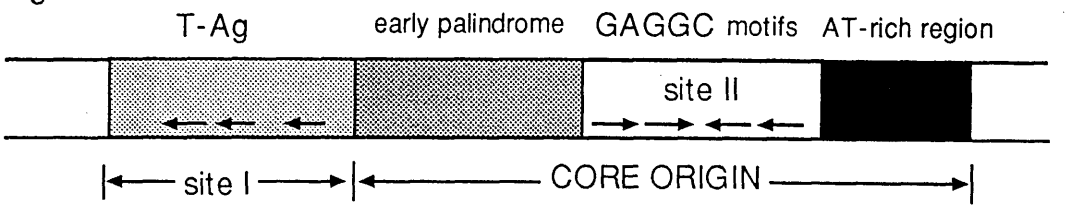


Figure 1.10 Features of the replication origins of E.coli, bacteriphage lambda and simian virus 40

(a) The E.coli minimal oriC region. The 13 mers of the AT-rich region (left [L], middle [M], right [R]) are shown by large solid arrows, with the consensus sequence shown below. The dnaA protein binding sites are shown by shaded boxes (dnaA A boxes) with the consensus sequence shown below.

(b) Part of the sequence of the bacteriophage lambda origin of replication. Overlined and capitalised regions I to IV signify the location and sequence of the bacteriophage lambda O protein binding sites located at the left hand side of the AT-rich region of the origin. Stippled underlined dA runs, four of which are located in the O protein binding sites, are proposed to be responsible for DNA bending (Zahn and Blattner, 1984).

(c) The SV40 core origin. The T antigen binding sites I and II, the early palindrome and AT-rich tract are marked. The orientation and approximate position of the GAGGC motifs in binding sites I and II are indicated by short arrows within the boxed regions.

Studies on the activity of the dnaA protein have established that it performs three crucial roles in initiating DNA replication. Firstly, the dnaA protein binds tightly to each of the four dnaA boxes in oriC (initial complex; Fuller and Kornberg, 1983; Fuller et al., 1984). A dnaA-oriC complex is then formed which consists of negatively supercoiled oriC DNA wrapped around a central core of 20-40 dnaA protein monomers (Funnell et al., 1987; Baker and Kornberg, 1988).

Secondly, in the presence of the protein HU (a histone-like DNA binding protein; Dixon and Kornberg, 1983) the dnaA protein bound to oriC, successively melts the three AT-rich 13 mer repeats to form an open complex, as evidenced by this region becoming susceptible to single-stranded nuclease digestion. Formation of this open complex requires bound ATP. The dnaA protein can stably bind to both ATP and ADP, slowly hydrolysing the former to the latter, but the ATP bound form alone has been shown to be active in oriC replication (Sekimizu et al., 1987).

Thirdly, the dnaA protein guides the dnaB-dnaC complex into this melted region to form a pre-priming complex. The origin DNA must unwind prior to actual initiation of DNA synthesis to allow the replication machinery access to this region in order to copy the individual DNA strands. The dnaB helicase is required to unwind DNA ahead of the replication fork (Baker et al., 1986,1987; Dodson et al., 1986). This protein is unable to initiate unwinding of a closed duplex molecule (LeBowitz and McMacken, 1986) hence the requirement for an open complex formed through the action of dnaA protein.

The mechanism by which the dnaA protein forms the open complex is not totally clear. The repeated 13 mer region which, following binding of dnaA to oriC, becomes hypersensitive to nuclease activity, has a nucleotide composition that naturally confers a low free energy cost for DNA unwinding i.e. reduced helical stability over this region in the absence of replication proteins. However, the AT-rich nature of this region alone is not responsible for the formation of an open complex as other extended AT-rich regions in oriC are not sensitive to single-strand-specific nucleases. Thus, the energetic requirement for unwinding

this sequence is dependent on base sequence as well as composition (Kowalski and Eddy, 1989). It has been postulated that dnaA therefore recognises part of the R-13 mer which could then concentrate unwinding of the supercoiled template to the remaining non-contacted 13 mers by virtue of the reduced helical stability of the region. This model accounts for the specific duplex opening of oriC without requiring dnaA interaction with all of the 13 mers and is consistent with mutational studies of oriC which showed that the R-13 mer but not the L and M-13 mers are required for dnaA/HU induced opening of oriC (Bramhill and Kornberg, 1988). In addition, the R-13 mer sequence and spacing from the leftmost dnaA box is completely conserved amongst all known Enterobacteriaceae origins whereas the L and M-13 mers have tolerated base substitutions. The L-13 mer can be replaced by a dissimilar DNA sequence with reduced helical stability (Asada et al., 1982; Kowalski and Eddy. 1989). Hence, the 13 mer repeat region is a complex genetic element that plays at least two essential overlapping roles; protein recognition by dnaA and confering helical instability.

In addition to its key role in initiating replication at oriC, dnaA may play some role in modulating its own transcription. Schaefer and Messer (1989) demonstrated that the presence of a dnaA box site in the middle of the open reading frame of the dnaA gene can lead to a transcriptional block of the dnaA gene as well as downstream gene expression. The alteration of a single base within the box reduces its ability to terminate transcription as seen by the corresponding overproduction of the dnaA protein. A dnaA box and 13 mer sequence are also found in the dnaA gene promoter region leading to autoregulation of the gene by dnaA.

2. Bacteriophage lambda O protein

Bacteriophage lambda initiates bidirectional replication at a unique site in the phage genome. The lambda origin contains two essential domains. The left domain consists of four 18 bp repeating units (iterons) of hyphenated dyad symmetry and the right domain adjacent to

the iterons contains a 49 bp region of high AT-content (71%) with almost all purines lying on the same strand {Schnos and Inman, 1970; Fig. 1.10(b)}.

Replication of phage is dependent on two phage-encoded proteins O and P and, except for dnaA and dnaC proteins, all of the replicative proteins of E.coli (Eisen et al., 1966; Friedman et al., 1984).

The O protein is regarded as the functional analogue of the dnaA protein. It binds, most likely as a dimer, to each iteron forming a characteristic DNA protein structure termed the 'O-some' which consists of O protein molecules with the DNA wrapped around (initial complex). Due to the tracts of adenosine residues between and within each iteron, the origin DNA is naturally bent in solution. Binding of the O protein induces further bending of this region (Zahn and Blattner, 1985a,b). The lambda origin/O protein complex differs from the dnaA/oriC complex in that it does not require bound ATP for helix destabilisation of the AT-rich region (Schnos et al., 1988). Binding of the O protein renders the AT-rich region sensitive to single-strand-specific nuclease digestion indicating the formation of an open complex. The modification of the DNA is absolutely dependent on supercoiled tension at the origin. The mechanism by which the free energy stored in the supercoiled DNA and/or statically bent origin is transferred into a localised and asymmetric strand separation remains to be investigated (Schnos et al., 1988; Dodson et al., 1989).

The O protein at the lambda origin interacts with P protein (analogous to dnaC; Furth et al., 1978) via its carboxy terminus (Wicker and Zahn, 1986) and the P protein in turn interacts with the dnaB helicase, dnaJ and dnaK proteins of E.coli (Friedman et al., 1984). In the presence of single-stranded DNA binding proteins and ATP, the origin is unwound by dnaB helicase. Similarily to oriC of E.coli, the binding of the O protein is the catalyst for this reaction as the dnaB helicase can only function on pre-formed open complexes (LeBowitz and McMacken, 1986). Functional studies have demonstrated that the AT-rich domain of the origin is indeed the target for entry and initial action of dnaB (Dodson et al., 1986).

3. SV40 T antigen

SV40 has proved to be an excellent model system for the study of eukaryotic mechanisms of DNA replication. The viral genome consists of a 5.2 kbp circular double-stranded DNA molecule that is complexed with histones to form a minichromosome, indistinguishable from cellular chromatin. Since SV40 encodes a single replication protein, T antigen, the virus uses much of the cellular replicative machinery (Tegtmeyer, 1972).

The primary step in initiation of SV40 DNA replication is the binding of T-ag to sequence elements within the origin. The SV40 T antigen (T-ag) is a multifunctional 82-kD phosphoprotein that catalyses pre-synthesis reactions at the SV40 origin and contains an ATP-dependent DNA helicase activity (Stahl et al., 1986). The protein binds strongly to two sites, I and II, shown in Fig. 1.10(c). Site II is located within the 64 bp sequence which marks the core origin. The pentameric motif 5'-G(A/G)GGC-3' which serves as the recognition site for T-ag is found in sites I and II although the number and orientation of the motifs and their spacing differ at each site. Site II contains four motifs arranged in a 27 bp perfect inverted repeat with two motifs on each arm of the palindrome (Tjian, 1978; DeLucia et al., 1983). At least one T-ag monomer can bind to each pentamer (Mastrangelo et al., 1985).

Through detailed analysis of base substitution mutations, three domains within the core origin have been identified as crucial for replication; the central GAGGC elements, a 10 bp region overlapping an imperfect inverted repeat (early palindrome) and a 17 bp AT-rich region (Fig.1.10(c) Deb et al., 1986a). Fragments containing the AT-rich region exhibit naturally occurring stable bends in the DNA (Deb et al., 1986b).

DNase I footprinting assays show that in the presence of ATP, T-ag covers the entire core origin sequence on all faces of the DNA helix (Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988a). EM studies have shown that the protein is arranged as a two-lobed structure at the core

origin which contains twelve monomers of protein. In the absence of DNA, ATP can cause T-ag to aggregate in hexameric clusters (Mastrangelo et al., 1989) which suggests that each lobe of T-ag bound to DNA contains six T-ag monomers.

The ATP-dependent binding of T-ag to core origin induces structural changes in the early palindrome region and the AT-rich element (Borowiec and Hurwitz, 1988b). Surprisingly, the early palindrome, rather than the AT-rich element appears to be the initial site of DNA melting. Parsons et al. (1990) demonstrated that high levels of T-ag can cause significant melting at this element in the absence of the other two elements. The sequence of the early palindrome is strongly biased toward purines on one strand and pyrimidines on the other. As these sequences are inherently unstable (Wells, 1988) this may facilitate melting in this region. Melting of the early palindrome does not require energy as ADP and nonhydrolysable analogues can effectively sustitute for ATP. This T-ag activity is distinct from its pentanucleotide binding, as the early palindrome does not contain GACCG motifs, and also its helicase activity which is energy dependent (Borowiec and Hurwitz, 1988b; Stahl et al, 1986; Parsons et al., 1990).

After formation of this open complex, the origin becomes completely denatured and the T-ag helicase function unwinds the duplex DNA in a bidirectional manner. In vitro, this unwinding reaction requires a cellular single-stranded DNA binding protein to maintain extensive regions of single-stranded DNA (Wobbe et al., 1987; Fairman and Stillman, 1988).

The requirement for a third factor, identical to the catalytic subunit of cellular protein phosphatase 2A ($PP2A_c$) has recently been demonstrated when using T-ag isolated from cells infected with a recombinant adenovirus vector expressing T-ag (Virship et al., 1989). The purified $PP2A_c$ dephosphorylates specific phosphoserine residues, consistent with the hypothesis that SV40 DNA replication is regulated by modulating the phosphorylation state of T-ag (McVey et al., 1989).

To summarise the key requirements for initiation of DNA replication in these three systems; each origin sequence has a lengthy AT-rich tract and multiple sequence repeats

that bind multiple units of an origin-binding protein forming an initial multimeric complex. The binding of the proteins causes structural changes in the origin region forming an open complex. These complexes, in conjunction with other replication proteins e.g. helicases and single-stranded DNA binding proteins, lead to regions of highly underwound DNA ultimately allowing entry and activity of the polymerising enzyme.

There are of course notable differences between the three systems. Firstly, while the dnaA and lambda O proteins are wrapped by DNA, in SV40, the DNA is surrounded by T-ag. Secondly, origin-binding and helicase functions are encoded by separate genes in E.coli and lambda whereas SV40 T-ag can carry out both functions. Thirdly, the AT-rich region in E.coli and lambda serves as the initial melting site. In SV40, a polypurine-polypyrimidine tract is the first region to be separated. SV40 T-ag does not require supercoiled DNA to form a pre-priming complex but can function effectively on linear DNA templates.

Although our knowledge of the initiation of HSV DNA synthesis has not reached the levels of sophistication exemplified by the above systems, several analogies can be drawn. For example, HSV has internal origins of DNA replication and these contain DNA palindromes, lengthy AT-rich tracts and sequence-specific recognition sites (Stow and McMonagle, 1983; Weller et al., 1985; Stow and Davison, 1986; Elias et al., 1986). The work described in this thesis aimed to provide further relevant information by identifying the HSV-1 encoded origin binding protein and investigating the role of its interaction with the origin in HSV DNA synthesis.

CHAPTER 2: MATERIALS AND METHODS

SECTION 2A: Materials

1. Chemicals

Chemicals were obtained from either BDH Ltd. or Sigma Chemical Co. Ltd. except for the following:

Beecham Research Labs. Ltd.: ampicillin (penbritin); Bio-Rad Labs. Ltd.: ammonium persulphate, TEMED [N,N,N'N' tetramethylethylenediamine], Bio-Rad protein assay dye reagent concentrate; Boehringer Mannheim: proteinase K, tris-[tris(hydroxymethyl-aminomethane)]; Du Pont UK Ltd.: En^{3}hance autoradiography enhancer; Fluka Chemicals Ltd.: HPLC grade dimethyl-sulphoxide, formamide, piperidine; James Burrough (FAD) Ltd.: absolute alcohol 100; Koch-Light Labs.: boric acid, caesium chloride; May and Baker Ltd.: acetic acid (glacial), chloroform, glycerol, hydrochloric acid; Pharmacia LKB Ltd.: DEAE-sephacel, ficoll 400, 2'-deoxyribonucleoside 5'-triphosphates, ribonucleoside 5'-triphosphates, gene 32 protein, ultra-pure dNTP set, `universal' and T7 promoter sequencing primers, diguanosine triphosphate sodium salt [G(5')ppp(5')G].

2. Miscellaneous Materials

dialysis membrane	Medicell International Ltd
nitrocellulose BA85	Schleicher and Schuell
rabbit reticulocyte lysate (N90)	Amersham International plc.
RNasin	Promega
SDS-7B pre-stained molecular weight markers	Sigma Chemicals Co. Ltd.
tissue culture plastics	Gibco Ltd. and Sterilin Ltd.
Trypsin (0.25% w/v)	Gibco Ltd.
wacker silane GF 38	Wacker Chemicals
X-ray film for autoradiography (XS1)	Kodak Ltd.

3. Enzymes

Restriction enzymes were manufactured by Bethesda Research Labs. and Northumbria Biologicals Ltd. Calf intestinal phosphatase was manufactured by Boehringer Mannheim, T4 kinase by New England Biolabs and T7 DNA polymerase by Pharmacia LKB Ltd.

4. Radiochemicals

Radiolabelled compounds were supplied by Amersham International plc.

	specific activity
(a) [^{35}-S]-L-methionine	around 800 mCi/mmol
(b) 5'[α-^{32}P]-deoxyribonucleoside triphosphates	3000 Ci/mmol (10 uCi/ul)
(c) 5'[α-^{32}P]-adenosine triphosphate	5000 Ci/mmol (10 uCi/ul)
(d) 5'[α-^{35}S]- deoxyadenosine thiotriphosphate	>1000 Ci/mmol (10 uCi/ul)

5. Synthetic oligonucleotides

These were synthesised using a model 8600 biosearch DNA synthesiser by Dr. J. McLauchlan. A list of the oligonucleotides used during the course of this study are given in Table 2.1.

6. Plasmids

(a) Cloning vectors

The pTZ range of phagemid expression vectors pTZ 19U, pTZ19R, pTZ18U and pTZ18R (Mead et al., 1986) were obtained from Dr. R. M. Elliott and are shown in Fig. 2.1. These plasmids were used for cloning, production of single-stranded DNA for site-directed mutagenesis and in vitro transcription.

Table 2.1 Double-stranded oligonucleotides used in this study

Oligonucleotide I :

5' GATCCGCGAAGCGTTCGCACTTCGTCCCA
GCGCTTCGCAAGCGTGAAGCAGGGTCTAG 5'

Oligonucleotide II :

5' GATCTGGGGCGAAGTGCGAGCACTTCGCG
ACCCCGCTTCACGCTCGTGAAGCGCCTAG 5'

Oligonucleotide III :

5' GATCTAAAAGAAGTCAGAACGCGA
ATTTTCTTCAGTCTTGCGCTCTAG 5'

Oligonucleotide pmI :

5' GATCCGCGAAGCGTTCTCACTTCGTCCCA
GCGCTTCGCAAGAGTGAAGCAGGGTCTAG 5'

Oligonucleotide X :

5' GATCGGATATGCTAATTAAATACAT
GCCTATACGATTAATTTATGTA 5'

pTZ18R and pTZ19R Multifunctional Phagemids

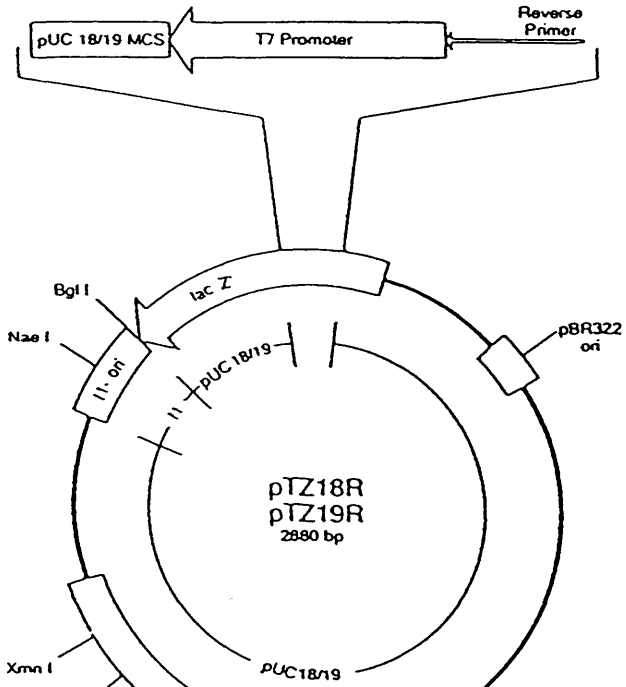


Figure 2.1 Diagram of the pTZ range of phagemid expression vectors

The key features of these plasmids include a multiple cloning site from pUC18/19, an ampicillin resistance gene, a lacZ gene, an f1 origin of replication for the production of single-stranded DNA and a T7 RNA polymerase promoter. Vectors pTZ18R and pTZ19R are identical except for the multiple cloning site (MCS) being in the reverse orientation in pTZ19R. Vectors pTZ18U and pTZ19U (not shown) are identical to pTZ18R and pTZ19R respectively, except the f1 origin is in the reverse orientation.

(b) Plasmids containing HSV-1 replication genes and IE-3 and IE-1 genes

Four plasmids containing HSV-1 (Glasgow strain 17) replication genes were obtained from Institute stocks and are as follows:

pGX25 - BamHI c fragment containing UL5 + UL8 cloned into pATl53 (Twigg and Sherratt, 1980)

pGXl25 - KpnI f fragment containing UL8 + UL9 cloned into pMKl6 (Kahn et al., 1979)

p9-4 - (derived from pGXl84) HindIII l fragment containing the UL42 gene cloned into pUC9 (Vieira and Messing, 1982) was obtained from Dr. C. MacLean

pGX94 - XhoI b fragment containing the UL52 gene cloned into pATl53

Plasmid pGX58 consists of the HSV-1 XhoI c fragment containing the IE-3 gene cloned into pMKl6. Plasmid pJR3 (obtained from Dr. J. Russell) consists of a PstI + SstI fragment containing the IE-1 gene cloned into a pUC9 derivative.

Plasmids pNNl and pNN3 were kindly provided by Dr. M. D. Challberg (Challberg, 1986). Both plasmids were constructed from a starting plasmid, pMCll9, which consists of the HpaI b fragment of HSV-1 DNA cloned into pUCl9. pNNl contains the UL29 gene encoding the major single-stranded DNA binding protein and pNN3 the UL30 gene, encoding the viral DNA polymerase.

(c) Plasmids containing HSV-1 and VZV ori_S sequence

Plasmid pSl consists of a 535 bp DNA fragment from the HSV-1 BamHI x fragment which contains a functional copy of ori_S, cloned into pATl53 (Stow and McMonagle, 1983).

Plasmid pSl9 was generated by deletion of pSl and consists of a 100 bp fragment which retains ori_S activity (Stow and McMonagle, 1983).

Plasmid pVO2 consists of a 259 bp fragment produced by RsaI plus ClaI cleavage of pVKr (nucleotides 5502 to 5760

of the TR_S/IR_S sequence of Davison and Scott, 1985) cloned between the EcoRI and ClaI sites of pAT153 following the ligation of an EcoRI linker to the RsaI terminus. This plasmid contains a functional copy of VZV ori_S (Stow and Davison, 1986).

(d) Plasmids containing segments of the UL9 gene

To express parts of the UL9 gene as fusion proteins in Escherichia coli, three plasmids containing in-frame segments of the UL9 gene were constructed by Dr. N. D. Stow. and are shown in Fig. 2.2. The parental vector, pRIT2T, was supplied by Pharmacia LKB Ltd. (Nilsson et al., 1980). An additional plasmid was constructed, pRIT2T-4, in which the EcoRI site in the multiple cloning region of pRIT2T was replaced by an ApaI site and a 4 bp deletion introduced. Plasmid pX8 encodes the N-terminal 10-524 amino acids of the UL9 gene cloned into pRIT2T-4, pB1 encodes the C-terminal 317 amino acids cloned into pRIT2T-4 and pP31 encodes the C-terminal 591 amino acids cloned into pRIT2T.

7. Cells

BHK 21 clone 13 (BHK) cells, a continous line derived from baby hamster kidney (MacPherson and Stoker, 1962) were routinely used for the growth of virus, preparation of nuclear extracts and plasmid amplification assays.

8. Viruses

HSV-1 Glasgow strain 17syn$^+$, HSV-1 strain KOS and HSV-2 strain HG52 were obtained from Institute stocks. HSV-1 strain 17syn$^+$ was the wild type virus used in most experiments. Seed stocks of HSV-1 DNA negative ts mutants were obtained from Mrs. M. Murphy and Dr. V. G. Preston (Table 2.2).

HSV-1 recombinant viruses tsK/UL5, tsK/UL8, tsK/UL9 and tsK/UL52 were obtained from Janice Calder in our laboratory. These viruses contain additional copies of the early genes UL5, UL8, UL9, and UL52 inserted under the control of the IE 3 promoter within the TK gene of tsK (Calder and Stow, unpulished results).

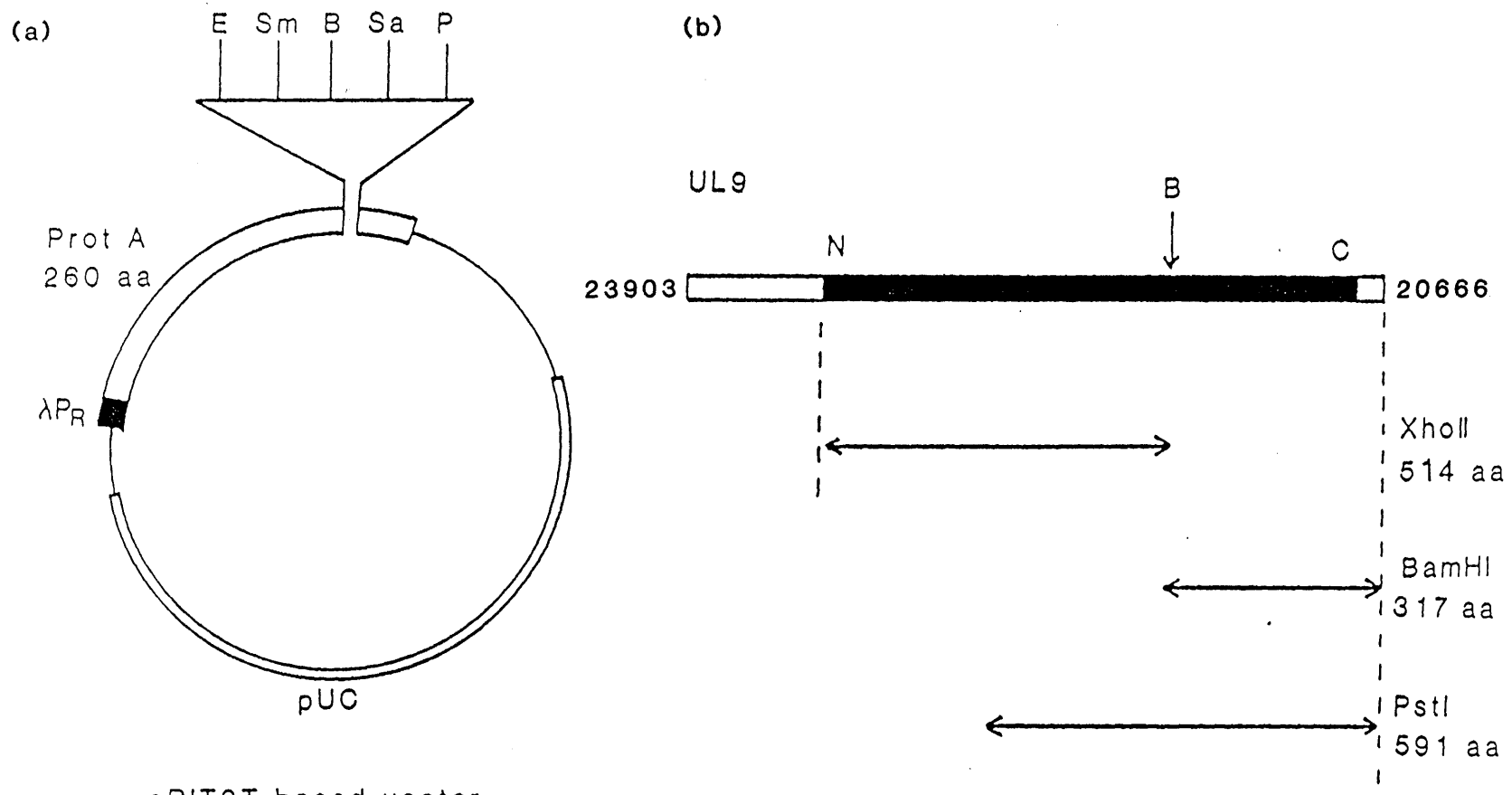

(a)
E
Sm
B
Sa
P
Prot A
260 aa
λP_R
pUC
pRIT2T based vector
(b)
UL9
B
N
C
23903
20666
XhoII
514 aa
BamHI
317 aa
PstI
591 aa

Figure 2.2 Construction of plasmids containing fragments of the UL9 gene for expression in E.coli

(a) Structure of plasmid pRIT2T. pRIT2T-4 is essentially similar except the EcoRI site in pRIT2T is replaced by an ApaI site (Weir et al., 1989). The positions of the phage lambda promoter, P_R, and coding sequences for the N-terminal region of the Staphylococcus aureus A protein are indicated.

(b) Fragments inserted into vectors. The upper line shows the SstI fragment containing the UL9 gene which was initially cloned into the EcoRI site of pUC8. The N- and C-terminal regions of the coding sequences (black bar) are indicated. Plasmids pX8, pB1 and pP31 contain the indicated fragment inserted in frame and in the appropriate orientation into pRIT2T-4, pRIT2T-4 and pRIT2T respectively. The C-terminal ends of the pB1 and pP31 inserts lie within the pUC8 polylinker. The nucleotide numbering is from McGeoch et al. (1988).

Table 2.2 HSV-1 ts mutants used in this study

ts mutant	lesion in:	complementation gp	References
tsK	Vmw175	1-2	3,4
tsO	UL5	1-10	5
tsS	UL9	1-36	1,2
ts1205	UL30	1-3	5
ts1206	UL52	1-37	5
ts1234	UL42	1-42	6
ts1235	UL29	1-1	6

References

1. Marsden et al. (1976)
2. Stow and Wilkie, (1978)
3. Preston, (1979a)
4. Preston, (1981)
5. Matz et al. (1983)
6. V.G. Preston (unpublished)

9. Tissue culture media

BHK cells were grown in Glasgow modified Eagle's medium (GMEM; Busby et al., 1964) supplemented with 10% new born calf serum, 10% tryptose phosphate broth, 100 units/ml penicillin and 100 ug/ml streptomycin (ETC10).

The following modified tissue culture media were also used:

GMEM wash	GMEM containing antibiotics only
ECx	GMEM containing x% calf serum
ECxHuy	GMEM containing x% calf serum y% human serum

10. Bacterial strains and bacteriophage

Three bacterial strains of E.coli were used: DH-1 (recA1, nalA, r_R^-, m_R^-, endoI$^-$, B$^-$, relA1 ; Hanahan, 1983), K12ΔHIΔtrp (Bernard et al., 1979) and BW313 (dut$^-$, ung$^-$, thi$^-$, relA1, spoT1, HfrKL16 ; Kunkel, 1985). The bacterial strain BW313 and the bacteriophage M13 R408 (Russel et al., 1986) were provided by Dr. R. Thompson.

11. Bacterial culture media

The following solutions were used:

L-Broth	10 g/l NaCl, 10 g/l Bactopeptone 5 g/l yeast extract
L-Broth agar	L-Broth plus 1.5% w/v agar
2 x YT broth	5 g/l NaCl, 16 g/l Bactopeptone 10 g/l yeast extract

Ampicillin, where appropriate, was added to L-Broth and L-Broth agar at 50 ug/ml.

12. Antisera

Rabbit antisera raised against decapeptides from

the C-termini of the UL5, UL8, UL9 and UL52 gene products and reactive with the corresponding polypeptides were kindly provided by Dr. M. D. Challberg (Olivo et al., 1988).

13. Commonly used solutions

50 x Denhardt's solution	1% polyvinylpyrrolidone, 1% BSA 1% Ficoll
Giemsa stain	1.5% Giemsa in glycerol, heated to 50°C for 2 h and diluted with an equal volume of methanol
Gel soak I	200 mM NaOH, 600 mM NaCl
Gel soak II	1 M Tris, 600 mM NaCl, pH adjusted to 8.0 with HCl
Hybridisation buffer	6 x SSC, 5 x Denhardt's solution, 0.05% SDS, 50 ug/ml denatured calf thymus DNA, 20 mM Tris.HCl (pH 7.5), 1 mM EDTA
Loening's buffer	36 mM Tris, 36 mM $NaH_2PO_4.2H_2O$, 1 mM EDTA
PBS A	170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.2)
PBS	PBS A supplemented with 6.8 mM $CaCl_2$ and 4.9 mM $MgCl_2$
Prehybridisation buffer	6 x SSC, 5 x Denhardt's solution, 0.1% SDS, 20 ug/ml denatured calf thymus DNA
STET buffer	8% (w/v) sucrose, 0.5% (v/v) TritonX-100, 50 mM Tris.HCl (pH 8.0) 50 mM EDTA
20 x SSC	3 M NaCl, 3 M trisodium citrate
TBE	90 mM Tris.HCl, 89 mM boric acid, 1 mM EDTA
TE	10 mM Tris (pH 8.0), 1 mM EDTA
TEN	TE plus 100 mM NaCl
versene	0.6 mM EDTA in PBS A plus 0.02% (w/v) phenol red

SECTION 2B: Methods

1. Cell culture

BHK cells were grown in ETC10 medium at 37°C in an atmosphere of 5% CO_2 (MacPherson and Stoker, 1962). Cultures were passaged every two to three days. Cells from confluent monolayers (850 cm^2 roller bottles) were harvested in 20 ml ETC10 after two versene washes and brief trypsinisation (0.25% trypsin) and remained viable for up to 5 days at 4°C.

2. Virus stocks

(a) production of virus stocks

Stocks of wt HSV-1 and HSV-1 ts mutants were propagated in BHK cells. Cell monolayers in glass roller bottles were infected with virus at a moi of 0.01 pfu per cell in 50 ml EC5. Infected cultures were maintained at 31°C for 3-4 days until the appearance of extensive cytopathic effect. The bottles were shaken vigorously to dislodge the cells into the medium. Cells were spun from the medium at 2,000 rpm (Sorval GS3 rotor) for 5 min, resuspended in 5 ml of EC5, sonicated in a Cole Palmer ultrasonic bath to release the virus and centrifuged at 2,000 rpm (Sorval RT6000B in universals) for 5 min at 4°C to remove cell debris. Cell-associated virus (CAV) in the supernatant was stored in aliquots at -70°C. The medium from infected cells was centrifuged at 12,000 rpm (Sorval GSA rotor) for 2.5 h at 4°C and the pellet of cell-released virus (CRV) resuspended in a small volume of EC5, sonicated and stored at -70°C. Sterility checks were performed on CAV and CRV stocks using blood agar plates.

(b) titration of virus

All virus stocks were titrated on BHK cell monolayers in 50 mm or 35 mm tissue culture dishes. Serial 10-fold dilutions of virus were made in GMEM wash and 0.1 ml samples of each dilution were inoculated onto cell monolayers from which the growth medium had been removed.

Following an absorption period of 1 h at permissive temperature (PT; 31°C for ts mutants; 31°C or 37°C for wt HSV), the cells were overlaid with EC2Hu5 to neutralise unadsorbed virus and prevent the formation of secondary HSV plaques. Incubation was continued at the appropriate temperature (2 days at 37°C, 3 days at 31°C). An equal volume of Giemsa stain was added to the medium for several minutes. The stain was washed off and the viral plaques, clearly visible under a dissecting microscope were counted.

3. Preparation of HSV-1 DNA

HSV-1 DNA was prepared essentially as described by Wilkie (1973). Infected cells were pelleted as described in 2(a). and resuspended in RSB buffer (10 mM Tris.HCl (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$) containing 0.5% Nonidet P40 and placed on ice for 10 min. Nuclei were removed by centrifugation at 2,000 rpm (Sorval GSA rotor) for 5 min at 4°C and the viral particles in the resulting supernatant (CAV) were pelleted at 12,000 rpm (Sorval GSA rotor) for 2.5 h at 4°C. CRV was pelleted from the growth medium as described in 2(a). The virus pellets from CAV and CRV were resuspended in TE and 1/25 vol of 250 mM EDTA and 1/40 vol of 20% SDS added to disrupt the particles. DNA was then sequentially extracted by gentle mixing, twice with phenol, once with phenol/chloroform (1:1) and once with chloroform. DNA was precipitated by the addition of 1/20 vol of 5 M NaCl and 2.5 vols of ethanol, pelleted, washed twice with ethanol lyophilised and resuspended overnight in TE.

Viral DNA was further purified by caesium chloride (CsCl) equilibrium gradient centrifugation. 1.25 g of CsCl was added for each ml of solution, to give a final density of 1.72 g/ml, dissolved by gentle inversion and the sample centrifuged in sealed plastic tubes at 40,000 rpm (Beckman Type 65 rotor) 48 h at 15°C. The bottom of each tube was punctured with a syringe needle and fractions collected in Linbro wells. 2 ul of each fraction were analysed on a mini-gel and fractions containing viral DNA were pooled and dialysed against TE for 3-4 h at 4°C. The viral DNA was subsequently stored at -20°C.

4. Gel electrophoresis

(a) non-denaturing agarose gel electrophoresis

Analysis and preparation of DNA restriction fragments used horizontal, 0.8% agarose gels in TBE or Loening's buffer containing 0.5 ug/ml of ethidium bromide. DNA samples were mixed with a solution of 5 x SB dyes {5 x TBE or Loenings buffer, depending on the electrophoresis buffer used, 50% sucrose and 0.2% bromophenol blue(BPB)}. After loading samples, gels were electrophoresed at 100 V for 3 h (TBE gels) or 30 V overnight (Loening's buffer gels).

For a rapid assessment of small amounts of DNA, mini-gels, consisting of 20 ml of 1% agarose were run in TBE at 50 V for approximately 30 min. In both cases, DNA bands were visualised by exposure to U.V. light and photographed on type 667 Polaroid film.

(b) non-denaturing polyacrylamide gel electrophoresis

To analyse or purify labelled DNA fragments, vertical slab gels were poured without a stacking gel using appropriate concentrations of acrylamide in TBE. The acrylamide stock solution used was normally 29:1 acrylamide:N,N'-methylene bisacrylamide and final solutions (50 ml) containing 8% polyacrylamide in TBE were polymerised with 200 ul of 25% (w/v) ammonium persulphate solution (APS) and 20 ul of TEMED.

(c) non-denaturing polyacrylamide gels for size estimation of protein-DNA complexes

Gels were set up exactly as described by van Huijsdnen *et al*. (1987). 20 ml of 25% and 5% acrylamide solutions were prepared using stock 30% acrylamide (28.5:1.5 acrylamide:bisacrylamide) in 0.5 x TBE with the 25% polyacrylamide solution also containing 3% sucrose. Linear 5-25% gels were poured using a gradient maker. The gels were run at 150 V for 36-48 h at 4^{o}C. After the run, the gel was fixed in 3.5% perchloric acid, dried and exposed to autoradiography film (Kodak XS1) at -70^{o}C.

(d) denaturing polyacrylamide gels

Sequencing gel mix (6% acrylamide, 8.3 M urea in 0.5 x TBE) was prepared as follows: 460 g of urea were dissolved in 150 ml of 40% acrylamide stock (acrylamide:N,N'-methylene bisacrylamide 19:1, previously deionised by stirring with 10 g of amberlite for 30 min and filtered) and 50 ml of 10 x TBE and adjusted to 1 l with H_2O. This solution was then stored at 4°C in the dark for up to one month. To polymerise the gel, 72 ul each of 25% APS and TEMED were added to 60 ml of sequencing gel mix. Gels were poured between sealed plates separated by 0.35 mm spacers, using a 25 ml syringe. Once set, the wells were rinsed throughly in 0.5 x TBE using a 10 ml syringe with needle. DNA samples were boiled for 2-3 min in 95% deionised formamide, 0.05% BPB and 0.05% xylene cy anol, 20 mM EDTA (pH 7.5) and 2-3 ul immediately loaded using a Hamilton syringe. Gels were electrophoresed at 40 W until the BPB reached the bottom of the gel (approximately 1.5-2 h) and the siliconised notched front plate removed. Gels were then soaked in 10% acetic acid for 30 min to remove urea, before drying onto the 'wackered' back glass plate at 80°C. Dried gels were exposed to autoradiography film at -70°C.

(e) SDS-PAGE polyacrylamide gel electrophoresis

SDS-PAGE polyacrylamide gel electrophoresis was carried out essentially as described in Marsden et al. (1976). Stocks of 30% acrylamide were prepared in water, with a ratio of acrylamide to N,N'-methylene bisacrylamide (cross-linking reagent) of 28.5:1.5 and 29.25:0.75. For the resolving gel mix (40 ml) solutions containing the appropriate percentage of acrylamide (normally 8% of 29.25:0.75) were prepared in a buffer of final concentration 375 mM Tris.HCl (pH 8.9), 0.1% SDS. To polymerise the gel solution, 0.6 ml of 10% APS and 40 ul of TEMED were added and the mixture was then poured into a sandwich of glass plates (including one notched plate) to within 3-4 cm from the top of the notched plate. Butan-2-ol was gently layered on top of the acrylamide solution and the gel allowed to polymerise. Following polymerisation, the butanol was rinsed off using unpolymerised stacking gel solution. Once removed, a 25 ml stacking gel solution consisting of 5%

acrylamide (28.5:1.5) in 122 mM Tris.HCl (pH 6.7), 0.1% SDS, to which had been added 200 ul of 10% APS and 10 ul TEMED, was prepared and layered on top of the resolving gel. Wells were formed using a teflon comb. Protein samples were boiled for 5 min in sample buffer at a final concentration of 50 mM Tris.HCl (pH 6.7), 2% SDS, 700 mM 2-mercaptoethanol and 10% glycerol with sufficient BPB to enable visualisation of a dye front. Electrophoresis was carried out in tank buffer (52 mM Tris, 53 mM glycine and 0.1% SDS) at 10-12 mA overnight.

Following electrophoresis, gels were fixed for 45 min in methanol:acetic acid:water (50:7:50), before gently shaking for 45 min in 3 vols En^{3}hance and then washed for 5 min in 5 vols of H_2O. The gel was then heat-dried under vacuum onto Whatman filter paper and exposed to autoradiography film at -70^{o}C.

5. Preparation of plasmid DNA

(a) growth of bacterial cultures

Overnight cultures of bacteria were set up using either 20 ul of a bacterial stock (stored in 50% glycerol or 7% DMSO) or from a single colony picked from an agar plate, and allowed to stand or shake overnight at 37^{o}C in 5 ml of L-broth containing 50 ug ampicillin, where appropriate.

(b) large scale preparation and purification of plasmid DNA

The propagation and purification of supercoiled plasmid DNA on caesium chloride/ethidium bromide gradients were essentially as described by Davison and Wilkie (1981). An overnight culture was transferred to a 1 l flask containing 400 ml of L-broth (containing 50 ug/ml of ampicillin where appropriate) and shaken at 37^{o}C until the optical density (O.D) had reached approximately 0.6 at 630 nm. Plasmid DNA was amplified by adding 1 ml of a chloramphenicol solution (34 mg/ml in absolute alcohol) to the culture and continuing shaking overnight before harvesting.

Bacterial cells were pelleted by centrifugation at 8,000 rpm (Sorval GS3 rotor) for 10 min, washed once by resuspending the pellet in 10 ml of TE and then pelleted

again at 5,000 rpm (Sorval SM24 rotor) for 5 min. The pellet was resuspended in 2 ml of 25% sucrose, 50 mM Tris.HCl (pH 8) and 2 mM $MgCl_2$ before adding 400 ul of 20 mg/ml lysozyme. The mixture was left at room temperature (RT) for 30 min before the addition of 800 ul of 250 mM EDTA and 3.2 ml of 50 mM Tris.HCl (pH 8), 62.5 mM EDTA, 0.5% TritonX-100. Following a further 15 min incubation, the mixture was centrifuged at 35,000 (Beckman Type 65 rotor in Oakridge tubes) for 30 min at 4°C. The supernatant, containing the extracted plasmid DNA, was decanted into a 15 ml Falcon tube and after adjusting the volume to 7.5 ml with H_2O, 7.5 g of caesium chloride were added to the DNA to give a final density of 1.55 to 1.60 g/ml. The caesium chloride was thoroughly mixed until dissolved and following the addition of 200 ul of 10 mg/ml ethidium bromide, samples were centrifuged in sealed plastic tubes at 44,000 rpm (Beckman Type 65 rotor) for 40 h at 15°C. Ethidium bromide bands were visualised under long wave U.V. light and the lower band containing the supercoiled plasmid DNA was collected from immediately below with a syringe.

The ethidium bromide was removed by successive extractions (usually x 4) with an equal volume of isoamyl alcohol until no ethidium bromide was detected under long wave U.V. light. The DNA was then dialysed against TE at 4°C for 2-3 h with one change of buffer and finally stored at -20°C.

(c) <u>quantitation of plasmid DNA preparations</u>

A small quantity of plasmid DNA was linearised with an appropriate restriction enzyme and resolved on an agarose gel along with a digested DNA sample of standard DNA of known concentration (previously quantified by UV absorbance). DNA bands were visualised under U.V. light and photographed using type 665 Polaroid film. DNA concentrations were obtained by densitometric analysis of the resulting band on the negative polaroid 665 film using the data system for the Hoefer GS-360 scanning densitometer. Within the ranges used, there was a linear relationship between the amount of DNA present and the area under the peak corresponding to the DNA band on the negative.

(d) small scale preparations of plasmid DNA

Transformed E.coli DH-1 colonies were picked and resuspended in 2 ml of L-broth and 50 ug/ml of ampicillin and shaken overnight at 37°C. 1.2 ml samples were pelleted in 1.5 ml Eppendorf tubes in a high speed SE microfuge (MSE microcentaur at 13,000 rpm) for 20-30 sec and the supernatant aspirated off. The pellet was dispersed by vortexing briefly before adding 100 ul of STET buffer and 16 ul of 10 mg/ml lysozyme. After vortexing, the mixture was heated at 100°C for 2 min, with caps off, and then centrifuged in a high speed microfuge for 15 min. The gelatinous pellet was removed with a sterile tooth-pick and 100 ul of isopropanol added to the supernatant. The mixture was briefly vortexed and incubated at RT for 10 min before spinning in a high speed microfuge for 3 min. The supernatant was poured off and the pellet lyophilised and resuspended in 50 ul of 3 mM EGTA.

6. Restriction enzyme digestion of DNA

DNAs were digested in H_2O containing the appropriate concentration of commercial restriction enzyme buffer (BRL) and the restriction enzyme (normally 1 unit/ug DNA). For the preparation and analysis of DNA fragments, the digestion was normally carried out in a 30 ul volume and incubated at 37°C for 3-4 h.

For the analysis of small scale plasmid DNA preparations, 3 ul of the sample were digested in a 10 ul volume also containing 10 ug/ml RNase A for 1 h at 37°C and electrophoresed on a mini-gel. For the digestion of high molecular weight cellular DNA and HSV DNA, digestion was carried out for a minimum of 6-8 h at 37°C.

7. Purification of DNA fragments from plasmids

(a) by agarose gel electrophoresis

Plasmids were cleaved with appropriate restriction enzymes and fragments separated by electrophoresis on a 1% agarose gel. The DNA was visualised under long wave U.V. light and the required band excised from the gel. The gel slice was placed in a bag prepared from boiled dialysis

tubing with 0.5 ml of 0.5 x TBE and immersed in a shallow layer of 0.5 x TBE in an electrophoresis tank. After electroelution at 200 V for 2-3 h, the current was briefly reversed to remove the DNA from the side of the bag. The DNA in solution was purified over a DEAE-sephacel column. A 0.5 ml DEAE-sephacel column was washed with TE + 0.1 M NaCl before applying the DNA in solution. The flow through was collected and re-applied to the column. The bound DNA was washed with 5 ml of TE + 0.1 M NaCl and eluted with two aliquots of 0.25 ml of TE + 1 M NaCl. The eluate was divided between two Eppendorf tubes and DNA precipitated with 2 vols of ethanol overnight at -20°C, spun down in a high speed microfuge for 10 min, washed in 70% ethanol, lyophilised and resuspended in TE.

(b) by low melting point (LMP) agarose gel electrophoesis

Digested DNA was electrophoresed in a 1% LMP agarose mini-gel in TBE at 4°C. DNA fragments were visualised under long wave U.V. light and the required gel slice was excised and placed in a 1.5 ml Eppendorf tube. Two volumes of TEN (w/v gel slice:TEN) were added to the gel slice and heated at 70°C for 10 min to melt the agarose. An equal volume of phenol, heated to 70°C, was added to the melted agarose and vortexed thoroughly for 2 min. After centrifugation in a high speed microfuge for 5 min, the aqueous phase was re-extracted with an equal volume of phenol at RT. Samples were then extracted once with chloroform, ethanol precipitated, lyophilised and resuspended in TE.

(c) by polyacrylamide gel electrophoresis

Small (100 bp) end-labelled fragments were resolved on 8% polyacrylamide gels. After electrophoresis, one glass plate was removed and the wet gel covered with cling film before exposure to autoradiography film at RT for 2-3 min. Alignment of the autoradiograph with the wet gel enabled the relevant band to be excised and placed in an Eppendorf tube. The gel slice was submerged in 0.5 ml of elution buffer (500 mM NH_4OAc, 10 mM $MgCl_2$, 0.1% SDS and 1 mM EDTA) and eluted overnight at 37°C with constant agitation. The buffer containing DNA was extracted sequentially with

phenol:chloroform (1:1) and chloroform, and precipitated in two volumes of ethanol in a dry ice bath for 10 mins. After pelleting the DNA for 15 min in a high speed microfuge, the pellet was washed in 100% ethanol, lyophilised and resuspended in H_2O.

8. Radioactive labelling of DNA fragments and oligonucleotides

(a) 3' end-labelling

Plasmid DNA (approximately 2 ug) was cleaved at the relevant restriction enzyme site to produce a 5' overhang, sequentially extracted with phenol and chloroform, ethanol precipitated and resuspended in an appropriate volume of H_2O. Synthetic oligonucleotides were synthesised such that on annealing complementary strands, a 5' overhang was formed. The DNA to be labelled was added to a reaction mix containing NTP buffer (500 mM Tris.HCl pH (7.8), 50 mM $MgCl_2$, 10 mM DTT), 20 uCi of the appropriate radiolabelled deoxyribonucleoside triphosphate, 40 nM of the other three unlabelled dNTPs and 2 units of the large fragment of E.coli DNA pol I, and left at RT for 30-45 min. Finally, unlabelled dNTP corresponding to the initial label was added to 40 nM and incubation continued for 15 mins to produce labelled fragments with blunt ends. End-labelled DNA was purified from unincorporated dNTPs by polyacrlyamide gel electrophoresis {7(c)}.

(b) 5' end-labelling of oligonucleotides

Oligonucleotides (200 ng) were resuspended in kinase buffer (50 mM Tris.HCl pH (7.6), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM spermidine, 5 mM DTT) containing 20 uCi of [γ-^{32}P]-ATP and 5 units T4 polynucleotide kinase and incubated at 37^{o}C for 30 min. The DNA was sequentially extracted with phenol and chloroform and precipitated with ethanol in a dry/ice ethanol bath. After pelleting the DNA for 15 min in a high speed microfuge, the pellet was lyophilised and resuspended in H_2O.

(c) internal labelling of plasmid DNA by nick translation

200 ng of plasmid DNA were radiolabelled (Rigby et

al., 1977) by the addition of 20 uCi of [α-^{32}P]dATP, 1 unit of E.coli DNA polymerase holoenzyme and 5 x 10^{-7} ug/ml of DNase I in a final volume of 20 ul of NTP buffer containing 40 nM each of dGTP, dCTP and dTTP. After a brief incubation at 37°C, the reaction mix was left at RT for 2-3 h. The reaction was stopped by the addition of 100 ul of TEN (pH 8.3), 10 ul of 5 x SB. To purify the labelled plasmid DNA from unincorporated dNTPs, the reaction was loaded on a Sephadex G-50 column. Sephadex beads were pre-swollen overnight in TE. Columns were prepared in plastic 5 ml pipettes with siliconised glass wool plugs and pre-run with 20 ml of TE. The column was loaded with the stopped reaction and the fastest eluting fraction, detected by monitering flow through with a Geiger counter, collected.

9. DNA cloning

(a) preparation of competent bacteria

The method used was based on that of Maniatis et al. (1982). A 1 ml aliquot from an overnight standing culture of bacteria was used to inoculate 40 ml of L-broth. The culture was then grown at 37°C to an O.D. of 0.2 at 630 nm. Bacteria were pelleted at 9,000 rpm (Sorval SS34 rotor) for 1 min and resuspended in 20 ml of 10 mM $CaCl_2$. After a brief vortex, cells were again pelleted before resuspension in 20 ml of 75 mM $CaCl_2$. Cells were incubated on ice for 20 min and pelleted. The cell pellet was resuspended in 1-2 ml of 75 mM $CaCl_2$ and divided into 200 ul aliquots. 50 ul of 80% glycerol was added to each aliquot, mixed and stored at -70°C.

(b) preparation of ampicillin plates

L-broth agar was melted and then allowed to cool until hand hot. Ampicillin was added to a concentration of 50 ug/ml and the agar poured into 90 mm sterile Petri dishes and allowed to set. The plates were dried overnight in an inverted position at 37°C before being stored at 4°C. Plates were normally used within three weeks.

(c) cloning of DNA fragments

DNA was cloned into the appropriate restriction

sites (normally BamHI-SalI) in the polylinker of pTZ19U. The vector was digested in the presence of 1 unit of calf intestinal phosphatase to minimise re-ligation of plasmid molecules. The DNA to be inserted was either purified from a starting plasmid by agarose gel electrophoresis {4(a)} or the plasmid was additionally digested with an enzyme which cleaves the ampicillin resistance gene. Prior to ligation reactions, the DNA was extracted sequentially with phenol and chloroform, precipitated with ethanol, lyophilised and resuspended in H_2O.

For ligation, DNA was incubated with BamHI-SalI digested pTZ19U (2 ug in 50 ul reaction) at a ratio of 5:2 insert:vector in ligase buffer (50 mM Tris.HCl pH (7.5), 10 mM $MgCl_2$, 1 mM ATP, 20 mM DTT, 50 ug/ml bovine serum albumin) containing 0.5 units of T4 DNA ligase and left overnight at RT.

(d) transformation of bacteria

A 200 ul aliquot of competent bacterial cells was thawed and placed on ice for 10 min. 10 ul of ligation mix were added to the cells, gently mixed and incubated on ice for a further 30 min. The cells were then heat-shocked at 42°C for 90 sec, placed back on ice for 60 sec before adding 400 ul of L-broth. The cells were further incubated at 37°C for 1 h. 100 ul of cells were spread onto ampicillin plates at various dilutions (usually 0, 10^{-1}, 10^{-2}) and incubated overnight at 37°C. Small scale DNA preparations were made from individual colonies transformed to ampicillin resistance {5(d)} and analysed on a mini-gel. Cultures were grown from positive clones and stored in 7% DMSO at -70°C.

10. Synthesis, purification and annealing of complementary oligonucleotides

Synthetic oligonucleotides were synthesised on a Biosearch 8600 DNA synthesiser using the macroscale synthesis procedure. The DNA was eluted from the column by resuspending the beads in 1 ml of ammonia. The ammonia solution was incubated at 55°C for 5 hr and removed by lyophilisation. The samples were resuspended in 40 ul H_2O, 20 ul of which was stored at -70°C and the remainder

lyophilised before resuspending in TBE containing 90% formamide. The sample was purified by denaturing polyacrylamide gel electrophoresis using a 15% polyacrylamide gel (29:1) containing 8 M urea in TBE. 2 ul of a formamide-dye mix {4(d)} was loaded in a separate well to act as a migration marker. The gels were electrophoresed at 250 V until the BPB had migrated 2/3 through the gel.

To visualise the DNA, the gel was removed from the glass plate onto cling film and viewed against a thin layer chromotography plate with an angled short wave U.V. lamp. The DNA in the gel absorbs the U.V. radiation and appears as a dark band against a uniform fluorescent background contributed by the chromotographic plate. The top band was excised and eluted as described in 7(c). Finally, the recovered DNA was resuspended in H_2O and the O.D. at 260 nm and 280 nm, determined. For synthetic oligonucleotides, the conversion factor is taken as 1 O.D. unit at 260 nm =20 ug/ml.

Based on O.D. calculations, approximately equal amounts of oligonucleotides were run on an agarose gel and photographed using Polaroid 665 film under U.V. illumination. The relative concentrations were calculated using the data program for the Hoefer GS-360 scanning densitometer. DNA solution volumes were re-adjusted (if necessary) to equalise the oligonucleotide concentrations of complementary strands.

Equal amounts of complementary strands (normally 2 ug of each) were mixed together in a 100 ul final volume with H_2O, heated in a boiling bath for 2-3 mins and incubated at 37°C for 30 min. To check the efficiency of annealing, one of the strands was 5' end-labelled before annealing to the complementary strand. Aliquots of labelled ss and annealed oligonucleotide were analysed on a non-denaturing polyacrylamide gel (Fig. 2.3). As the annealing efficiency was greater than 95%, 100% efficiency was assumed for calculating the final concentration of ds oligonucleotides.

11. Expression of Staphylococcus aureus protein A:UL9 fusion proteins in E.coli

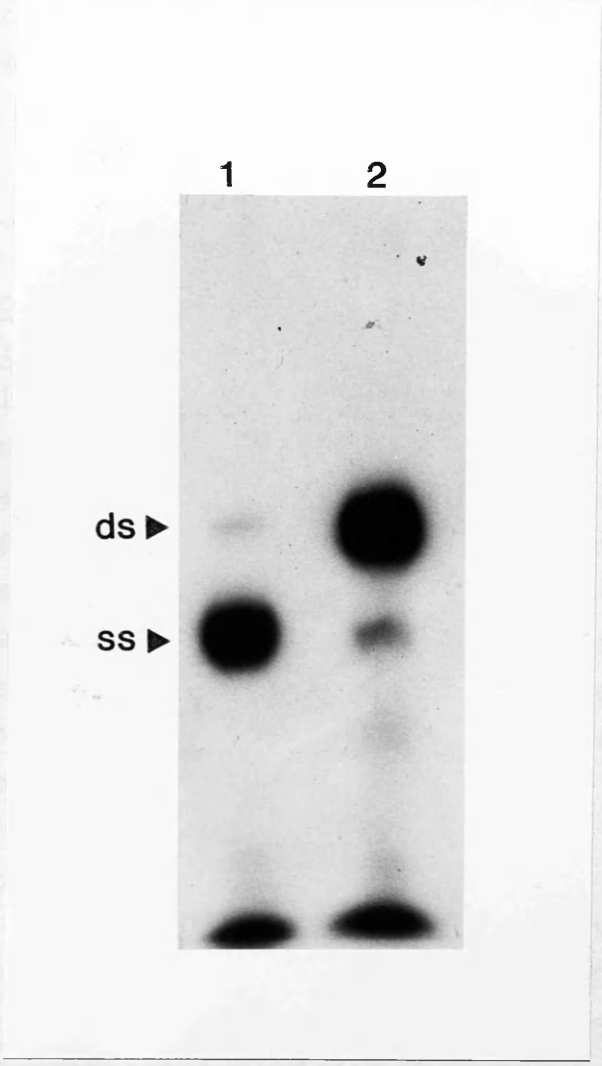


Figure 2.3 Efficiency of annealing of complementary oligonucleotides

A single-stranded oligonucleotide;

[A]= 5' GATCCGCGAAGCGTTCGCACTTCGTCCCA

was 5' ^{32}P-labelled and annealed to an equimolar amount of its complementary strand;

[B]= GCGCTTCGCAAGCGTGAAGCAGGGTCTAG 5'

Aliquots of oligonucleotide [A] before (track 1) and after (track 2) annealing were resolved on a 8% non-denaturing polyacrylamide gel.

E.coli strain K12ΔH1Δtrp cells (Bernard et al., 1979) containing the fusion protein constructs (see materials) were propagated at 28°C. To induce synthesis of the fusion proteins, bacteria were grown to an O.D. of 0.4 at 630 nm in 50 ml L-broth and rapidly shifted to 42°C for 75 min. Total cellular extracts were prepared as described in 12(b).

12. Preparation of extracts for DNA binding studies

(a) preparation of nuclear extracts from cells infected with virus or transfected with viral DNA

BHK cell monolayers were infected with 5 pfu/cell of virus and incubated at 37°C or 38.5°C for 8 h or 31°C for 12-16 h. For transfection experiments, approximately 10 ug of HSV-1 digested DNA, or plasmids containing HSV-1 replication genes (0.5 ug of each), were made up to a total of 15 ug of DNA with calf thymus carrier DNA, transfected onto BHK cells as described in section 14 and incubated at 37°C for 24 h. Nuclear extracts were prepared using the following methods:

Method A - Extracts were prepared essentially as described by Piette et al. (1985). Infected cells from 3 x 90 mm plates were harvested by scraping into ice-cold PBS and pelleted by centrifugation at 2,000 rpm (Sorval SM24 rotor) for 5 min. All subsequent procedures were carried out at 4°C. The cell pellet was resuspended in 3 ml of buffer A (10 mM Hepes (pH 8.0), 50 mM NaCl, 0.5 M sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 7 mM 2-mercaptoethanol, 0.5% Triton X-100) and disrupted using 12 strokes of a Dounce homogeniser with a B-type pestle. After centrifugation at 2,000 rpm (Sorval SM24 rotor) for 10 min, nuclei were resuspended in 1 ml of buffer B (10 mM Hepes (pH 8.0), 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 7 mM 2-mercaptoethanol) containing 100 mM NaCl and after 15 min of gentle agitation, centrifuged at 5,000 rpm for 10 min. The supernatant was removed and the nuclei were resuspended in 1 ml of buffer B containing 600 mM NaCl. After gentle agitation for 30 min, the nuclei were pelleted at 15,000 rpm (Sorval SS34 rotor using Eppendorf

tube adaptors) for 15 min and the supernatant brought to 45% saturation using solid $(NH_4)_2SO_4$. Proteins were precipitated by centrifugation at 15,000 rpm (Sorval SS34 using Eppendorf tube adaptors) for 15 min. The pellet was resuspended in 100 ul of buffer B containing 100 mM NaCl. Aliquots of the resulting extract were flash frozen in a dry ice/ethanol bath and stored immediately at -70°C.

Method B - Extracts were prepared essentially as described by Dignam et al. (1983) and modified by Preston et al. (1988). Cells from 3 x 90 mm plates were harvested as described above and washed with PBS. The cell pellet was resuspended in 200 ul 90 of lysis buffer A (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% NP40, 0.5 mM DTT and 0.5 mM PMSF) and gently pipetted at 3 min intervals for 10 min. Nuclei were pelleted by successive centrifugation at 2,000 rpm for 5 min and 12,000 rpm (Sorval SS34 using Eppendorf tube adapters) for 15 min, and resuspended in 100 ul of buffer C (20 mM Hepes (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT) containing 600 mM NaCl. Proteins were eluted by gentle pipetting at regular intervals for 30 min before centrifugation at 15,000 rpm for 20 min. The supernatant, designated nuclear extract, was flash frozen in aliquots using a dry ice/ethanol bath and immediately stored at -70°C.

(b) preparation of extracts from bacterial cells

Heat-induced bacterial cells were pelleted at 4,000 rpm (Sorval SS34 rotor) for 7 min, washed twice in 10 ml of TE and finally resuspended in 1 ml of TE and spun down in a high speed microfuge. The pellet was resuspended in 400 ul of buffer C (see method B) containing 600 mM KCl. The suspension was sonicated using a probe sonicator at 70 W, four times for 1 sec with 30 sec intervals on ice and centrifuged in a high speed microfuge for 10 min at 4°C. The resulting supernatant was aliquoted, flash frozen in a dry ice/ethanol bath and stored at -70°C for use in gel retardation assays.

13. Estimation of protein concentration

Estimates of total protein concentration in nuclear extracts were obtained using Bio-Rad protein assay dye reagent. 5 ul of cell extract was diluted in 1 ml of H_2O and 800 ul of the diluted protein sample mixed with 200 ul of dye reagent and left at RT for 10 min. The absorbance of the solution was measured at 595 nm and converted to milligrams of protein by comparison to a standard curve produced by using known quantities of BSA made up in H_2O and diluted as described for the cell extract.

14. Plasmid amplification assay (Stow, 1982)

(a) transfection of cells with DNA and virus superinfection

Monolayers of BHK cells in 50 mm Petri dishes were transfected with supercoiled plasmid containing HSV-1 ori_S fragments in the presence of 10 ug of calf thymus carrier DNA using the calcium phosphate technique (Graham and van der Eb, 1973). The DNAs were mixed in 1 ml of HeBS buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM D-glucose, 21 mM Hepes, pH adjusted to 7.05 with NaOH). 2 M $CaCl_2$ was added to the DNA solution to give a final concentration of 125 mM and 500 ul of the fine suspension which formed were added to cell monolayers from which growth medium had been removed. The plates were incubated at 37°C for 45 min and overlaid with 4 ml of EC5. Each monolayer received an amount of plasmid DNA calculated to contain the same number of plasmid molecules as present in 0.24 ug pAT153 (Stow and McMonagle, 1983). The cells were given a dimethyl sulphoxide boost (Stow and Wilkie, 1976) 4 h after tranfection. The growth medium was removed from the plates and cells washed with 4 ml of GMEM wash. 1 ml of a 25% DMSO solution (diluted in HeBS) was added to the plates for 4 min. The DMSO was removed, plates washed and overlaid with EC5 and incubated at 37°C. Six h after transfection, the cells were either mock-infected or superinfected with wt HSV-1 or ts mutants at a moi of 5 pfu/cell and incubated in EC5 at the appropriate temperature.

(b) preparation of total cellular DNA

Growth medium was removed from plates 24 h post transfection and the cells incubated at 37°C for 5 h with 2

ml of lysis buffer (0.6% SDS, 10 mM EDTA, 10 mM Tris.HCl (pH 7.5) containing 500 ul of proteinase (type XIV from Streptomyces griseus) per ml. NaCl was added to a final concentration of 200 mM followed by sequential extraction with phenol and chloroform. DNA was precipitated in ethanol overnight at -20°C, pelleted by centrifugation at 7,000 rpm (Sorval SM24 rotor) for 20 min, lyophilised and resuspended in TE containing RNase A (10 ug/ml).

1/15 of the total DNA extracted from a 50 mm plate was digested with DpnI and EcoRI. DpnI cleaves the sequence GATC provided the A residue is methylated (Lacks and Greenberg, 1977). Plasmid DNA which has been methylated by the dam methylation system of E.coli, can be digested into several fragments. Following replication of the vector sequences in eukaryotic cells, GATC sequences containing unmethylated A residues are produced which are resistent to DpnI. EcoRI cleaves the replicated plasmid DNA (in the form of long concatemeric molecules; Stow, 1982) into unit length plasmid molecules.

(c) Southern transfer of DNA to nitrocellulose

Digested DNA samples for Southern transfer (Southern, 1975) were run on a 1% agarose gel in Loening's buffer. Following electrophoresis, the gel was gently shaken, first in gel soak I for 45 min, then in gel soak II for 45 min. The DNA was blotted overnight onto a pre-soaked nitrocellulose filter using 6 x SSC, a wick of Whatman 3 mm paper and a weighted capillary stack of absorbent paper towelling. The filter was then air dried and baked at 80°C for 2 h in a vacuum oven prior to hybridisation.

(d) DNA:DNA hybridisation

Nitrocellulose filters were incubated in 20 ml of prehybridisation buffer for 2-3 h at 65°C with constant agitation. 10^7-10^8 cpm of nick translated probe DNA was denatured by the addition of 1/10 vol of 2 M NaOH for 10 min then neutralised with 2 M HCl before adding to 10 ml of hybridisation buffer. The prehybridisation buffer was removed and replaced by hybridisation mix containing the denatured labelled DNA. Hybridisation was carried out at 65°C overnight with constant agitation. The filter was then

washed twice for 1 h in 2 x SSC, 0.25% SDS at 65°C. The filter was blotted onto tissue paper before exposure to autoradiography film at -70°C.

15. In vitro transcription and translation of pTZ plasmids

The pTZ derived plasmids were linearised 3' to the coding sequence with an appropriate restriction enzyme, extracted with phenol and chloroform and ethanol precipitated.

In vitro transcription was performed using the Riboprobe system (Promega Biotech) following the manufacturer's protocol. 1 ug of linearised DNA was mixed with 5 ul 5 x SP6 buffer (200 mM Tris.HCl (pH 7.5), 30 mM $MgCl_2$, 10 mM spermidine, 0.01% BSA, 10 mM DTT, 0.4 mM each of ATP, UTP, CTP, GTP, 0.5 mM G(5')ppp(5')G, 40 units of RNasin and 10 units of T7 RNA polymerase in a total volume of 25 ul. The reaction mixture was incubated at 37°C for 1 h.

In vitro translation was carried out by adding 2.5 ul of the transcription reaction mixture to 20 ul of rabbit reticulocyte lysate. Duplicate samples were incubated at 31°C for 90 min, either in the absence or presence of 25-50 uCi [^{35}S]-methionine in a 25 ul volume. The samples were treated with 200 ug/ml RNase A, 20 mM EDTA for 15 min at 31°C. Non-radioactive samples were used immediately in gel retardation assays (described below) and radiolabelled translation products were analysed by SDS-polyacrylamide gel electrophoresis {section 4(e)}.

16. Preparation of sonicated calf thymus DNA

Approximately 50 mg of calf thymus DNA were resuspended in 5 ml of H_2O and sonicated 3 times using a probe sonicator at 100 W for 30 sec with 30 sec intervals on ice. The sonicated DNA was sequentially extracted with phenol (x 3) and ether (x 3), then dialysed overnight against 1 L of H_2O. The O.D. 260 nm was measured and the volume of DNA adjusted to give a final concentration of 1 mg/ml before storing at -20°C.

17. Gel retardation assay

The method used was essentially as described by Schneider et al., (1986). Probe fragments, 3' end-labelled with [α-^{32}P] deoxyribonucleoside triphosphates were eluted from 8% non-denaturing polyacrylamide gels and extracted sequentially with phenol and chloroform and precipitated with ethanol. Either 1 ng of labelled oligonucleotide (44 fmoles) or 5 ng of labelled 100 bp fragment (75 fmoles) were incubated at 25^{o}C with 2 ug of sonicated calf thymus DNA and 5 ug of extract in a 20 ul reaction mix containing buffer C plus 100 mM NaCl. After 20 min, 5 ul of loading buffer (25% glycerol, 10 mM DTT and 0.01% BPB in TBE) were added and the samples loaded onto 5% polyacrylamide gels (55:1 acrylamide:N,N'-methylene bisacrylamide) containing TBE. Gels were run in TBE at 25 mA for 1.5-2 h, fixed in 10% acetic acid, dried and exposed to autoradiography film at -70^{o}C.

For assays containing proteinase or antisera, the reagents were added 10 min after the extract and incubation continued for a further 10 min. 1 ul of antiserum was added to the 20 ul binding reactions.

18. Plasmid DNA sequencing

Plasmid DNA was sequenced following a dideoxy sequencing protocol using denatured plasmid templates described by Hattori and Sakaki (1986).

(a) preparation of denatured plasmid templates

15 ml overnight cultures from single transformed colonies were pelleted at 10,000 rpm (Sorval SS34 rotor) for 15 min and resuspended in 1 ml of lysozyme solution (0.4% lysozyme, 25 mM Tris.HCl (pH 7.5), 100 mM EDTA, 50 mM glucose) and after 5 min at RT, mixed with 2 ml of 0.2 M NaOH, 1% SDS, vortexed and incubated on ice for 5 min. 1.5 ml of potassium acetate solution (3 M KOAc, 11.5% glacial acetic acid) was added to the sample which was vortexed and incubated on ice for a further 5 min. The sample was centifuged at 10,000 rpm (Sorval SS34 rotor) for 15 min at 4^{0}C and the supernatant extracted once with phenol/chloroform (1:1) and precipitated with 2 vols of ethanol at RT for 15-30 min. The DNA was pelleted at 10,000 rpm (Sorval SS34 rotor) for 15 min at 4^{o}C, washed in 70%

ethanol and lyophilised. The DNA pellet was resuspended in 100 ul TE containing 2 ul of 10 mg/ml RNase A solution at 37°C for 30 min. The DNA was precipitated by the addition of 60 ul of 20% polyethylene glycol 6000, 2.5 M NaCl for 1 h at 4°C. The sample was centrifuged in a high speed microfuge for 5 min, the pellet washed in ethanol and lyophilised before resuspending in 18 ul TE.

2 ul of 2 M NaOH was added to the 18 ul DNA sample and left at RT for 5 min. The denatured DNA was precipitated by the addition of 8 ul of 5 M ammonium acetate and 100 ul of ethanol for 5 min at -70°C. The DNA was pelleted in a high speed microfuge for 10 min, washed in 70% ethanol and lyophilised. The pellet was resuspended in 20 ul of H_2O and 3 ul were used for dideoxy sequencing reactions.

(b) dideoxy sequencing

The sequencing reactions were performed using T7 DNA polymerase following the manufacturer's protocol (Pharmacia). 3 ul of denatured DNA sample were incubated with 5 ul of H_2O, 1 ul of TM buffer (280 mM Tris.HCL (pH 7.5), 150 mM $MgCl_2$, 350 mM NaCl) and 1 ul (5 ng) of universal or T7 promoter primer at 37°C for 30 min to allow annealing of the primer to the DNA template. In the meantime, labelling reactions were set up in Eppendorf tubes such that for one set of reactions (i.e. G, A, T and C) the mix contained 1 ul of 100 mM DTT, 2 ul of labelling mix (2 uM dGTP, dCTP, TTP) 20 uCi [$-^{35}$S]dATP and 3 units of T7 DNA polymerase.

Termination mixes contained 150 uM dGTP, dATP, dCTP, TTP and 15 uM of either dideoxy (dd) ATP (A-mix), or ddCTP (C-mix), or ddGTP (G-mix), or ddTTP (T-mix), 20 mM $MgCl_2$, 40 mM Tris.HCl (pH 7.5) and 50 mM NaCl. 2.5 ul of termination mix was added to Eppendorf tubes labelled G, A, T or C and incubated at 37°C. After the 30 min annnealing reaction, the labelling reaction (5.5 ul) was added to the annealing mix and 3.5 ul of the mixture added to each of the G,A,T,C tubes containing the corresponding termination mix. The samples were incubated at 37°C for 15 min and the reaction stopped by adding 4 ul of stop dyes (95% deionised formamide containing 20 mM EDTA (pH 7.5), 0.05% (w/v) xylene

cyanol and 0.05% BPB). The samples were boiled for 2-3 min at 100°C and 2-3 ul of each reaction resolved on a 6% denaturing polyacrylamide sequencing gel {4(b)}.

19. DNase I footprinting assays

(a) chemical sequencing reactions

3' end-labelled DNA fragments were cleaved using the chemical methodology of Maxam and Gilbert (1980). This involves the modification of bases, in reactions that are relatively base-specific, followed by cleavage at the modified bases by piperidine. As these reactions served as markers for locating the bases protected from DNase I digestion by DNA binding protein, only G and G+A reactions were used.

Uniquely end-labelled DNA restriction fragments were generated by 3' end-labelling a restriction enzyme site, inactivating the large fragment polymerase by heating to 70°C for 10 min and cutting the plasmid with a second enzyme to release the uniquely end-labelled fragment. The fragment was purified on a non-denaturing polyacrylamide gel as described in 4(b) and 10^5 cpm aliquots reacted to modify purine bases. The end-labelled DNA was first heated to 90°C for 1 min in the presence of 1 ug sonicated carrier DNA and chilled on ice.

(i) G reaction

The labelled DNA (10-15 ul) was incubated with 200 ul of G buffer (50 mM sodium cacodylate (pH 8), 10 mM $MgCl_2$ and 1 mM EDTA) and 1 ul of dimethyl sulphate at RT for 10 min. The reaction was stopped by the addition of 50 ul of DMS stop buffer (1.5 M NaOAc, 1 M 2-mercaptoethanol, 40 ug/ml sonicated carrier DNA) and DNA precipitated with 750 ul of ethanol in a dry ice/ethanol bath and pelleted for 5 min in a high speed microfuge. The DNA was resuspended in 250 ul of 2 M NH_4OAc, 0.1 mM EDTA, precipitated with 750 ul of ethanol in a dry ice/ethanol bath, pelleted, washed and lyophilised.

(ii) G+A reaction

The labelled DNA (10 ul) was mixed with an equal

volume of H_2O, 3 ul of pyridinium formate (4% formic acid pH adjusted to 4 by the addition of piperidine - 15ul/ml of solution) and incubated at 37°C for 25 min. The sample was chilled in a dry ice/ethanol bath, lyophilised, resuspended in 20 ul H_2O, and lyophilised again.

(iii) piperidine cleavage reaction

10 ul of 5 M NH_4OH was added to all dried modified DNA samples, without mixing, and lyophilised. Then, 100 ul of 1 M piperidine was added to each sample and heated at 90°C for 30 min. Samples were chilled and transferred to a new tube containing 100 ul of 2 M NH_4OAc, 0.1 mM EDTA. DNA was precipitated by the addition of 800 ul of ethanol in a dry/ice ethanol bath, pelleted, washed and lyophilised.

(b) DNase I footprinting assay

These assays were performed essentially as described by Dynam (1987) and Preston et al. (1988). DNA binding reactions were set up as described in section 17, using 10^5 cpm of labelled probe. After incubation at 25°C for 20 min, $MgCl_2$ and DNase I were added to final concentrations of 5 mM and 6 ug/ml respectively. After further incubation at 25°C for 3 min, digestion was terminated by the addition of EDTA to a final concentration of 10 mM. Samples were loaded onto an 8% non-denaturing polyacrylamide gel. After electrophoresis, the wet gel was exposed to autoradiography film for 1 h at 4°C. Bands containing complexed and free DNA were excised and the DNA recovered by electroelution was extracted sequentially with phenol and chloroform and precipitated with ethanol. Recovered DNA fragments were analysed on 6% denaturing polyacrylamide gels together with markers obtained by G and G+A sequencing reactions performed on both strands of the DNA as described in part (a). Prior to electrophoresis, all lyophilised samples were resuspended in stop dyes {section 18(b)} and heated to 100°C for 2 min.

In some experiments, the binding reactions were directly extracted with phenol and chloroform and then ethanol precipitated. The mixture of bound and free DNA recovered was analysed on a denaturing polyacrylamide gel as described above.

20. Oligonucleotide-directed mutagenesis

Specific deletions and point mutations were introduced into the pST19 ori_S-containing fragment essentially as described by Mead et al. (1986) and Kunkel (1985). The method of Kunkel employs a labile uracil-rich parental DNA strand isolated from an E.coli strain which lacks the enzymes uracil DNA glycosylase (ung^-) and dUTPase (dut^-). When these bacteria are transformed with pTZ phagemid vectors and subsequently grown and superinfected with helper phage in medium containing a high uridine concentration, uracil is incorporated into newly synthesised ssDNA. Following the annealing of the mutagenic oligonucleotide to extracted uracil-rich ssDNA and conversion to dsDNA in vitro, the DNA molecules are transformed back into an ung^+, dut^+ strain (e.g. DH1). In this host the uracil rich strand undergoes extensive repair synthesis, hence enriching for the plasmid molecules containing the specific mutation.

(a) procedure for obtaining and purifying uracil-rich ssDNA

E.coli BW313 (ung^-, dut^-) were transformed with pST19 and 12.5 ml of an overnight culture used to inoculate 250 ml of 2 x YT broth containing 0.25 mg/ml of uridine. Once the bacteria had grown to an O.D. of 0.3 at 630 nm, the culture was infected with phage M13 R408 at a moi of 10. After an 8 h infection, the culture was pelleted at 9,000 rpm (Sorval GSA rotor) for 10 min. The phage containing ssDNA in the supernatant could be stored at 4°C. The phage were precipitated by adding 1/4 vol of 20% polyethylene glycol 6000, 3.5 M NH_4OAc, incubating on ice for 30 min and then pelleted at 12,000 rpm (Sorval SS34 rotor) for 15 min. The tube was well drained and the pellet lyophilised thoroughly before resuspension in 200 ul of TE. The DNA was extracted with phenol/chloroform (1:1) until no interface was visible (approximately 6-7 times). Following ethanol precipitation and lyophilisation, the pellet was resuspended in 20 ul H_2O.

(b) site-directed mutagenesis

The appropriate mutagenic oligonucleotide was 5' phosphorylated by incubating 100 ng of oligonucleotide in ligase buffer {9(c)} with 2 units of polynucleotide kinase at 37°C for 30 min. Half of the reaction was annealed to the complementary uracil-rich ssDNA at a molar ratio of 10:1 by incubating at 65°C for 10 min and allowing to cool slowly to RT. Ds DNA was synthesised using the Klenow fragment of DNA polymerase I and T4 DNA ligase in the presence of 100 uM of all four dNTPs, 1 mM ATP and 100 ug/ml of gene 32 protein. The reaction mix was incubated at 15°C and 1/4 of the products used to transform E.coli DH1 (ung^+, dut^+) to ampicillin resistance. Individual colonies were picked and plasmids carrying the desired mutations were identified by sequencing the complete insert as described in section 18.

21. Computing

Computer analysis of DNA and protein sequence were performed on the MicroVAX 11 and PDP 11/14 computers in this department, using primarily the University of Wisconsin Genetics Computer Group software package.

CHAPTER 3 : RESULTS

SECTION 3A : Origin binding activity in cells infected with wt HSV-1

As described in the introduction, Elias et al. (1986) demonstrated the presence of a protein in HSV-1 infected cell nuclear extracts that bound to a specific 18 bp sequence at the left hand end of the HSV-1 ori_S palindrome (Fig. 1.6). The origin binding activity was detected using nuclear extracts from large numbers of infected cells (20-30 g) which had been partially purified on a phosphocellulose column. Fractions from the column were incubated with an ori_S-containing DNA fragment and binding activity measured using nitrocellulose filter-binding assays. Any unbound double-stranded DNA passes through the filter while protein-DNA complexes are retained.

My primary aim was to identify the viral gene encoding this origin binding activity. As it was envisaged that this would involve assaying many different nuclear protein preparations (e.g. from transfected cells) for origin binding activity, it was important to develop a more convenient assay which would not require such large numbers of cells and could be performed on unfractionated nuclear extracts.

My initial experiments therefore attempted to develop a sensitive gel retardation assay which would allow identification of origin binding activity from a small number of infected cells. This technique (developed by Fried and Crothers, 1981) involves the incubation of protein extracts with DNA fragments or oligonucleotides containing putative protein binding sites. Mixtures are loaded onto native polyacrylamide gels and protein-DNA complexes separated from unbound DNA on the basis of the lower electrophoretic mobility of the former species (Garner and Revin, 1981). It is possible to detect sequence-specific binding activity, even in crude extracts, by adding an excess of unlabelled carrier DNA to the binding reactions

with which other DNA binding proteins will preferentially interact (Garner and Revin, 1981; Schneider et al., 1986).

1. Detection of ori$_S$ binding activity in nuclear extracts from HSV-1 infected cells

(a) cloning of a functional ori$_S$ into plasmid pTZ19U

The family of pTZ vectors is well suited for carrying out a variety of molecular manipulations such as site-directed mutagenesis, in vitro transcription and dideoxy sequencing since these plasmids include a T7 promoter and a phage origin of replication which allows the production of single-stranded DNA (Mead et al., 1986). As it was envisaged that these features would prove useful throughout the course of this project, these vectors were routinely used. A 100 bp BamHI-SalI ori$_S$ fragment from plasmid pS19, which was previously shown to contain a functional origin of replication (Stow and McMonagle, 1983) was therefore re-cloned into the corresponding restriction sites of plasmid pTZ19U, yielding plasmid pST19. This fragment was chosen as it was a convenient size for use as a probe in gel retardation assays and was known to contain the 18 bp protein binding site identified by Elias et al. (1986).

To verify that the viral origin within pST19 could still function, a plasmid amplification assay was carried out in which the replicative ability of pST19 was tested together with a plasmid, pS1, which contains a known functional 535 bp ori$_S$-fragment (Stow and McMonagle, 1983) and the vector plasmid pST19U. Plasmids were transfected into BHK cells which were subsequently either mock-infected or superinfected with wt HSV-1 to provide helper functions. Samples of DNA extracted from cells 16 h post infection (pi) were cleaved with EcoRI and DpnI and replicated (DpnI-resistant) plasmid molecules were detected by Southern blotting using ^{32}P-labelled pAT153 DNA as probe (pAT153 detects DNA sequences of the pTZ family of vectors since these plasmids contain the same ampicillin resistance gene and plasmid origin of DNA synthesis).

The results of this assay are shown in Fig. 3.1. As expected, HSV-1 encoded functions facilitated the replication of both pS1 and pST19 (tracks 2 and 4) but not pTZ19U (track 6). The level of replication achieved with pST19 which contains the minimal ori_S sequence was lower than that obtained with pS1 which is consistent with published observations for plasmid pS19 which show that sequences flanking the core origin have an enhancing effect on origin activity (Stow and McMonagle, 1983). Nevertheless, this assay demonstrates that the 100 bp fragment within pST19 retains its ability to function as an origin of DNA replication.

Dideoxy sequencing verified that the insert in pST19 had a sequence identical to that published for the pS19 insert sequence (Stow and McMonagle, 1983; see Fig. 3.27; section 3C). Plasmid pST19 was therefore used as a source of HSV-1 ori_S sequence in subsequent experiments. The BamHI-SalI fragment was routinely end-labelled, purified and used as probe in gel retardation assays.

(b) demonstration of ori_S binding activity in cells infected with wt HSV-1

In initial experiments, nuclear extracts of HSV-1 infected BHK cells were prepared 8 h pi using a method of Piette et al. (1988) {method A}. Proteins from either mock-infected or infected nuclei were eluted in 600 mM NaCl, precipitated in $(NH_4)_2SO_4$ and redissolved in buffer B containing 100 mM NaCl. These extracts were incubated with the end-labelled ori_S-containing BamHI-SalI fragment of pST19, in the presence of sonicated calf thymus DNA which competes for any non-sequence-specific binding activity. Reaction mixtures were analysed by polyacrylamide gel electrophoresis.

Fig. 3.2 shows the pattern of retarded bands obtained using this assay. No binding activity was detected in extracts from mock-infected cells (track 1). Using HSV-1 infected cell extracts, a major retarded band, A, and a weaker band, B, were evident which represent protein-DNA complexes (track 2). In addition, some smeared binding was seen at the top of the gel. When the amount of infected

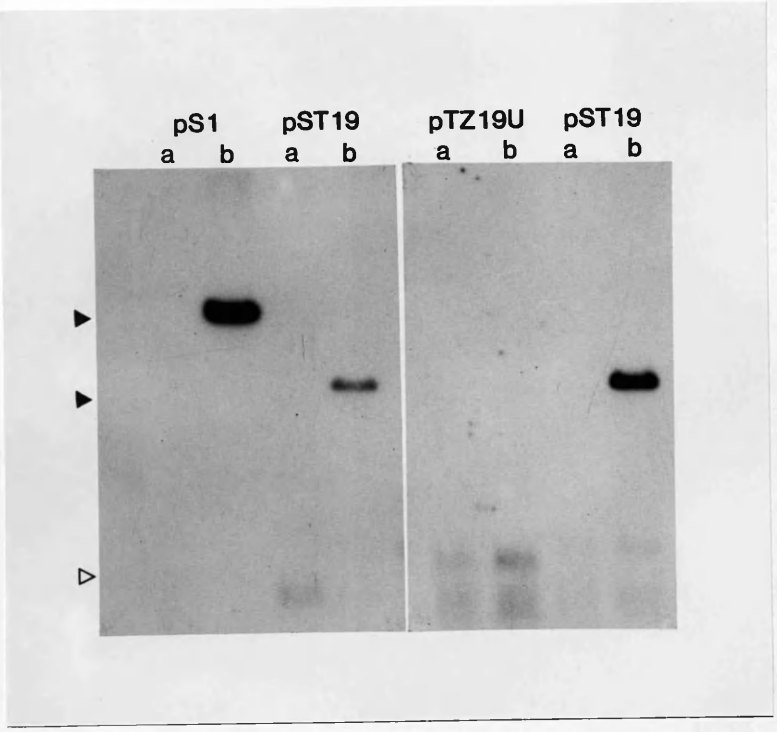


Figure 3.1 Replication of plasmids containing HSV-1 ori_S fragments

Monolayers of BHK cells (50 mm Petri dishes) were transfected with pS1 (which contains a 535 bp ori_S-containing fragment from HSV-1 BamHI x cloned into pAT153), pTZ19U or pST19 (which consists of a 100 bp ori_S fragment (Stow and McMonagle, 1983) cloned into pTZ19U) and either mock-infected (tracks a) or superinfected with HSV-1 (tracks b) at 37°C for 16 h. Samples containing 1/15 of the DNA recovered from each monolayer were digested with EcoRI plus DpnI and the fragments separated by electrophoresis, transferred to nitrocellulose and hybridised to ^{32}P-labelled pAT153. An autoradiograph of the washed filter is shown. Solid arrowheads indicate DpnI resistant replicated plasmid DNA (unit length pS1 -4.2 kbp and pST19 -3 kbp) and the open arrowhead indicates DpnI digested input plasmid DNA.

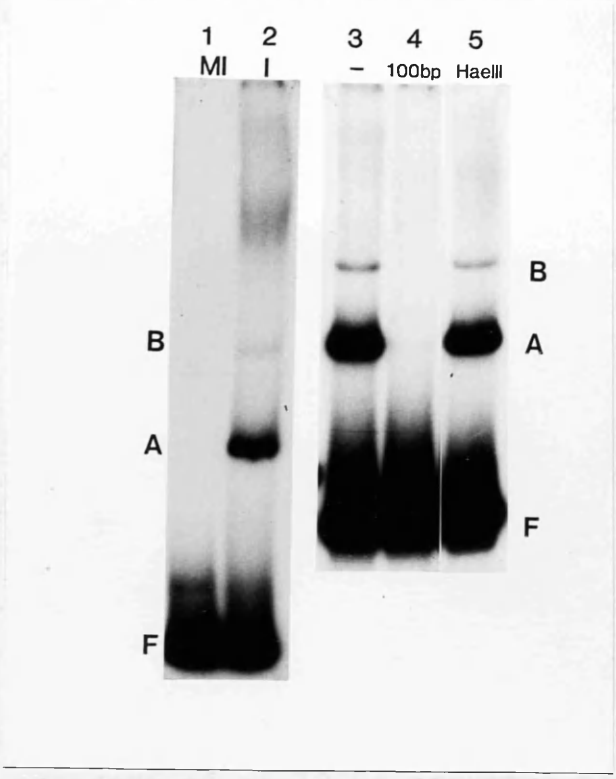


Figure 3.2 Gel retardation analysis of extracts of wt HSV-1 infected cells

Nuclear extract was incubated with ^{32}P end-labelled 100 bp pST19 ori_S fragment either in the absence of competitor DNA (tracks 2 and 3) or in the presence of 100-fold molar excesses of unlabelled 100 bp ori_S fragment (track 4) or HaeIII fragment from the HSV-1 Bam y region containing sequence unrelated to ori_S sequence (track 5). Track 1 contains nuclear extract from mock-infected BHK cells and tracks 2-5, extract from cells infected with wt HSV-1.* The products were separated by electrophoresis in a 8% non-denaturing polyacrylamide gel. "A" and "B" indicate major retarded complexes and "F", the free probe.

* Tracks 1 and 2 contained 5 ug of nuclear extract and tracks 3-5 contained 7.5 ug of nuclear extract.

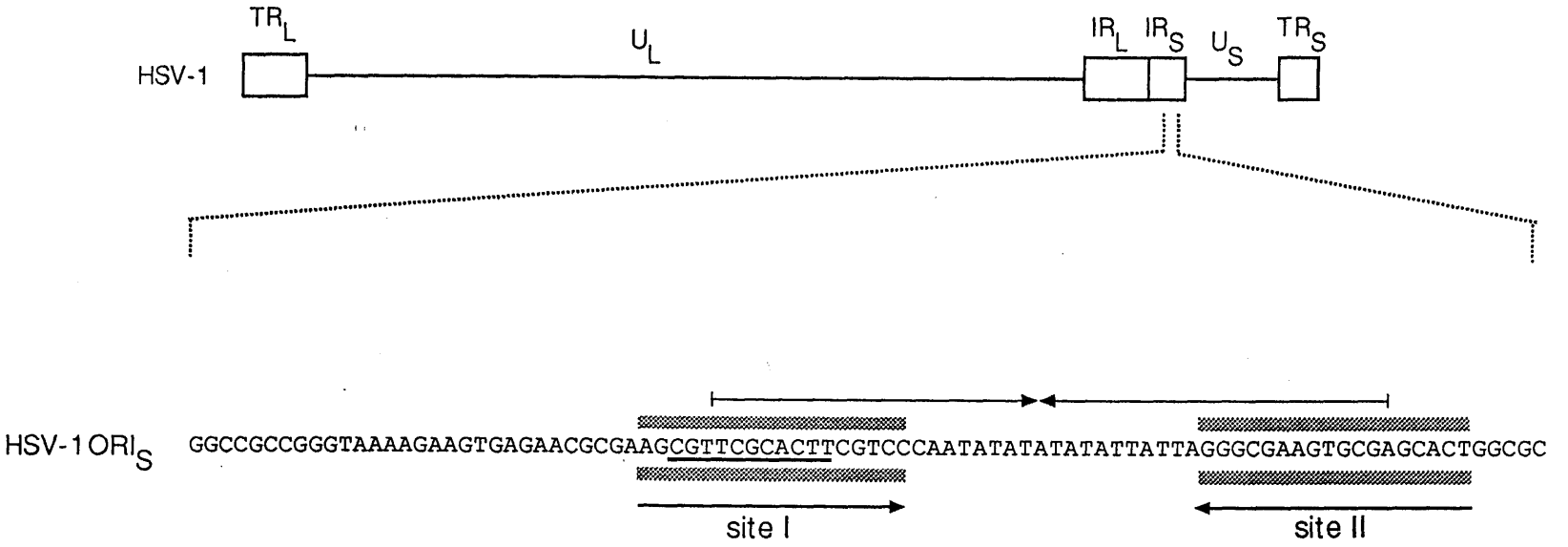
cell extract was increased, band B appeared more prominent (track 3). To verify the specificity of binding, a 100-fold molar excess of the unlabelled ori_S fragment or of a similarily sized HaeIII fragment from the HSV-1 Bam y region containing sequence unrelated to HSV-1 ori_S was added to the assay to compete for specific binding (track 4 and 5). The ori_S but not the HaeIII fragment effectively eliminated bands A and B indicating that these complexes were specific for the ori_S fragment. Thus, the gel retardation assay can identify origin-specific binding using unfractionated nuclear extracts from small numbers of cells.

2. Identification of protein binding sites in HSV-1 ori_S

(a) binding of an HSV-1 induced protein to two sites in ori_S

Using DNase I footprinting, Elias et al. (1986), demonstrated the presence of an 18 bp sequence at the left hand end of the HSV-1 ori_S palindrome which was protected by an HSV-1 encoded protein(s) (see Fig. 3.3 site I). A related sequence is also present in an inverted orientation at the right hand side of the palindrome (Fig. 3.3 site II). To determine whether the complexes A and B in Fig. 3.2 resulted from protein binding to site I and/or site II, double-stranded oligonucleotides containing site I and II sequences were synthesised and tested for their ability to act as competitor DNAs.

The results of these assays are shown in Fig. 3.4. Both site I (track 2) and site II (track 3) oligonucleotides were able to compete for binding to ori_S, resulting in the disappearance of retarded complexes A and B (track 1). This indicates that both sites I and II within ori_S are recognised by the origin binding protein. To further substantiate that binding can occur at sites I and II within ori_S, the double-stranded oligonucleotides were end-labelled and used as probes in a gel retardation assay. Fig. 3.5 demonstrates that both oligonucleotides contain sequence-specific protein binding sites. A single retarded



HSV site I AGCGTTCGCACTTCGTCC

HSV site II AGTGCTCGCACTTCGCCC

Figure 3.3 Location of a further sequence within the HSV-1 ori_S region related to the binding site identified by Elias et al. (1986)

90 bp from the ori_S region are shown (Stow and McMonagle, 1983) and the 45 bp palindrome is indicated by arrows. The shaded region overlapping the left end of the palindrome represents an 18 bp sequence (HSV site I) which is protected from DNase I digestion by an HSV-1 encoded protein(s) (Elias et al., 1986). A related sequence is also found in an inverted orientation at the right end of the palindrome (HSV site II). Site I and site II aligned sequences are shown below with mismatches marked by dots. The underlined region in site I represents an 11 bp sequence which is conserved in HSV-1 ori_L, HSV-2 ori_S, HSV-2 ori_L and VZV ori_S.

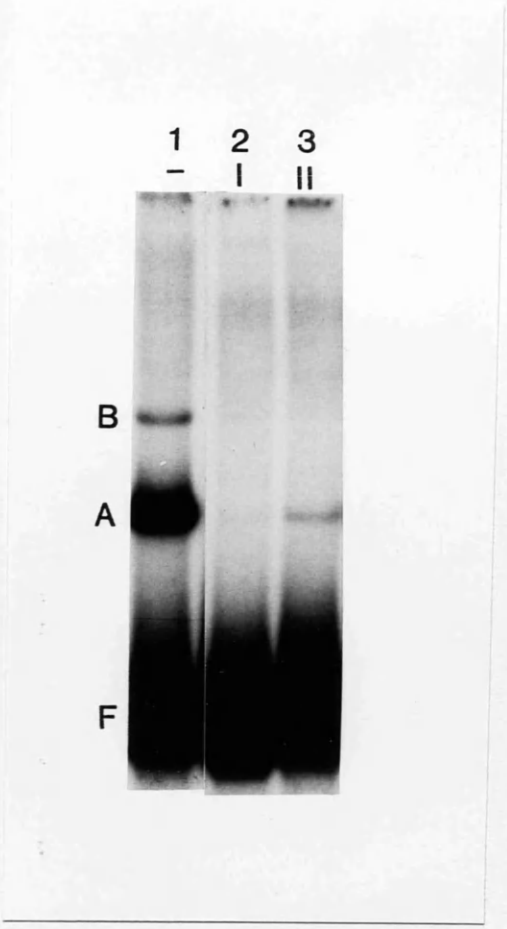


Figure 3.4 Competition of binding of HSV-1 encoded protein to HSV-1 ori_S by ds oligonucleotides

Nuclear extract from cells infected with wt HSV-1 was incubated with ^{32}P-labelled pST19 ori_S fragment in the absence of competitor DNA (track 1) or in the presence of 100-fold molar excesses of unlabelled ds oligonucleotides I or II containing sequences from ori_S site I (track 2) or site II (track 3) respectively.

Oligonucleotide I : 5' GATCCGCGAAGCGTTCGCACTTCGTCCCA
GCGCTTCGCAAGCGTGAAGCAGGGTCTAG 5'

OLigonucleotide II : 5' GATCTGGGGCGAAGTGCGAGCACTTCGCG
ACCCCGCTTCACGCTCGTGAAGCGCCTAG 5'

Underlined residues correspond to those shown in Fig. 3.3. The products were separated by electrophoresis in a 8% non-denaturing polyacrylamide gel. "A" and "B" indicate retarded complexes and "F", the free probe.

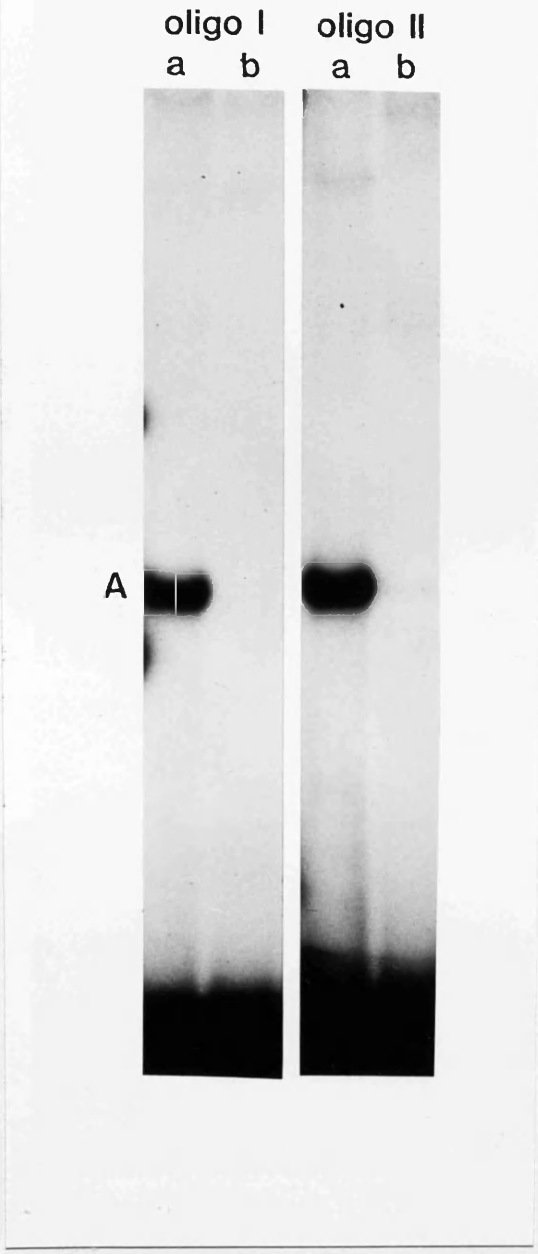

oligo I
a b
oligo II
a b
A

Figure 3.5 Gel retardation analysis of extracts of wt HSV-1 infected cells using oligonucleotide probes

Nuclear extract was incubated with ^{32}P-labelled oligonucleotide I (Fig. 3.4) or oligonucleotide II (Fig. 3.4) either in the absence (tracks a) or in the presence of 100-fold molar excess of unlabelled oligonucleotide I (tracks b). The products were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. "A" indicates a retarded complex observed with both oligonucleotides

complex was seen with both oligonucleotide I and oligonucleotide II (tracks 1 and 3). Adding a 100-fold molar excess of the 100 bp ori_S fragment effectively competed for binding to both sites (tracks 2 and 4). These results (Weir et al., 1989) map a binding site (site II) not initially identified by Elias et al. (1986) and are in agreement with subsequent work by Olivo et al. (1988), Elias et al. (1988) and Koff and Tegtmeyer (1989). Furthermore, they suggest that complexes A and B observed with the 100 bp ori_S fragment represent binding to a single site (either site I or II) and both sites respectively.

(b) comparison of the binding affinity of sites I and II

Competition experiments were carried out to determine the relative binding affinity of the ori_S binding activity observed in HSV-1 infected cells for sites I and II. In these experiments, a different protocol was used for the preparation of nuclear extracts. This procedure, essentially as described by Dignam et al. (1983) and modified by Preston et al. (1988) {method B}, avoided $(NH_4)_2SO_4$ precipitation of extracted proteins and was found to give higher reproducibilty and consistency in the yield and activity of ori_S binding protein. It was used for all subsequent experiments unless otherwise stated.

Nuclear extract from HSV-1 infected cells was incubated with labelled oligonucleotides I or II in the presence of increasing amounts of either unlabelled oligonucleotide. Fig. 3.6 shows competition of binding to labelled site I oligonucleotide in the presence of a 2.5, 25, 50 and 250-fold molar excess of unlabelled oligonucleotides I and II (tracks 1-10). Using oligonucleotide I as competitor, a 25-fold molar excess almost eliminated the signal intensity of complex A (track 3) while a 250-fold molar excess of oligonucleotide II was required to produce a similar reduction in signal intensity (track 10). These results indicate that the origin binding protein has a higher affinity for binding site I than site II. Comparison of the two competitors therefore suggests an approximate 10-fold difference in binding affinity between

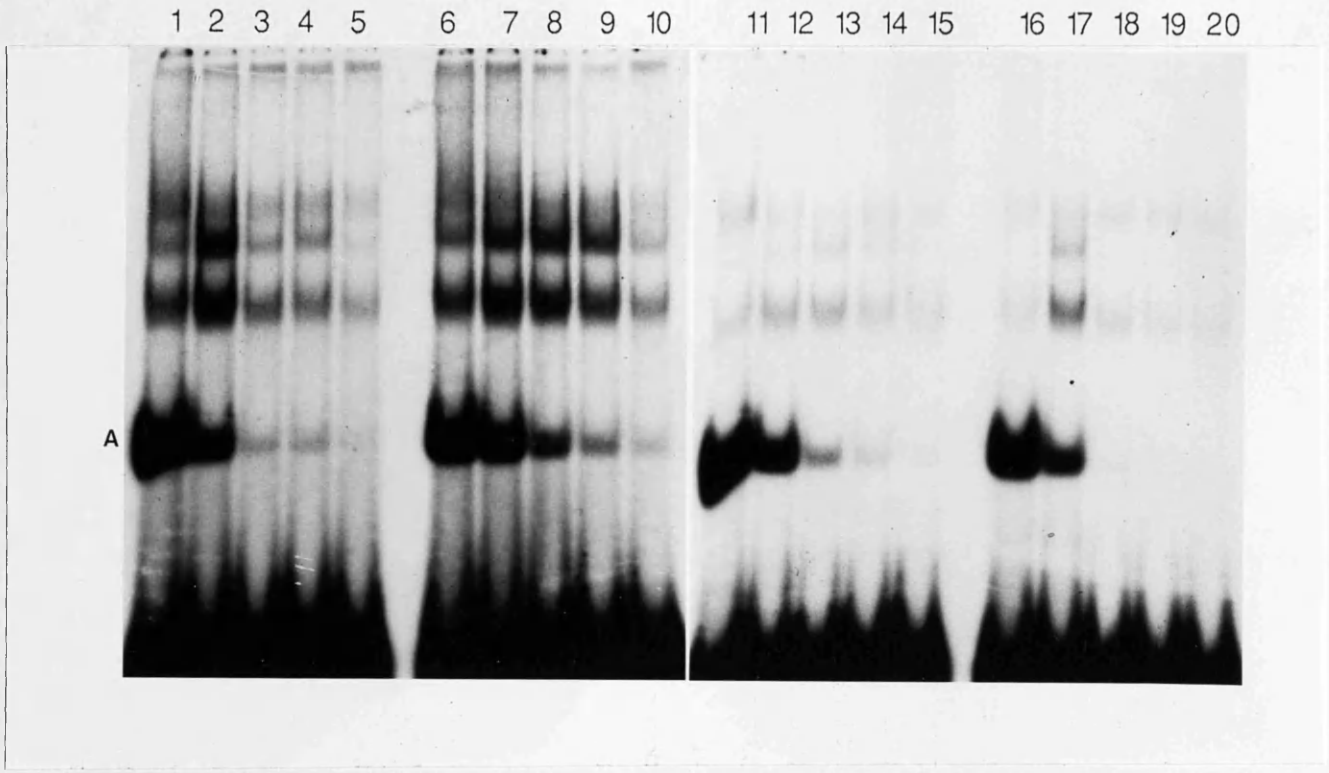

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
A

Figure 3.6 Comparison of the relative binding affinity of the origin binding activity in wt HSV-1 infected cells for sites I and II

(a) Nuclear extract was incubated with ^{32}P-labelled oligonucleotide I either in the absence of competitor DNA (tracks 1 and 6) or in the presence of increasing amounts of unlabelled oligonucleotide I (tracks 2-5) or II (tracks 7-10): tracks 2 and 7 =2.5-fold molar excess; tracks 3 and 8 =25-fold molar excess; tracks 4 and 9 =50-fold excess; tracks 5 and 10 =250-fold excess.

The products were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. "A" indicates retarded complexes.

(b) The reciprocal experiment was carried out in which nuclear extract was incubated with ^{32}P-labelled oligonucleotide II either in the absence of competitor DNA (tracks 11 and 16) or in the presence of increasing amounts of unlabelled oligonucleotide II (tracks 12-15) or I (tracks 17-20): tracks 12 and 17 =2.5-fold molar excess; tracks 13 and 18 =25-fold molar excess; tracks 14 and 19 =50-fold excess; tracks 15 and 20 =250-fold excess. The assays were electrophoresed as described in part (a).

site I and II.

Similar results were obtained in the reciprocal experiment where competition of protein binding to site II labelled sequences was investigated (tracks 11-20). A 250-fold molar excess of unlabelled oligonucleotide II was required to almost eliminate the formation of complex A whereas a 25-fold molar excess of oligonucleotide I produced a similar effect (tracks 15 and 18).

Lower mobility complexes were also present in these assays and probably arise as a consequence of the modification in the preparation of nuclear extracts. These lower mobility bands do appear to be affected by the presence of a specific competitor. In addition, the relative amount of these bands varied considerably from extract to extract whereas the major small retarded fragment (complex A) was always prevalent. The significance of these other complexes will be discussed in section 4.

3. Characterisation of origin binding activty

(a) time course of origin binding protein (OBP) production in HSV-1 infected cells

To ascertain at which point in the virus lytic cycle the origin binding activity was synthesized, BHK cells were either mock-infected or infected with HSV-1 strains 17*syn*$^+$ or KOS and cell extracts made at various time intervals after infection. These extracts were assayed for binding activity using oligonucleotide I as probe. Two HSV-1 strains were tested to ensure that the binding activity observed with Glasgow strain 17*syn*$^+$ was not due to some unique property of this virus strain. Because other laboratories (e.g. Elias *et al.*, Challberg *et al.*) were examining origin binding activity of strain KOS, this strain was used for comparison with strain 17 *syn*$^+$.

Fig. 3.7 shows the binding activity in mock-infected and infected cells harvested at 3, 6, 9, 12, 18 and 24 h after virus addition. No significant differences were observed in the binding pattern obtained with the two viruses. Complex A was detected at 3 h after

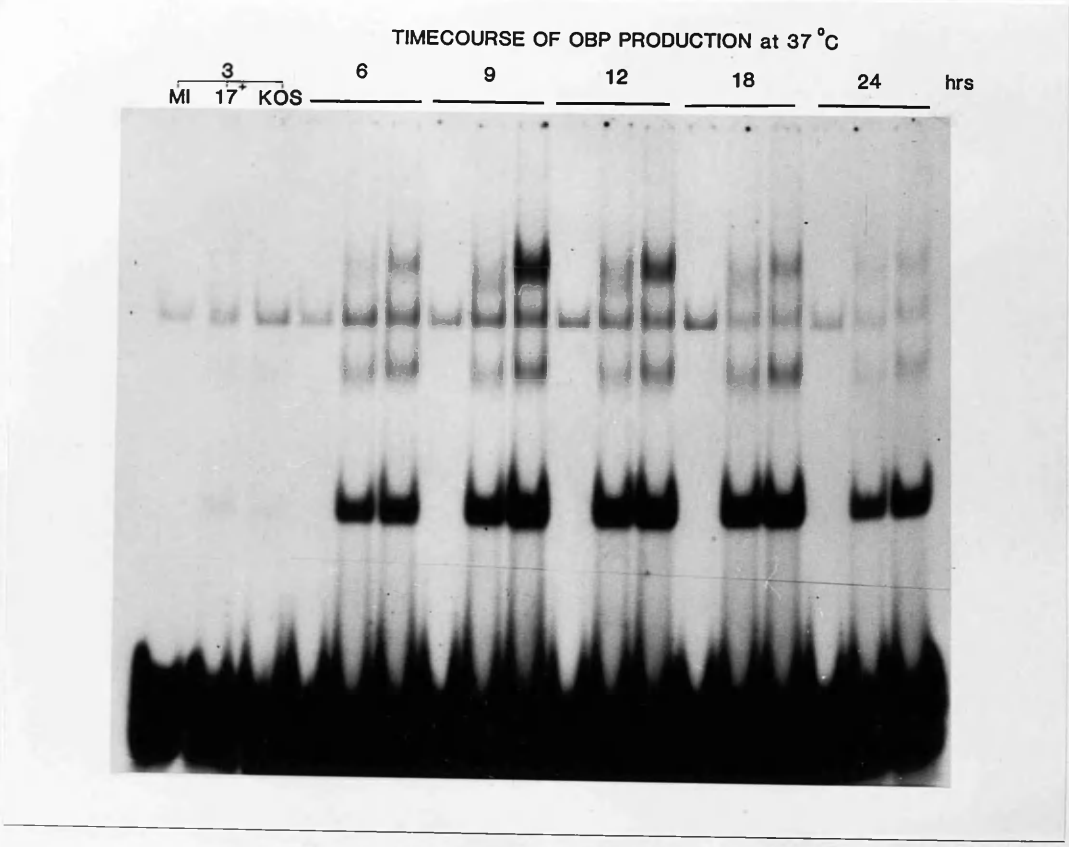


Figure 3.7 Gel retardation analysis of extracts from wt HSV-1 strain 17 syn$^+$ and KOS at various times after infection

Nuclear extracts were prepared from either mock-infected (MI) cells, or cells infected with wt HSV-1 Glasgow strain 17 syn$^+$ (17$^+$) or wt HSV-1 strain KOS (KOS) at 3, 6, 9, 12, 18 and 24 h after infection at 37^{o}C. The extract was incubated with ^{32}P-labelled oligonucleotide I and the products separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. The order of the three samples for the 6, 9, 12, 18 and 24 h time points is the same as for 3 h.

* The slowest migrating complex in KOS extracts appeared to be more prevalent and migrated more slowly than the slowest migrating complex in 17 $\underline{syn}^+$ tracks. This might reflect a difference in the level of proteolytic activity between KOS and 17 syn^+ extracts.

virus addition and was produced in maximum amounts at 6-9 h. The activity subsequently remained at a stable level throughout the time course with a slight drop in activity at 24h. Of the lower mobility complexes previously observed (Fig. 3.6), the middle band appears to be host specific while the others are virus induced.* The early detection of origin binding activity suggests that the binding activity may be encoded by an IE or E gene.

(b) the origin binding activity is not synthesised in cells infected with tsK at NPT

At NPT, the HSV-1 mutant tsK (Preston, 1979a) overproduces IE polypeptides and RR1 yet fails to activate later classes of viral genes due to the synthesis of a non-functional Vmw175 polypeptide. Nuclear extracts were made (using method A) from either mock-infected cells or cells infected with tsK at 31°C and 38.5°C and analysed using a gel retardation assay. The results are shown in Fig. 3.8(a). At the permissive temperature, binding activity was detected in tsK infected cells (track a; 31°C). However, at 38.5°C no activity was detected in tsK-infected cells (track a; 38.5°C) whereas binding activity is observed in cells infected with wt at 38.5°C (see Fig. 3.11 track 2 at 38.5°C). This indicates that the binding activity is not due to one of the functional IE polypeptides synthesised in tsK-infected cells and suggests that it is probably encoded by an early or late viral gene or may possibly be a function of the Vmw175 polypeptide. The early appearance (3 h pi) of binding activity and its kinetics of synthesis suggest that the protein may be synthesised as an early gene product, consistent with a presumed role in DNA synthesis.

(c) binding activity in HSV-2 infected cell extracts

Extracts made from cells infected with HSV-2 strain HG52 were analysed for binding to site I oligonucleotide. Fig. 3.8(b) shows that a retarded complex is observed in HSV-2 infected cell extract which exhibits the same mobility as complex A formed with HSV-1 infected cell extract. The origin binding activity is therefore encoded by both virus

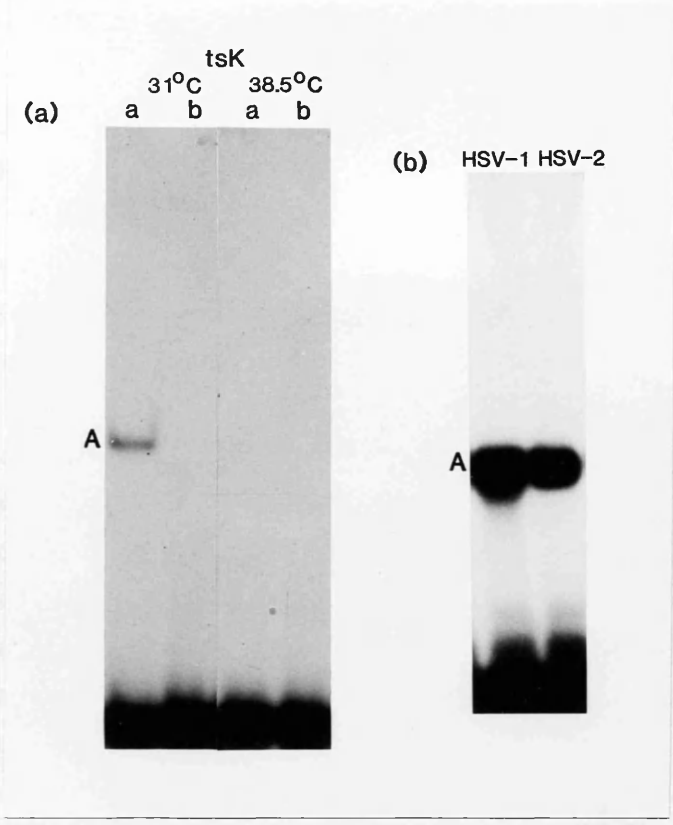


Figure 3.8 Gel retardation analysis of extracts from cells infected with tsK at 31°C and 38.5°C and HSV-1 and HSV-2 at 37°C.

(a) Nuclear extract was prepared from cells infected with tsK at 31°C and 38.5°C and incubated with ^{32}P-labelled oligonucleotide I either in the absence (tracks a) or in the presence of 100-fold molar excess of unlabelled oligonucleotide I (tracks b). The products were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. "A" represents a retarded complex.

(b) Nuclear extract was prepared from cells infected with wt HSV-1 Glasgow strain 17syn$^+$ or wt HSV-2 strain HG52 at 37°C and incubated with ^{32}P-labelled oligonucleotide I. The products were electrophoresed as above.

types. The failure to detect a mobility difference between HSV type 1 and 2 complexes unfortunately ruled out the possibilty of using intertypic recombinants to map the gene encoding the origin binding activity.

(d) estimation of the molecular weight of the OBP complex in HSV-1 infected cells

A technique involving electrophoresis of protein-DNA complexes on non-denaturing polyacrylamide gels of graded porosity has previously been used to obtain crude estimates of the molecular weights of protein-DNA complexes (Huet and Sentenac, 1987; van Huijsduijnen et al., 1987). The principle of the separation technique is that the protein/DNA complex migrates through progressively smaller pores (regulated by the gel concentration) and finally stops and concentrates where the pore size is too small to allow further migration. Separation at this stage is related to size, although in the earlier stages of the gel, the separation is based on both charge and size (Anderson et al., 1972).

This method was used to estimate the size of complex A. HSV-1 infected cell extract was incubated with labelled oligonucleotide I and complexes separated on a 5-25% gradient gel along with pre-stained molecular weight markers. Electrophoresis was continued until the protein-DNA complexes stopped migrating (approximately 36 h at $4^{o}C$). Under these conditions the unbound DNA consistently ran off the bottom of the gel. By comparison with gels which were electrophoresed for shorter time periods, the fastest migrating complex shown in Fig. 3.9 corresponds to complex A. The other bands located towards the top of the gel represent the slow migrating complexes previously seen in Fig. 3.7. By comparison with the molecular weight markers, the migration of complex A corresonds to it having an approximate molecular weight of 45,000. Given that approximately 20kD is contributed by the weight of the double-stranded oligonucleotide, a crude estimate of the molecular weight of the protein fraction of this complex is around 25,000.

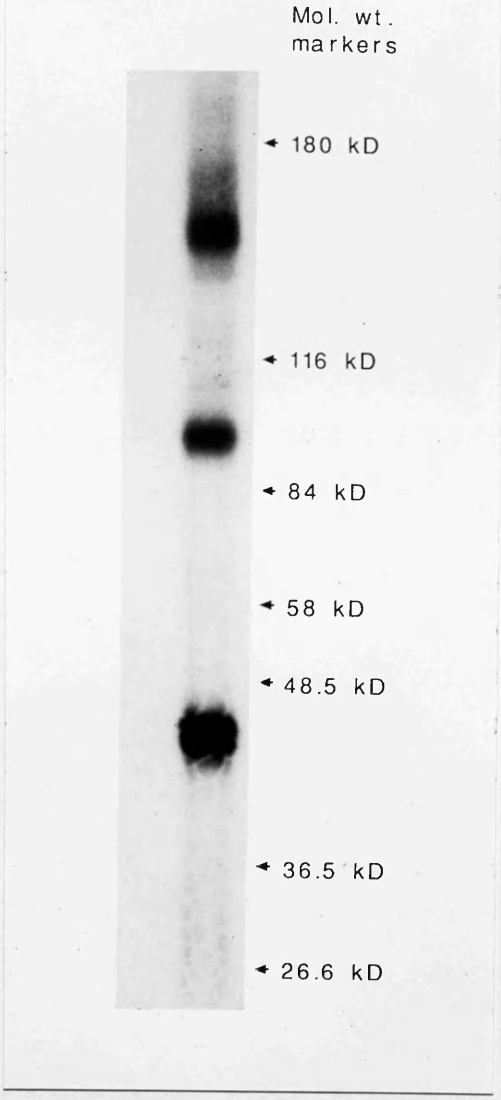

Mol. wt.
markers
180 kD
116 kD
84 kD
58 kD
48.5 kD
36.5 kD
26.6 kD

Figure 3.9 Estimation of the molecular weight of the OBP complex in HSV-1 infected cells

Nuclear extract from HSV-1 infected cells was incubated with ^{32}P-labelled oligonucleotide I and the products were separated by electrophoresis in a 5-25% gradient non-denaturing polyacrylamide gel along with pre-stained molecular weight markers which were as follows: 26.6 kD - triosephosphate isomerase; 36.5 kD - lactic dehydrogenase; 48.5 kD - fumarase; 58 kD - pyruvate kinase; 84 kD - fructose-6-phosphate kinase; 116 kD - beta-galactosidase; 180 kD - alpha$_2$macrogobulin. The products were electrophoresed until the protein-DNA complexes stopped migrating (approximately 36 h at 4°C) and the gel was then fixed and exposed to autoradiography film. Under the conditions of electrophoresis, the free DNA ran off the bottom of the gel, hence the fastest migrating complex corresponds to complex A (see Fig. 3.7). The positions of the molecular weight markers in the gel are indicated by arrowheads.

SECTION 3B : Identification and expression of the HSV-1 gene encoding OBP

Having developed the gel retardation assay for detecting OBP, I next attempted to identify the HSV-1 gene which encoded this activity. At about this time (mid 1987) I learned that Mark Challberg's laboratory had shown that a set of seven HSV-1 genes (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) were necessary and sufficient for replication of an HSV ori$_S$-containing plasmid (subsequently published by Wu et al., 1988). It seemed likely that OBP might play an essential role in HSV DNA replication and be encoded by one of these seven genes. Furthermore, previous studies on the products of UL29 (dbp), UL30 (pol) and UL42 (dsDBP) (see section 1C) suggested that these proteins did not function as sequence-specific DNA binding proteins. Thus, the most likely candidates for encoding OBP were UL5, UL8, UL9 and UL52, the products of which, at that time, had not been identified in HSV infected cells. Several approaches were adopted to identify which, if any, of the genes essential for DNA replication encoded the origin binding activity.

1. Unsuccessful attempts at identifying the HSV-1 gene encoding OBP

(a) analysis of HSV-1 DNA negative ts mutants with lesions in six of the seven replication genes

Six DNA negative ts mutants were available whose lesions mapped to the following replication genes: tsO (UL5), tsS (UL9), ts1205 (UL30; pol), ts1206 (UL52), ts1234 (UL42) and ts1235 (UL29; dbp). No ts mutant mapping to the UL8 gene was available. I decided to assay nuclear extracts from cells infected with these mutants at permissive and NPT for origin binding activity. The success of this approach would depend upon the presence of a ts mutation within the gene encoding OBP leading to the synthesis at NPT of a polypeptide affected in DNA binding activity.

To confirm their DNA negative phenotypes, the ts mutants were tested for their ability to amplify plasmid pST19 (containing a functional HSV-1 ori_S sequence) at permissive (31°C) and non-permissive (38.5°C and 39.5°C; NPT) temperatures. Cells were transfected with pST19 and either mock-infected or superinfected with each ts mutant or wt HSV-1 to provide helper functions. Samples of DNA extracted from the cells 16 h pi were cleaved with EcoRI and DpnI. Replicated plasmid molecules were detected by Southern blotting using ^{32}P-labelled pAT153 DNA as probe. Each mutant was able to replicate pST19 as well as wt HSV-1 at 31°C (Fig. 3.10 tracks 2-8 at 31°C). At 38.5°C, tsO, tsS and ts1235 were unable to support amplification of pST19 (tracks 3, 4 and 8 at 38.5°C) while ts1205, ts1206 and ts1234 produced low levels of plasmid amplification compared to wt (tracks 5, 6, 7 and 2 at 38.5°C). At 39.5°C, only the ts mutant mapping to UL52, ts1206 exhibited detectable amplification (track 6 at 39.5°C) albeit at a lower level than wt HSV-1 (track 2 at 39.5°C). Thus, all ts mutants were clearly impaired in DNA synthesis at NPT but some exhibited a slightly leaky phenotype at 38.5°C.

Nuclear extracts from cells infected with individual ts mutants at 31°C, 38.5°C and 39.5°C were incubated with labelled oligonucleotide I and resolved on polyacrylamide gels (Fig. 3.11). Nuclear extracts from cells infected with ts mutants at 31°C contained similar levels of binding activity to wt HSV-1 (tracks 2-8 at 31°C). At 38.5°C, all mutants exhibited some binding activity although, in each case, this was lower than that seen with an extract from cells infected with wt HSV-1 at 38.5°C (tracks 2-8 at 38.5°C). A similar situation was observed at 39.5°C (tracks 2-8 at 39.5°C).

These results suggest that either:

(1) none of the mutants tested has a lesion in the gene specifying origin binding activity i.e. the gene not tested, UL8, may encode this function, or

(2) one of the mutations does lie in the gene encoding OBP but the lesion does not disrupt its binding activity. This might occur if the polypeptide

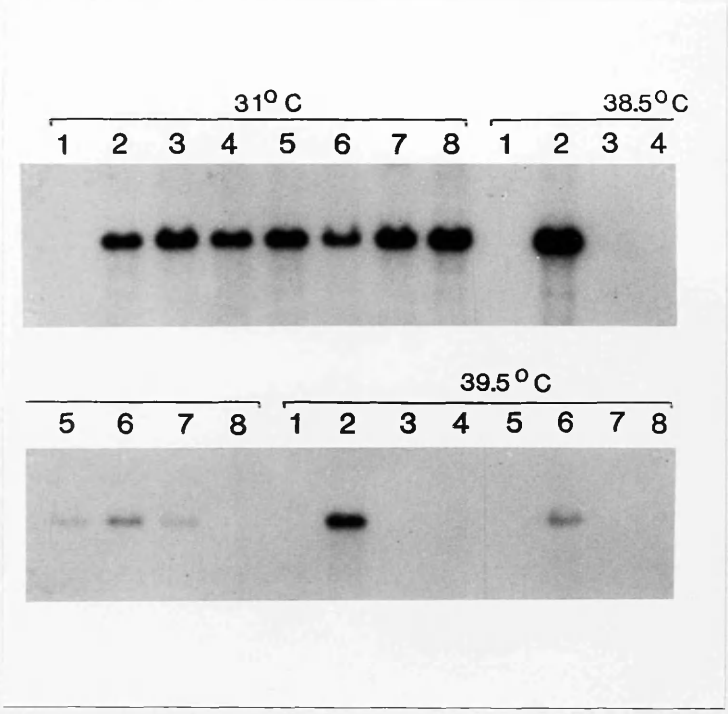


Figure 3.10 Replication of an ori_S-containing plasmid by trans-acting products from HSV-1 DNA negative ts mutants

BHK cells were transfected with pST19 and either mock-infected (tracks 1) or superinfected with wt HSV-1 or HSV-1 DNA negative ts mutants as follows: wt HSV-1 (tracks 2); tsO [UL5] (tracks 3); tsS [UL9] (tracks 4); ts1205 [UL30] (tracks 5); ts1206 [UL52] (tracks 6); ts1234 [UL42] (tracks 7); ts1235 [UL29] (tracks 8). Cells were subsequently incubated for 16 h at 31°C, 38.5°C or 39.5°C as indicated. 1/15 of the DNA recovered from each monolayer was digested with EcoRI plus DpnI and the fragments separated by electrophoresis, transferred to nitrocellulose and hybridised to ^{32}P-labelled pAT153. An autoradiograph of the washed filter is shown.

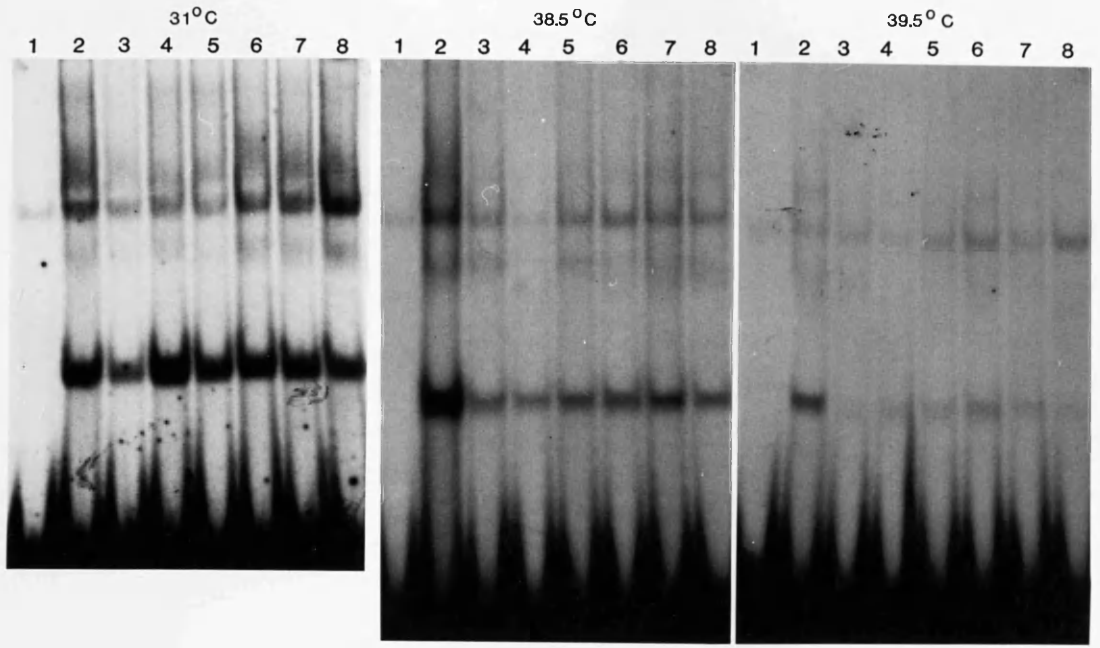

31°C
1 2 3 4 5 6 7 8
38.5°C
1 2 3 4 5 6 7 8
39.5°C
1 2 3 4 5 6 7 8

Figure 3.11 Gel retardation analysis of extracts from cells infected with individual HSV-1 DNA negative ts mutants

Nuclear extracts were prepared from either mock-infected cells (tracks 1) or cells infected with wt HSV-1 or HSV-1 DNA negative ts mutants: wt HSV-1 (tracks 2); tsO (tracks 3); tsS (tracks 4); ts1205 (tracks 6); ts1234 (tracks 7); ts1235 (tracks 8). Cells were subsequently incubated at 31°C, 38.5°C and 39.5°C for 8 h as indicated. Extracts were incubated with ^{32}P-labelled oligonucleotide I and the products separated by electrophoresis in a 5% non-denaturing polyacrylamide gel.

synthesised at NPT regained some activity under the conditions of the binding assay or if the ts lesion disrupted some function of the polypeptide other than its ability to bind to ori_S.

Attempts were made to test the thermal stability of the OBP present in extracts from cells infected with wt HSV-1 and ts mutants at 31°C, but no significant differences between the ts mutants were observed in these experiments. Attempts to increase the binding temperature to 39°C were unsuccessful and wt origin binding activity could not be consistently detected under these conditions.

(b) detection of origin binding activity in cells infected with XbaI-digested HSV-1 DNA

Challberg (1986) demonstrated that a set of five plasmids, containing XbaI fragments of HSV-1 DNA, enabled amplification of an ori_S-containing plasmid in transfected tissue culture cells. Thus, XbaI did not inactivate any of the replication genes or IE genes required for early gene expression. His laboratory then sub-cloned these fragments and seven plasmids containing individual HSV-1 genes were identified, which contained all necessary trans-acting functions required to replicate an origin-containing plasmid, provided that plasmids encoding IE gene products were supplied to allow their expression (Wu et al., 1988).

I therefore performed transfection experiments to determine whether ori_S-binding activity could be detected in nuclear extracts from cells transfected with plasmids containing individual replication genes and activated by one or more IE genes. To test the validity of this approach, cells were transfected with 10 ug of XbaI-digested HSV-1 DNA and nuclear extracts prepared 24 h later. Fig. 3.12 shows the formation of complex A with labelled oligonucleotide I in the presence of nuclear extract from these cells (track 3). This activity was competed by an excess of unlabelled oligonucleotide I (track 4). No specific activity was seen in control transfected cell extracts (track 1). To check that the HSV-1 DNA was digested to completion, samples of digested DNA were analysed on agarose gels. In addition, no

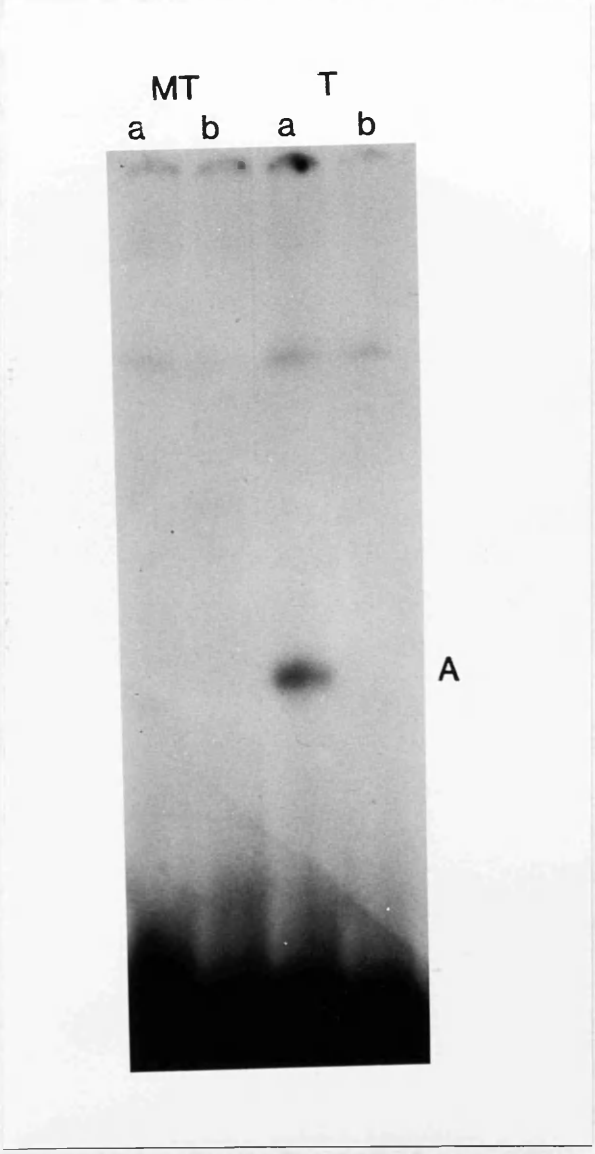


Figure 3.12 Gel retardation analysis of extracts from cells transfected with XbaI-digested HSV-1 DNA

BHK cells were transfected with 15 ug of calf thymus carrier DNA (MT) or 5 ug of calf thymus DNA plus 10 ug of XbaI-digested DNA (T) and nuclear extracts prepared 24 h later. The extract was incubated with ^{32}P-labelled oligonucleotide I either in the absence (tracks a) or presence of a 100-fold excess of unlabelled oligonucleotide I (tracks b). The products were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. "A" indicates a retarded complex.

progeny virus was detected on plates transfected in parallel, therefore the OBP activity observed in Fig. 3.12 was not due to the replication and spread of progeny virus.

Having established that it was possible to detect origin binding activity in cells transfected with XbaI-digested HSV-1 DNA, experiments were carried out whereby cells were co-transfected with plasmids pGX58 and pJR3 (IE-3 and IE-1 gene) and pGX25 (UL5 gene + UL8 gene) or pGX125 (UL8 gene + UL9 gene) or pNN1 (UL29 gene) or pNN3 (UL30 gene) or p9-4 (UL42 gene) or pGX94 (UL52 gene). Despite testing various transfection conditions, no origin binding activity was detected with any of these combinations. Possible explanations for the failure to observe activity using this approach are discussed in section 4.

2. Origin binding activity induced by tsK recombinant viruses

(a) identification of the HSV-1 gene encoding OBP

Four tsK recombinant viruses, tsK/UL5, tsK/UL8, tsK/UL9 and tsK/UL52 were obtained from J. Calder in our laboratory. The construction of these viruses is briefly outlined in Fig. 3.13. The genes, UL5, UL8, UL9 and UL52 were individually recombined into the genome of the HSV-1 ts mutant, tsK, under the control of the IE-3 gene promoter. At 38.5°C, tsK accumulates IE polypeptides but is unable to induce the expression of early and late genes (Preston, 1979a; Watson and Clements, 1980). Labelled polypeptides synthesised at 38.5°C by the recombinant viruses are indistinguishable from tsK, except for a single additional polypeptide corresponding to the product of the inserted gene (J.M. Calder, unpublished results). Since these viruses overexpress the UL5, UL8, UL9 and UL52 gene products (and had in fact initially allowed their identification) I decided to test whether any of these viruses might induce origin binding activity. This approach was possible because tsK is negative for OBP at NPT {Fig. 3.8(a)}.

BHK cells were infected with wt HSV-1, tsK or the

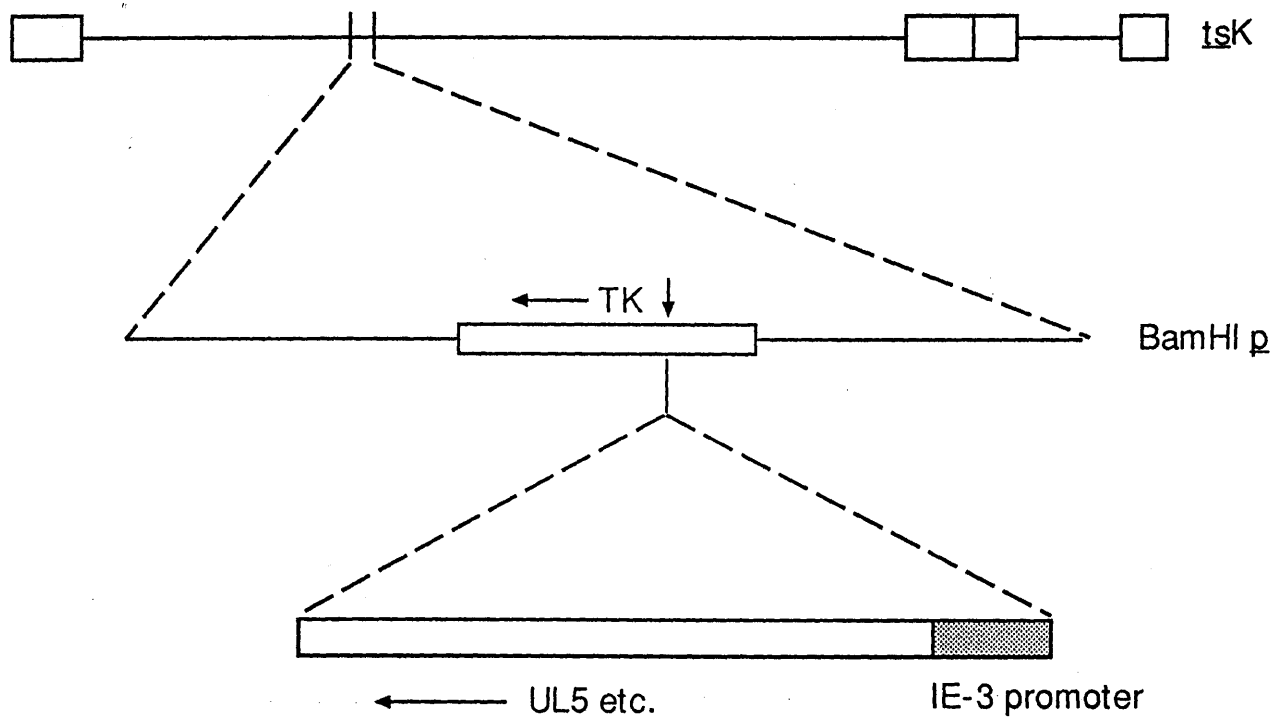

tsK
TK
BamHI p
UL5 etc.
IE-3 promoter

Figure 3.13 Construction of tsK recombinant viruses
(J. M. Calder)

The figure shows a schematic diagram of the construction of tsK recombinant viruses individually expressing four of the HSV-1 replication genes: UL5, UL8, UL9 and UL52. The coding sequences of the genes were individually linked to a copy of the IE-3 promoter and placed within the coding sequences of a plasmid copy of the HSV-1 TK gene (contained within HSV-1 BamHI p) which was then recombined with tsK DNA at the permissive temperature. TK minus recombinants were isolated and screened for the presence of the inserted gene. Labelled polypeptides synthesised at 38.5°C by the recombinant viruses are indistinguishable from tsK, except for a single additional polypeptide corresponding to the product of the inserted gene (J.M. Calder, unpublished results).

four recombinant viruses at 38.5°C and nuclear extracts prepared 8 h later. The extracts were analysed in a gel retardation assay using labelled oligonucleotide I as probe {Fig. 3.14(a)}. No DNA binding activity was observed in either mock-infected or tsK-infected cell extracts (tracks 1 and 3) apart from a weak host band which was evident in all tracks. As previously observed, extracts from cells infected with wt HSV-1 generated a major retarded complex, A, and some minor bands (track 2). The only other extract which caused retardation of the probe was from cells infected with the tsK/UL9 recombinant virus (track 6). Despite the difference in binding pattern observed between this virus and wt HSV-1 (i.e. the presence of material which failed to enter the gel and a smeared retardation throughout the length of the track), similar patterns were consistently observed with the tsK/UL9 recombinant alone. As with wt HSV-1 extract, diffuse bands of lower mobility were observed whereas a band co-migrating with complex A was notably absent.

Fig. 3.14(b) shows that the pattern of binding seen with the tsK/UL9 recombinant virus is sequence-specific. The addition of a 100-fold excess of unlabelled oligonucleotide I but not oligonucleotide X (which is of similiar size to oligonucleotide I but of unrelated DNA sequence) efficiently competed for binding to labelled DNA (tracks 2 and 3).

As the tsK/UL9 recombinant differs from tsK only in its ability to express the UL9 gene product at the NPT, these results indicate that the UL9 polypeptide is responsible for the observed sequence-specific binding to oligonucleotide I. However, because the binding pattern was different from wt HSV-1, it was not possible to conclude that UL9 was responsible for formation of complex A.

(b) effect of protease digestion on the binding activity from cells infected with the tsK/UL9 recombinant

It was rather surprising that the pattern of retardation observed using extracts from cells infected with the tsK/UL9 recombinant was very different from that seen

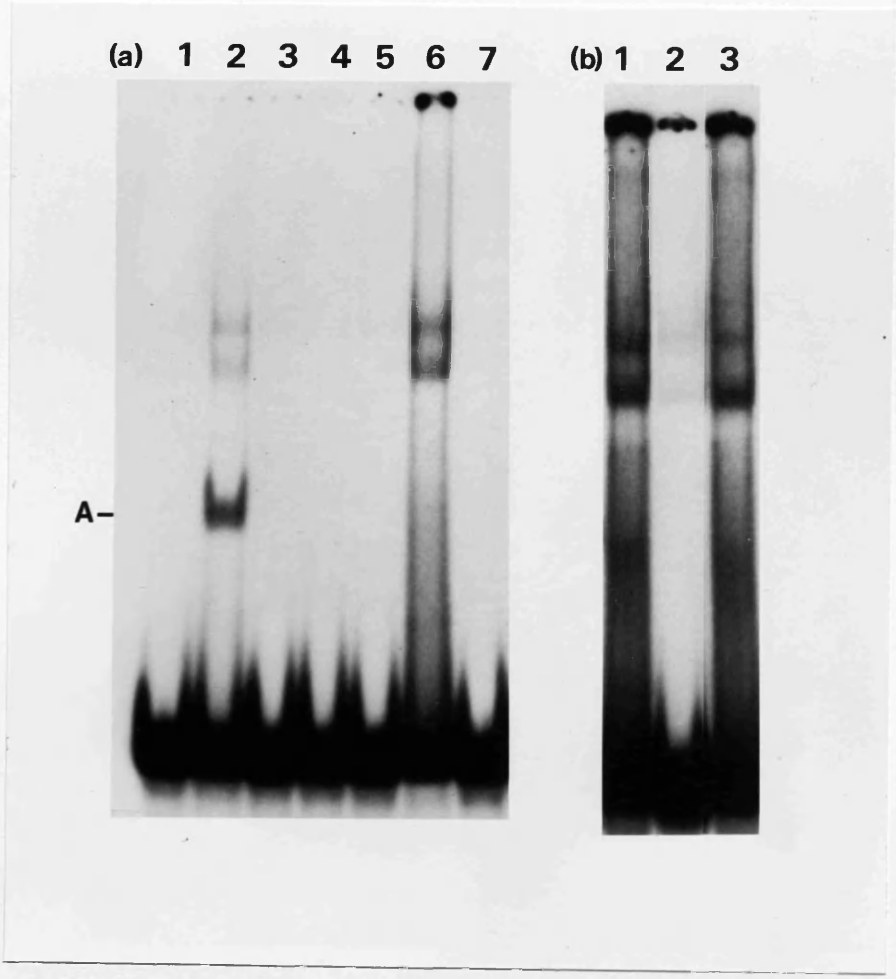

(a) 1 2 3 4 5 6 7
A–
(b) 1 2 3

Figure 3.14 Gel retardation analysis of extracts from cells infected with tsK recombinant viruses

(a) Nuclear extract was prepared from cells incubated at 38.5°C and reacted with ^{32}P-labelled oligonucleotide I. Track 1, mock-infected cells; tracks 2-7, cells infected with wt HSV-1, tsK, tsK/UL5, tsK/UL8, tsK/UL9 or tsK/UL52 respectively. Products were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. "A" indicates the major complex detected with wt HSV-1 extract.

(b) Nuclear extract from cells infected with tsK/UL9 was incubated with ^{32}P-labelled oligonucleotide I either in the absence of competitor DNA (track 1) or in the presence of 100-fold molar excess of oligonucleotide I (track 2) or oligonucleotide X containing an unrelated sequence (track 3).

Oligonucleotide X : 5' GATCGGATATGCTAATTAAATACAT
GCCTATACGATTAATTTATGTG 5'

with extracts from wt HSV-1 infected cells. However, experiments using pore-gradient gel electrophoresis, to ascertain the molecular weight of complex A, had suggested a molecular weight of around 45,000, clearly inconsistent with the binding of an intact UL9 polypeptide (predicted molecular weight of 94,000) to oligonucleotide I. One possible explanation was that complex A might contain only a fragment of the UL9 polypeptide. To examine this possibility, proteinase K was added to binding reactions containing tsK/UL9 nuclear extract and labelled oligonucleotide I and the resulting complexes analysed as before (Fig. 3.15). Addition of proteinase K to the binding reactions caused a reduction in the amount of lower mobility complexes and the simultaneous appearance of a faster migrating species (tracks 4 and 5). This species had a similar electrophoretic mobility to that of complex A (track 7). No such faster migrating complex was detected when extracts from mock-infected cells or cells infected with tsK were similarily treated with proteinase K (data not shown). These experiments therefore suggest that the addition of proteinase K to extracts containing the UL9 polypeptide results in the cleavage of the polypeptide and production of a relatively protease-resistant fragment, which retains DNA-binding activity, and produces a complex of comparable size to the complex found using extracts from cells infected with wt HSV-1.

(c) demonstration that complex A formed with wt HSV-1 infected cell extract contains the UL9 polypeptide

Antibodies to UL5, UL8, UL9 and UL52 gene products were kindly provided by Dr. M. D. Challberg. The rabbit antisera were raised against decapeptides from the carboxy termini (C-termini) of the gene products and were known to be reactive with the corresponding polypeptides (Olivo et al., 1988). Following a 10 min incubation of nuclear extract from cells infected with wt HSV-1 with labelled oligonucleotide I, antibodies reactive with UL5, UL8, UL9 or UL52 polypeptides were added to the reactions and after a further 10 min incubation, the products were analysed on a native polyacrylamide gel.

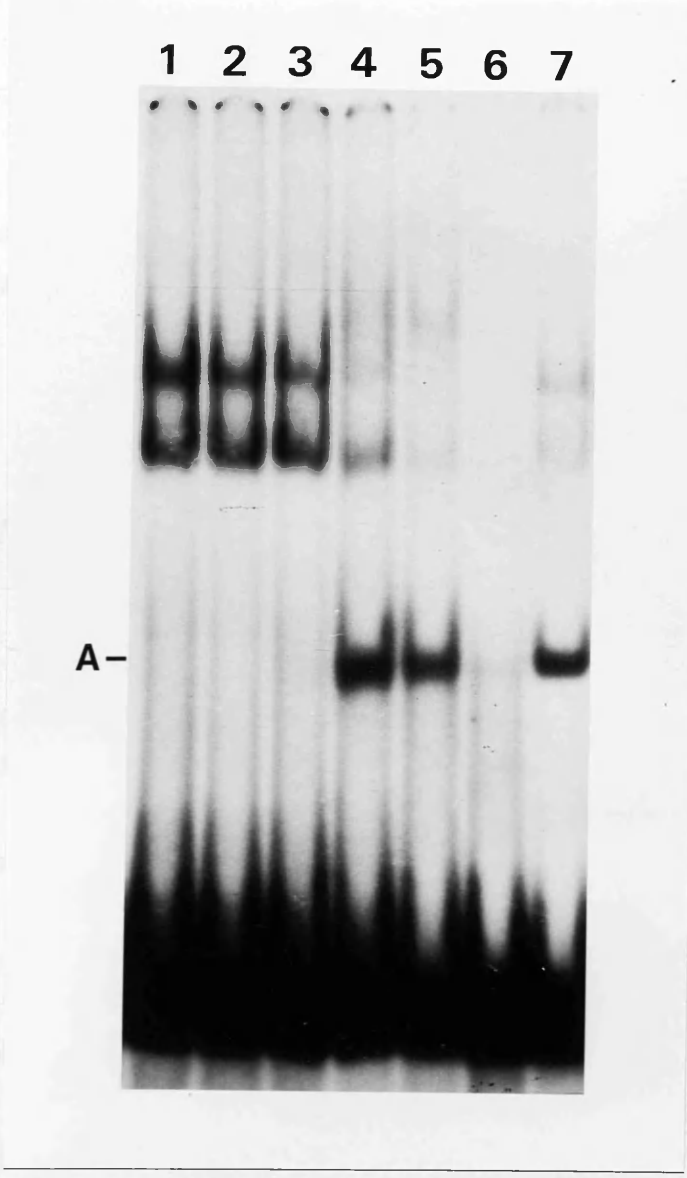


Figure 3.15 Effect of proteinase K on the tsK/UL9 complex

Nuclear extract from cells infected with recombinant tsK/UL9 was incubated with ^{32}P-labelled oligonucleotide I either in the absence of proteinase K (track 1) or in the presence of 1, 10, 100, 1000 or 2000 ng of proteinase K (tracks 2-6 respectively). wt HSV-1 extract was assayed in parallel in the absence of proteinase (track 7). The products were separated in a 5% non-denaturing polyacrylamide gel. "A" indicates the major complex formed with wt extract.

Fig. 3.16 track 1 shows wt HSV-1 DNA binding activity in the absence of added antibody. Antibodies to UL5 (track 2), UL8 (track 3), and UL52 (track 5) had a minimal effect on the pattern of retarded fragments. However, the addition of antibody to the UL9 gene product resulted in the disappearance of the major retarded complex A and its replacement by a more slowly migrating form, A' (track 4). This result indicates the interaction of anti-UL9 antibody molecules with complex A and demonstrates that at least part of the UL9 polypeptide must be present in complex A. As the antibody was raised against a C-terminal decapeptide, this region of the protein is included in the complex.

3. Expression of fragments of the UL9 gene in E.coli

The above experiments strongly implicate the UL9 protein (and possibly its C-terminal region) in binding to specific sequences (sites I and II) within HSV-1 ori_S. To ensure that this interaction was not due to the involvement of any other host or viral protein, it was decided to attempt to express the binding activity encoded by UL9 in heterologous systems. In addition, it was hoped that by expressing specific domains of the UL9 gene, further information on the binding site could be obtained.

To determine whether origin binding activity could be expressed in E.coli and to attempt to locate the DNA binding domain, three plasmids were obtained from Dr. N. D. Stow which contained fragments of the UL9 gene inserted into an expression vector. Details of the plasmids are shown in Fig. 2.2 section 2A. Plasmid pB1 contains approximately the C-terminal 1/3 of the gene (encoding 317 amino acids), plasmid pP31, the C-terminal 2/3 (encoding 591 amino acids) and plasmid pX8, the amino (N) terminal 2/3 (encoding 524 amino acids) cloned in frame into the vector pRIT2T. This expression vector contains 780 bp (encoding 260 amino acids) of the Staphylococcus aureus protein A gene under the control of the phage lambda P_r promoter. The constructs were transformed into the E.coli strain K12ΔH1Δtrp which contains a ts lambda cI repressor. To induce synthesis of

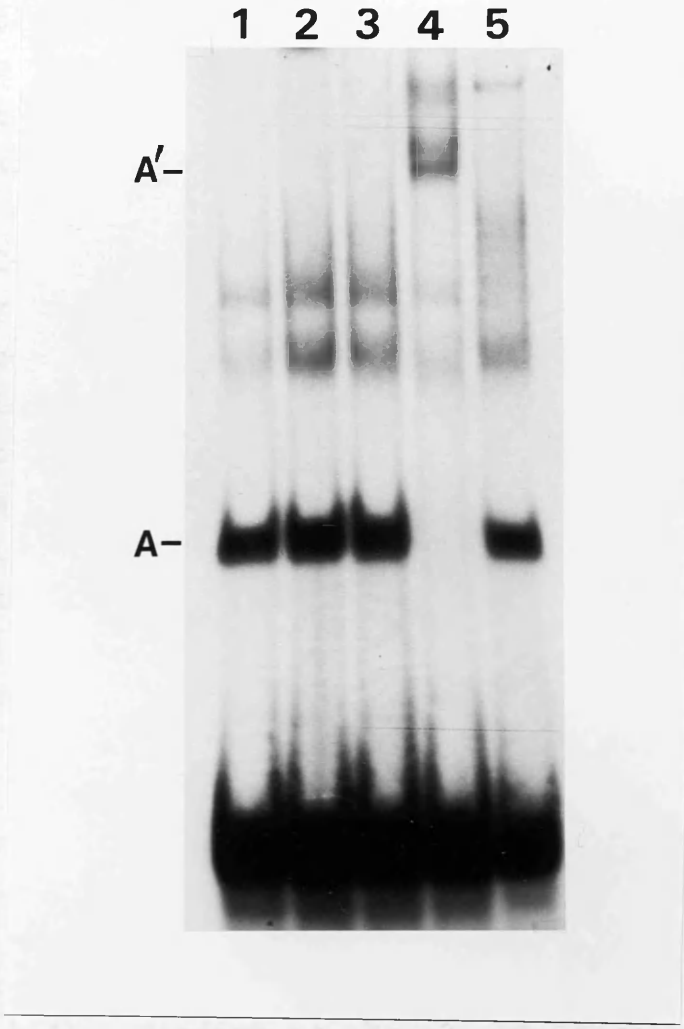


Figure 3.16 Effect of antisera on the wt HSV-1 complex

Nuclear extract from cells infected with wt HSV-1 was incubated with ^{32}P-labelled oligonucleotide I either in the absence of added antiserum (track 1) or in the presence of antisera reactive with the UL5, UL8, UL9 or UL52 gene products (tracks 2, 3, 4 and 5 respectively). The products were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. "A" and "A'" indicate the major complexes formed in the absence of antiserum or the presence of antiserum against the UL9 gene product respectively.

the protein A/UL9 fusion proteins, bacterial cultures, propagated at 28°C, were rapidly shifted to 42°C and incubation continued for 75 min. Extracts prepared from heat-induced bacteria were tested for origin binding activity using labelled oligonucleotide I as probe.

No binding activity was detected in extracts from the host bacteria alone (KIL) or from bacteria containing the vector plasmid pRIT2T (KR2){Fig. 3.17 KIL and KR2 (a)}. Of the three hybrid plasmids, pB1 {KB1 (a)} and pP31 {KP31 (a)} but not pX8 {KX8 (a)} encoded DNA binding activity. Adding an excess of unlabelled oligonucleotide I to KB1 and KP31 assays effectively competed for binding {KB1 and KP31 (a)}. The C-terminal 317 amino acids of the UL9 polypeptide specified by plasmid pB1 therefore contain all the amino acid sequences required for sequence-specific binding to site I within ori_S.

Interestingly, the binding activity encoded by pP31, expressing 2/3 of the UL9 polypeptide was reminiscent of the binding activity seen with an extract from cells infected with the tsK/UL9 recombinant i.e. a smeared pattern of retardation towards the top of the gel with little distinct formation of protein-DNA complexes. In contrast, the binding activity in KB1 extract formed a distinct complex, C.

The activity encoded by pB1 also bound to the 100 bp ori_S-containing fragment, isolated from pST19, and was efficiently competed by the addition of unlabelled oligonucleotide I and II but not oligonucleotide X (Fig. 3.18 tracks 1-4). Binding to the 100 bp fragment produced a major complex (marked C in Fig. 3.18) and a fainter lower mobility complex (marked D). When the amount of KB1 extract added to the assay was increased, the signal intensity of band D increased to a level similar to that of band C (track 5). This suggests that band C represents binding to a single site and band D, binding to sites I and II. Hence, adding more UL9 protein increases the proportion of probe fragment which has two occupied binding sites. The fainter higher mobility bands visible in most tracks probably result from the presence of a small amount of partially degraded fusion protein in the extract.

Fig. 3.18 also demonstrates that the UL9 fusion

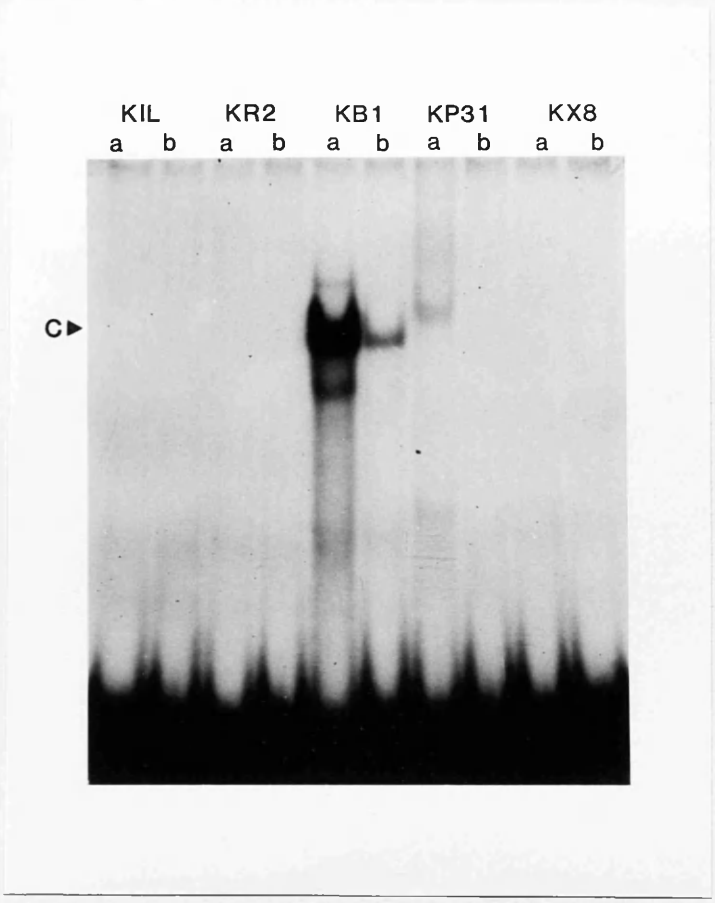


Figure 3.17 Gel retardation analysis of the Staphylococcus aureus protein A encoded fusion protein

Extracts were prepared from untransformed heat-induced bacteria (KIL), or from heat-induced bacteria transformed with the vector pRIT2T (KR2) or one of the three hybrid plasmids containing fragments of the UL9 gene; pB1 (KB1), pP31 (KP31) or pX8 (KX8). Extracts were incubated with ^{32}P-labelled oligonucleotide I in the absence of competitor DNA (tracks a) or in the presence of 100-fold molar excess of oligonucleotide I (tracks b). Products were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. "C" indicates the major complex formed with KB1 extract.

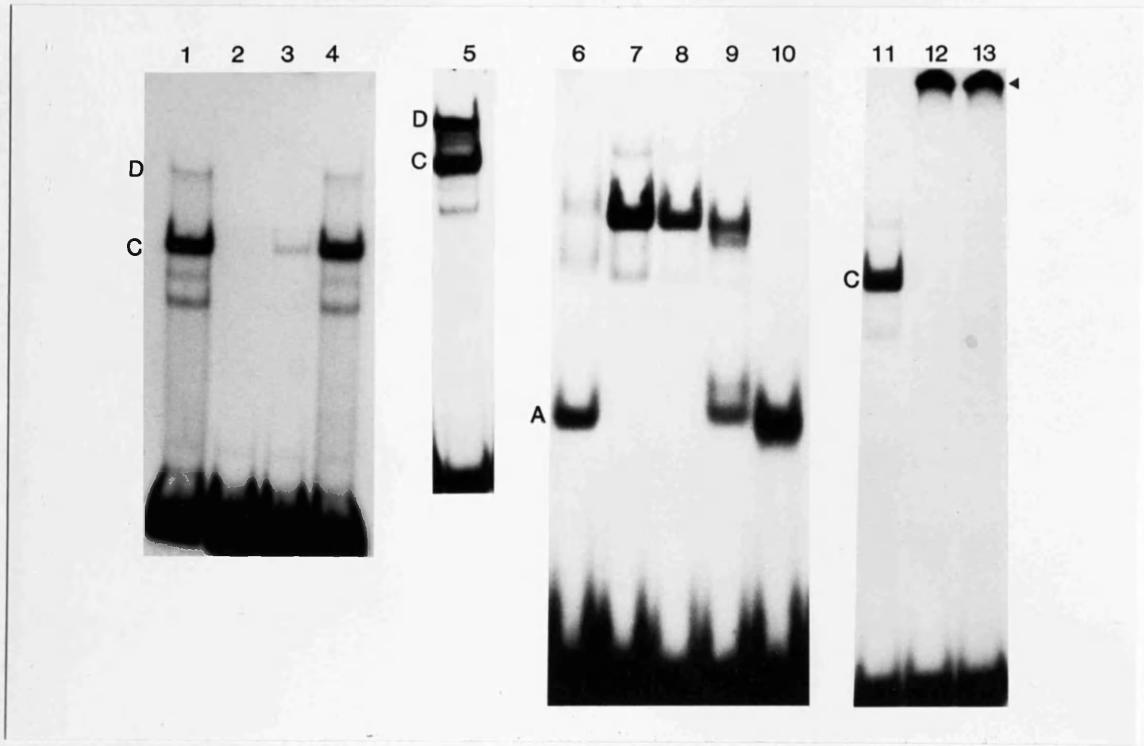

1
2
3
4
5
6
7
8
9
10
11
12
13
D
C
D
C
A
C

Figure 3.18 Gel retardation analysis of the pB1 encoded fusion protein

Binding reactions were performed with extract from heat induced bacteria transformed with pB1 (tracks 1-5 and 7-13) or from cells infected with wt HSV-1 (track 6). The probe fragments were ^{32}P end-labelled pST19 100 bp ori_S fragment (tracks 1-5) or ^{32}P-labelled oligonucleotide I (tracks 6-13). A 20 fold excess of unlabelled oligonucleotide I (track 2), oligonucleotide II (track 3) or oligonucleotide X (track 4) was added as competitor DNA. The products were separated by electrophoresis in an 8% non-denaturing polyacrylamide gel. "C" and "D" indicate major complexes observed with KB1 extract and "A", the major complex from HSV-1 infected cell extract. Increasing the amount of KB1 extract in the assay (track 5; 8 ug of total protein) increased the signal intensity of band D (compare tracks 1 and 5).

Tracks 7-10 show the effect of adding proteinase K on the KB1 complex. KB1 extract was incubated with probe either in the absence of proteinase K (track 7) or in the presence of 1, 10 or 100 ng of proteinase K (tracks 8-10).

Antiserum reactive with UL9 or UL52 was added to binding reactions (tracks 12 and 13) resulting in the disappearance of complex C (track 11) and the appearance of a retarded complex at the top of the gel indicated by the bold arrowhead.

protein encoded by pB1 (predicted molecular weight 63,000) forms a complex with a significantly lower mobility than complex A formed using extracts from cells infected with wt HSV-1 and oligonucleotide I (tracks 6 and 7). This is in agreement with complex A containing only a proteolytic fragment of the UL9 polypeptide. As with tsK/UL9 infected cell nuclear extract, adding proteinase K to KB1 extract reduced the amount of slowly migrating complex C and resulted in the concomitant appearance of a faster migrating species which exhibited a mobility similar to that of complex A (tracks 7-10). The mobility of the KB1 complex C, was further retarded by the addition of antibodies reactive with the UL9 and UL52 polypeptides to the binding reaction (tracks 11-13). The ability of both antibodies to recognise complex C is due to the presence of an IgG binding domain within the protein A moiety of the fusion protein and confirms that the fusion protein is responsible for the formation of complex C.

4. Expression of the DNA binding domain of the UL9 gene by in vitro transcription and translation

Earlier attempts to express the intact UL5, UL8, UL9 and UL52 gene products by in vitro transcription and translation using pTZ vectors proved largely unsuccessful. However, independent verification that the C-terminal 317 amino acids of the UL9 gene product contained DNA binding activity was obtained by in vitro transcription and translation of this region using a fusion protein construct with the N-terminal 68 amino acids originating from the Vmw65 protein (encoded by UL48). The construction of this chimeric gene is outlined in Fig. 3.19. Plasmids pMC.in4 and pMC.in17 were obtained from Dr. C. Ace. The plasmids contained a copy of the UL48 gene, encoding Vmw65 (491 amino acids), with 12 bp BamHI linker insertions, 222 bp and 1431 bp downstream from the start of the coding sequence respectively. These plasmids were known to enable efficient transcription and translation of Vmw65 in vitro. The polypeptide product of the Vmw65 gene does not contain any intrinsic DNA binding activity and therefore was not

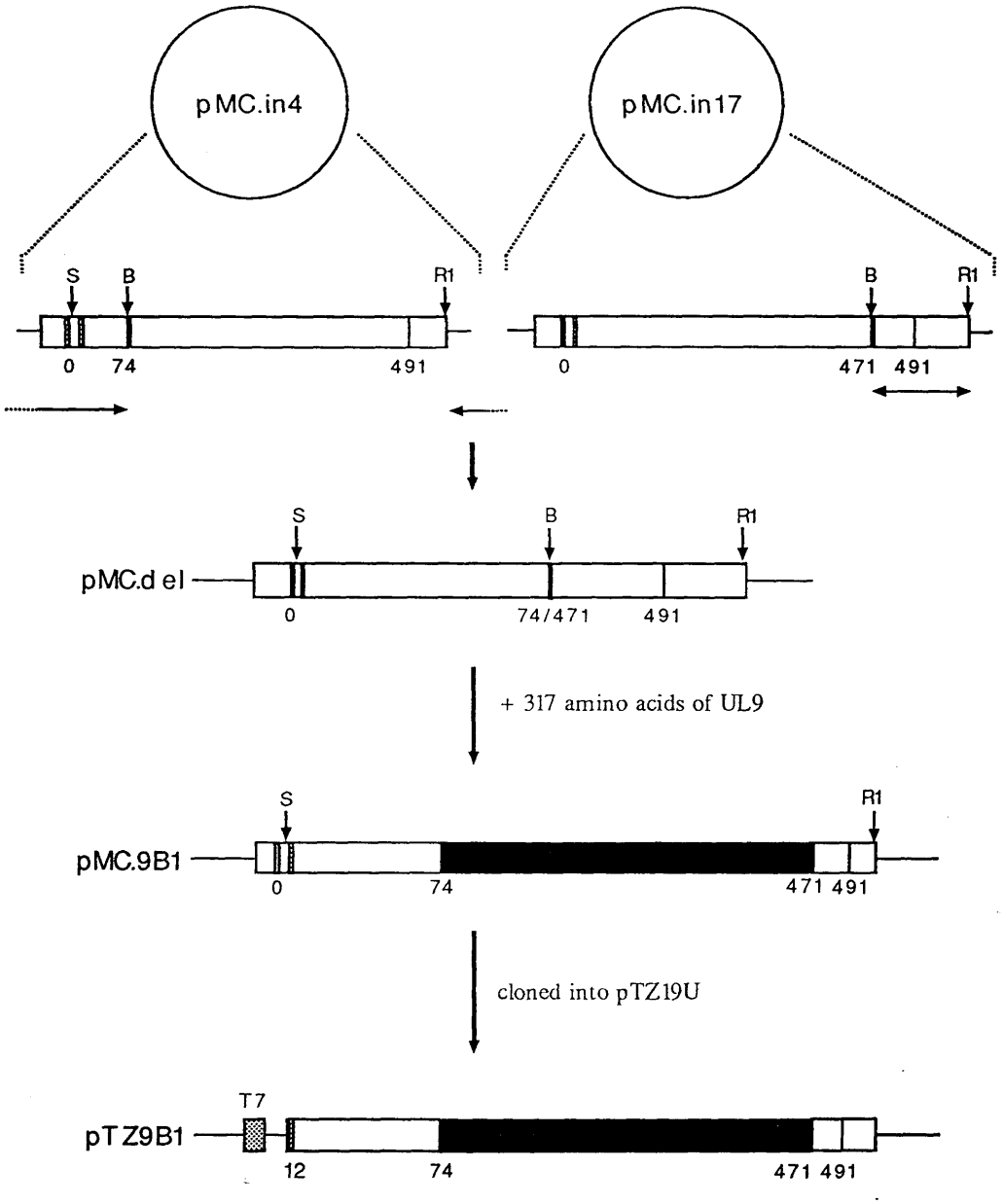

pMC.in4
pMC.in17
S
B
R1
0
74
491
B
R1
0
471
491
S
B
R1
pMC.del
0
74/471
491
+ 317 amino acids of UL9
S
R1
pMC.9B1
0
74
471
491
cloned into pTZ19U
T7
pTZ9B1
12
74
471
491

Figure 3.19 Contruction of plasmid pTZ9B1

Plasmids pMC.in4 and pMC.in17 were obtained from Dr.C. Ace. Each plasmid contained a copy of the UL48 gene (encoding Vmw65; 491 amino acids) with 12 bp BamHI linker insertions, 74 and 471 amino acids downstream from the start of the protein coding sequence (marked O). The UL48 gene has two AUG start codons either side of a SalI restriction enzyme site (hatched boxes). Both codons are utilised in the initiation of *in vitro* translation of the protein coding sequence (C. Ace, pers comm).

pMC.in17 was cleaved with BamHI plus EcoRI as shown and the C-terminal fragment (indicated by arrows) encoding 20 amino acids of Vmw65 and the polyadenylation signals was cloned into BamHI-EcoRI digested pMC.in4 yielding pMC.del which contains a unique BamHI site at position 74/471. A BamHI fragment encoding the C-terminal 317 amino acids of the UL9 gene (see Fig. 2.2, section 2A) was cloned, in frame, into the unique BamHI site of pMC.del. The resulting plasmid, pMC.9B1 was cleaved with SalI plus EcoRI and the resulting fragment cloned into SalI-EcoRI digested pTZ19U yielding pTZ9B1. This construct contains a single AUG start codon 15 nucleotides downstream from the SalI site.

expected to interfere with the DNA binding activity of the UL9 gene. Plasmids pMC.in4 and pMC.in17 were cleaved with BamHI and EcoRI and fragments containing the promoter and polyadenylation signals of the gene were isolated and ligated to form plasmid pMC.del (Fig. 3.19). The C-terminal 317 amino acids of the UL9 gene were cloned, in frame, into the unique BamHI site of pMC.del. The resulting plasmid, pMC.9B1 was cleaved with SalI and EcoRI and cloned into SalI-EcoRI digested pTZ19U containing a T7 promoter, yielding pTZ9B1.

For the in vitro transcription assay, 2ug of plasmid DNAs were linearized with EcoRI and incubated with all four NTPs (A, G, C, U), T7 RNA polymerase and synthetic 5'mRNA caps at 37°C. An aliquot of the transcription products was added to rabbit reticulocyte lysate containing ^{35}S-methionine and labelled protein products were analysed by SDS-PAGE gel electrophoresis {Fig. 3.20(a)}. Labelled proteins were detected corresponding to the expected products of plasmid pTZ9B1 (9B1) and the intact Vmw65 gene itself (Vmw65). No additional band was observed with the intact UL9 gene plasmid compared to the absence of any added plasmid (UL9 and C). The Vmw65/UL9 fusion protein which utilizes the stop codon of the UL9 gene contains 385 amino acids (predicted molecular weight of 43,000). As expected the Vmw65/UL9 fusion protein had a higher electrophoretic mobility than Vmw65.

Unlabelled in vitro translation products were tested in a gel retardation assay using labelled oligonucleotide I as probe. Sequence-specific binding activity was observed only with the Vmw65/UL9 fusion protein (Fig. 3.20(b) 9B1). As expected, this complex had a higher electrophoretic mobility than the bacterial fusion protein (predicted moleculer weight of 63,000). This confirms the previous finding that the C-terminal region of the UL9 gene encodes a DNA binding activity specific for ori$_S$ sequences.

5. Interaction of the UL9 DNA binding domain with ori$_S$ as analysed by DNase I footprinting assays

Ori$_S$ sequences protected from DNase I digestion by

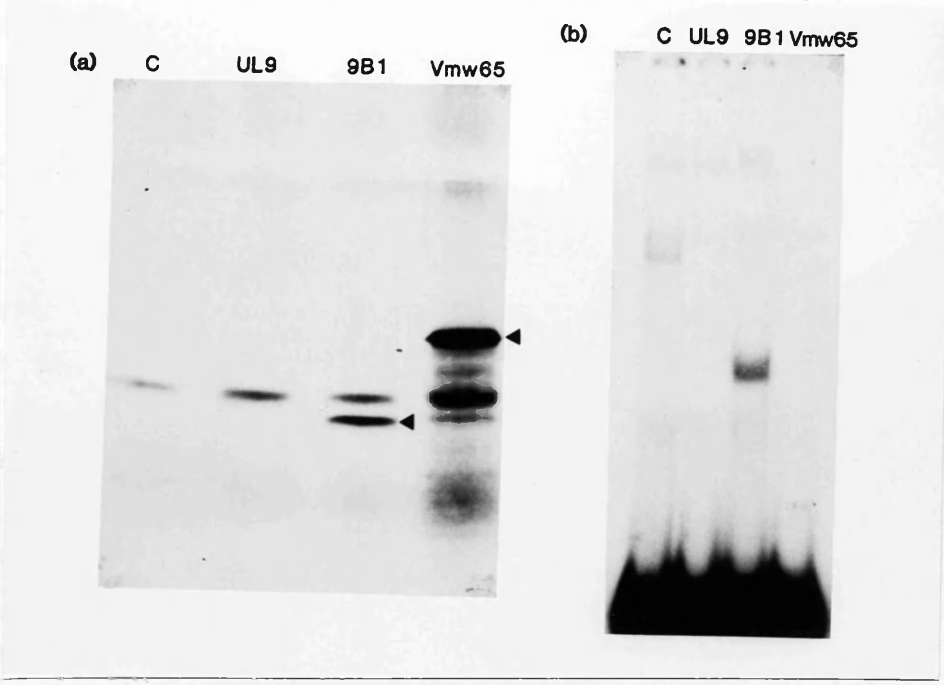


Figure 3.20 In vitro transcription and translation of the C-terminal region of UL9

(a) In vitro transcription assays were carried out using pTZUL9 [UL9] (the intact UL9 gene cloned into pTZ19U), pTZ9B1 [9B1] (see Fig. 3.19), pTZVmw65 [Vmw65] (the intact UL48 gene cloned into pTZ19R) or no plasmid DNA (C). An aliquot of the transcription product was added to rabbit reticulocyte lysate containing ^{35}S-methionine and labelled protein products were analysed by SDS-PAGE gel electrophoresis. Labelled proteins corresponding to the expected products of pTZ9B1 and Vmw65 are indicated by arrowheads.

(b) Gel retardation analysis of unlabelled in vitro transcription and translation products. An aliquot of the in vitro transcription assays described in part (a) were added to reticulocyte lysate without ^{35}S-methionine. Unlabelled protein products were incubated with ^{32}P-labelled oligonucleotide I and analysed in a 5% non-denaturing polyacrylamide gel.

the intact UL9 protein have been determined by Elias and Lehman (1988) and Olivo et al. (1988) using the DNase I footprinting method developed by Galas and Schmitz (1978). Having shown that the C-terminal 317 amino acids of the UL9 protein contained ori_S-specific DNA binding activity, it was of interest to compare the interactions of the protein A/UL9 fusion protein and intact UL9 protein with ori_S.

The principle of the DNase I footprinting technique is that following addition of DNase I to a protein-DNA binding reaction, the protein bound to a uniquely end-labelled DNA fragment will protect its binding site from digestion by the enzyme. Thus, when digested DNAs are extracted and run on a denaturing polyacrylamide gel, digested fragments corresponding to cleavage within the binding site will be under-represented in the gel compared with DNA digested in the absence of protein. By comparison with Maxam and Gilbert sequencing reactions of the probe fragment, the residues protected by the protein can be determined.

The 100 bp ori_S fragment was digested with either BamHI or SalI and 3' end-labelled using the Klenow fragment of DNA pol I. Labelled DNAs were extracted and re-digested with either SalI or BamHI respectively and the excised 100 bp fragment separated from labelled vector sequences on a polyacrylamide gel. These uniquely 3' end-labelled fragments were incubated with increasing amounts of KB1 extract under the conditions used for gel retardation assays. DNase I was added to the reactions and following a brief period of digestion, DNAs were extracted and analysed on a denaturing polyacrylamide gel together with G and G+A Maxam and Gilbert sequencing reactions performed on the probe fragment, and probe digested in the absence of protein extract. Protection of sequences in the region of binding site I was detected on both strands and weak protection of site II on the SalI labelled strand {Fig. 3.21(a)}. Sites hypersensitive to DNase I digestion were evident within the AT-rich region of the probe digested both in the presence and absence of KB1 extract and also to the top of the protected region covering binding site I. Surprisingly, there were many more bands in tracks in which the probe had been treated with DNase I in the presence of bacterial

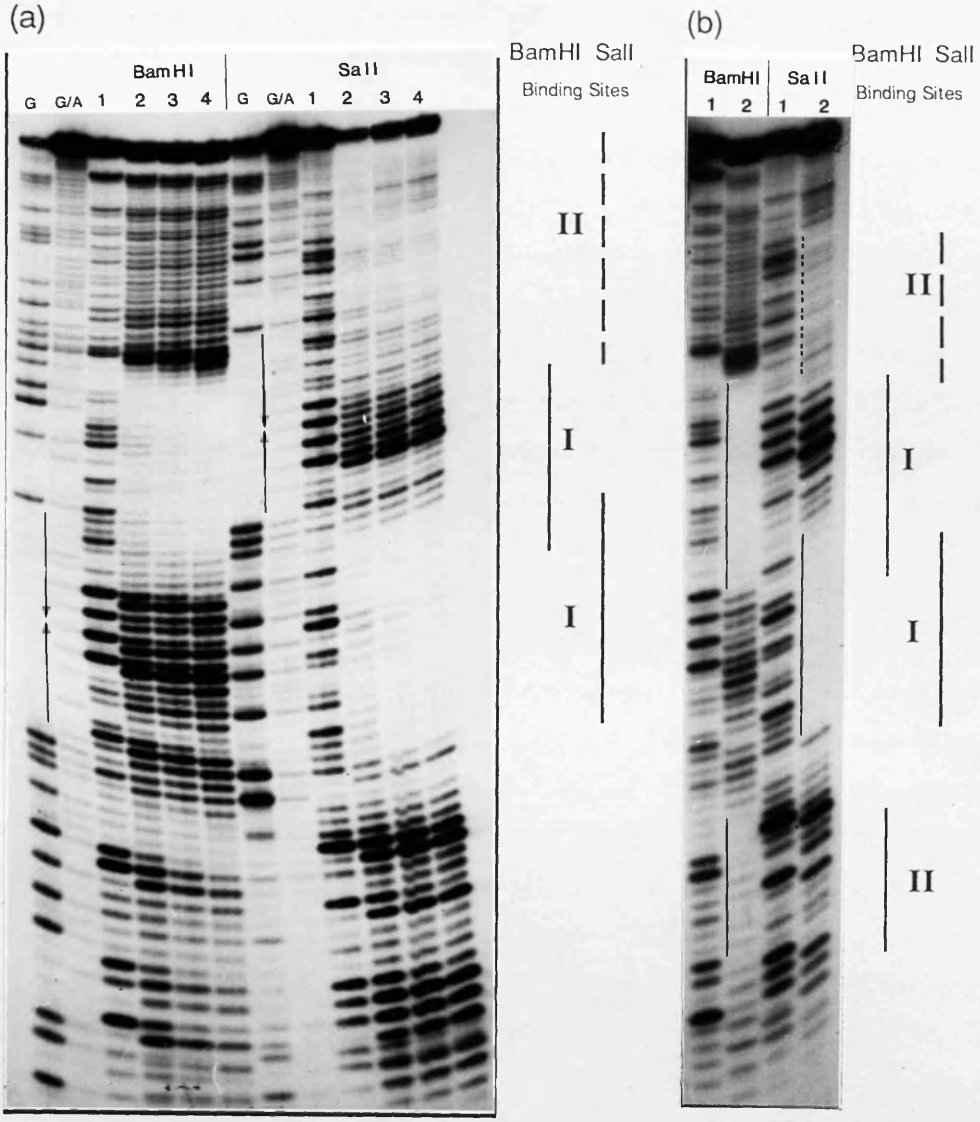

(a)
BamHI
SalI
G G/A 1 2 3 4 G G/A 1 2 3 4
BamHI SalI
Binding Sites
II
I
I
(b)
BamHI
SalI
1 2 1 2
BamHI SalI
Binding Sites
II
I
I
II

Figure 3.21 DNase I protection of HSV-1 ori_S by the UL9 DNA binding domain

(a) The pST19 100 bp ori_S insert was uniquely 3' end-labelled at either the BamHI or SalI terminus as indicated and incubated with increasing amounts of extract from heat induced bacteria transformed with pB1; 2.5 ug (track 2), 5 ug (track 3) or 7.5 ug (track 4) of total protein. Following a brief period of digestion with DNase I, DNAs were extracted and analysed on a 6% denaturing polyacrylamide gel together with markers generated by partial cleavage at G residues (G) or at G and A residues (G/A) and probe digested with DNase I in the absence of protein extract (track 1). Arrows indicate the AT-rich region of the ori_S palindrome. To the right of the autoradiograph, protected regions corresponding to binding sites I on BamHI and SalI labelled strands are marked by bold lines and weaker protection of binding site II on the SalI labelled strand by dashed lines.

(b) Reactions were set up as described in part (a) using 7.5 ug of KB1 extract or extract from heat induced bacteria transformed with control vector pRIT2T (KR2). Partial digestion with DNase I was performed prior to the resolution of complexes on a non-denaturing gel. DNA was extracted from bands corresponding to free DNA in the KR2 assay (track 1) and the upper KB1 complex (track 2) [equivalent to complex D in Fig. 3.18 (track 5)] and resolved on a 6% denaturing polyacrylamide gel together with markers as described in part (a) (not shown).

To the right of autoradiograph, clearly protected regions corresponding to binding sites I and II on the BamHI labelled strand and site I on the SalI labelled strand are shown as solid lines. A weakly protected region corresponding to binding site II on the Sal I labelled strand is shown as a dashed line.

extract than in its absence. Also, careful examination suggests that the presence of bacterial extract results in the shifting of more than 50% of the label in major bands produced by DNase I in the absence of extract to a position equivalent to the removal of one nucleotide (exemplified by the four major bands in the AT-rich region; tracks 3 and 4).

Using the above method incomplete protection resulted in weak footprinting, probably due to the failure of the protein to saturate all binding sites. Therefore in an attempt to improve the quality of the footprinting assay, DNase I treated binding reactions were first applied to a preparative non-denaturing gel and after brief autoradiography of the wet gel, gel slices containing major complexes were excised (corresponding to complex D in Fig. 3.18 track 5), electroeluted and analysed on a denaturing polyacrylamide gel. In an attempt to eliminate the differential effect on the mobility of bands caused by KB1 extract (above), probe fragment was incubated with an equivalent amount of control extract, KR2, and also electrophoresed on the preparative gel. Hence, using this method, the free DNA band has undergone similar DNase I digestion in the presence of bacterial extract to the protein/DNA complex. Fig. 3.21(b) shows clearer protection of sequences corresponding to binding site I and II on the BamHI labelled strand and site I on the SalI labelled strand also weak protection of site II on the SalI labelled strand. Again, the bands arising from the protein/DNA complex migrated slightly faster through the gel than the corresponding bands from free DNA, despite both fragments having been incubated initially with protein extract. The reason for this is unknown but it may reflect a property of the truncated UL9 protein or the differential expression of a host function in the KB1 cells. Ideally, the extent of complex formation should be adjusted so that bands corresponding to protein/DNA complexes and free probe can be obtained from a single binding reaction.

Nevertheless, these footprints confirm that the retarded complexes observed in gel retardation assays are due to the UL9 fusion protein binding to specific sequences within ori_S. Moreover, the protected sequences shown in Fig. 3.21(b) are very similar to those described by Elias

and Lehman (1988) using purified UL9 and Koff and Tegtmeyer (1989) who used nuclear extract from HSV-1 infected Vero cells.

SECTION 3C : The importance of the UL9 protein binding sites within ori_S for HSV DNA synthesis

The above sections identified OBP as the product of the UL9 gene. Since the results of Wu et al. (1988) had shown that UL9 protein was essential for DNA synthesis, and two binding sites for the protein had been identified within ori_S, I further investigated the importance in DNA replication of UL9 binding to these two sites.

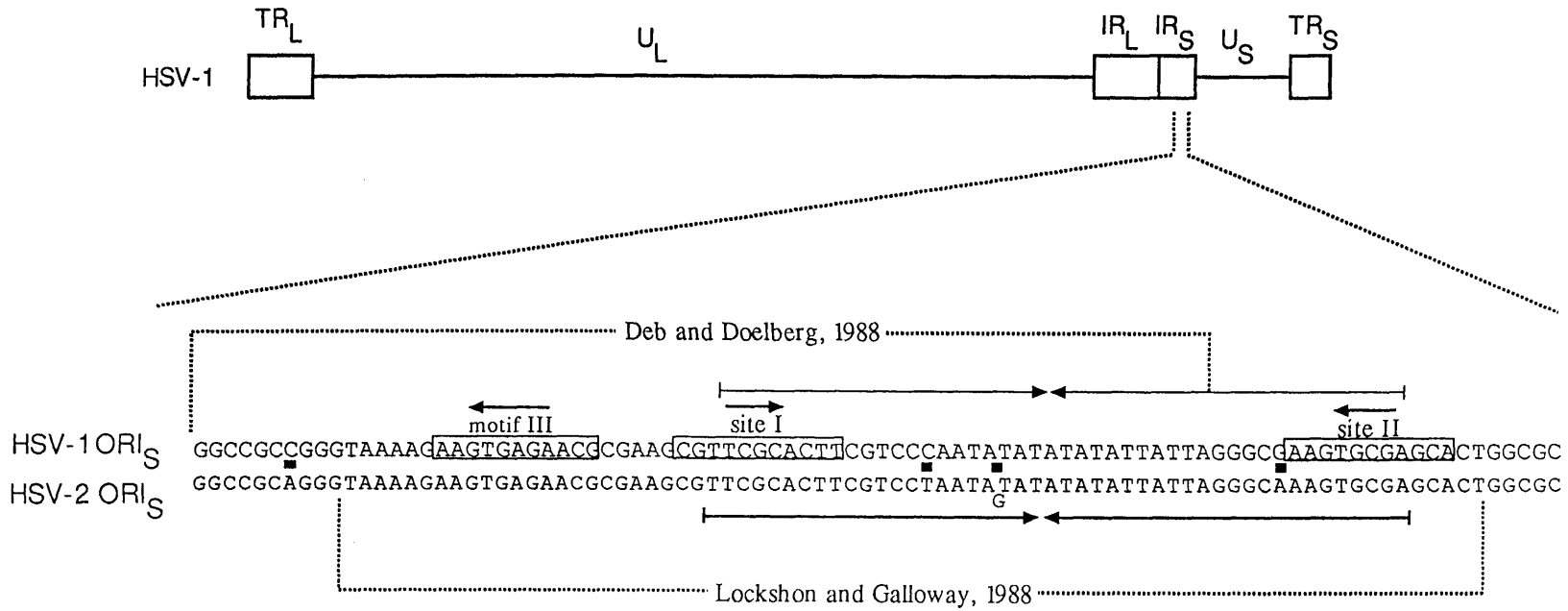
1. The role of UL9 binding sites I and II for ori_S activity

Published reports mapping the core ori_S sequences of HSV-1 and HSV-2 have brought into question the requirement for the second UL9 binding site, site II. Fine mapping of the core ori_S of HSV-2 identified an essential region of 75 bp {Fig. 3.22(a)}. Removing 7 bp from the left hand side of this region or 4 bp from the right, caused a significant reduction in ori_S activity (Lockshon and Galloway, 1988) suggesting that an intact binding site II was required for efficient ori_S activity, as were sequences at the left hand side of binding site I. Examination of these sequences to the left of site I reveals the presence in inverted orientation of an 11 bp element, differing in only one position from the corresponding 11 bp sequence present within binding site I and also conserved in VZV ori_S (section 1D). This sequence is designated motif III {Fig.3.22(b)}.

In contrast, Deb and Doelberg (1988) reported that a 67 bp origin fragment lacking binding site II {Fig. 3.22(a)} replicated as well as a wt 100 bp ori_S-containing plasmid suggesting that site II is unimportant for ori_S activity.

In an attempt to resolve this conflict, I therefore used site-directed mutagenesis of plasmid pST19 to investigate the role of binding sites I and II and sequence motif III in origin activity.

(a)


TR_L
U_L
IR_L
IR_S
U_S
TR_S
HSV-1
Deb and Doelberg, 1988
motif III
site I
site II
HSV-1 ORI_S
GGCCGCCCGGGTAAAAGAAGTGAGAACGCGAACGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGCGC
HSV-2 ORI_S
GGCCGCAGGGTAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCTAATATATATATATTATTAGGGCAAAGTGCGAGCACTGGCGC
G
Lockshon and Galloway, 1988

(b)

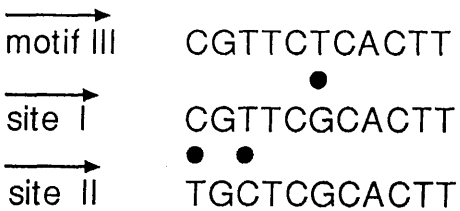

motif III
CGTTCTCACTT
site I
CGTTCGCACTT
site II
TGCTCGCACTT

Figure 3.22

(a) DNA sequence of the regions specifying ori_S activity in HSV-1 and HSV-2

The DNA sequence of the 90 bp region specifying ori_S activity in HSV-1 is shown (Stow and McMonagle, 1983). The insert in plasmid pST19 extends an additional 10 residues to the right. The corresponding region in HSV-2 is shown below with sequence differences marked by solid bars. The core HSV-2 ori_S sequence defined by Lockshon and Galloway (1988) is contained within the dotted lines. The core HSV-1 ori_S region defined by Deb and Doelberg (1988) is similarily marked. The 45 bp palindromes are indicated by arrows.

The 11 bp conserved sequence located within binding sites I and related sequences corresponding to site II and motif III, are boxed. These 11 bp sequences were deleted from plasmid pST19 by site-directed mutagenesis yielding plasmids pST19delI, pST19delII and pST19delIII respectively.

(b) Alignment of the three deleted sequences

The sequences deleted from site I, site II and motif III are represented in the same orientation. Mismatches are indicated by dots.

(a) site-directed deletion mutagenesis of ori_S

In order to examine the requirement for UL9 protein binding sites in initiating replication from HSV-1 ori_S, specific deletions were introduced into pST19. The method of Kunkel et al. (1987) employing a labile uracil-rich parental DNA strand was used to enrich for the required mutant plasmids. The oligonucleotides used to delete corresponding 11 bp sequences from within site I, site II and motif III contained 15 bases from either side of the region to be deleted. These oligonucleotides were phosphorylated and annealed to single-stranded uracil-rich pST19 DNA. Oligonucleotide primed *in vitro* DNA synthesis of the complementary strand of pST19 was carried out using the Klenow fragment of DNA pol I. Following ligation, the plasmids were used to transform *E.coli* strain DH1. The parental uracil-rich pST19 strand is extensively degraded in these bacteria and repair synthesis of the DNA strand complementary to the strand carrying the mutation occurs *in vivo*. Plasmids containing the required 11 bp deletions from within site I, II and motif III were identified by DNA sequencing of plasmid DNA from individual colonies (Fig. 3.23). The resulting plasmids, pST19delI, pST19delII, and pST19delIII contain 11 bp deletions from within sites I, II and motif III respectively {Fig. 3.22(b)}.

(b) ori_S activity of deleted plasmids

The replicative activities of the mutated viral origins were compared with those of the parental plasmid pST19 and the vector pTZ19U. Plasmids were transfected into BHK cells which were subsequently either mock-infected or superinfected with HSV-1 to provide helper functions. Samples of DNA extracted from the cells 16 h pi were cleaved with EcoRI and DpnI and replicated plasmid molecules were detected by Southern blotting using ^{32}P-labelled pAT153 DNA as probe. HSV-1 encoded functions facilitated the replication of pST19 but not pTZ19U {Fig. 3.24; ST19(b) and pTZ(b)}. Replication of pST19delIII was slightly reduced {dIII(b)} and pST19delII {dII(b)} was greatly reduced in

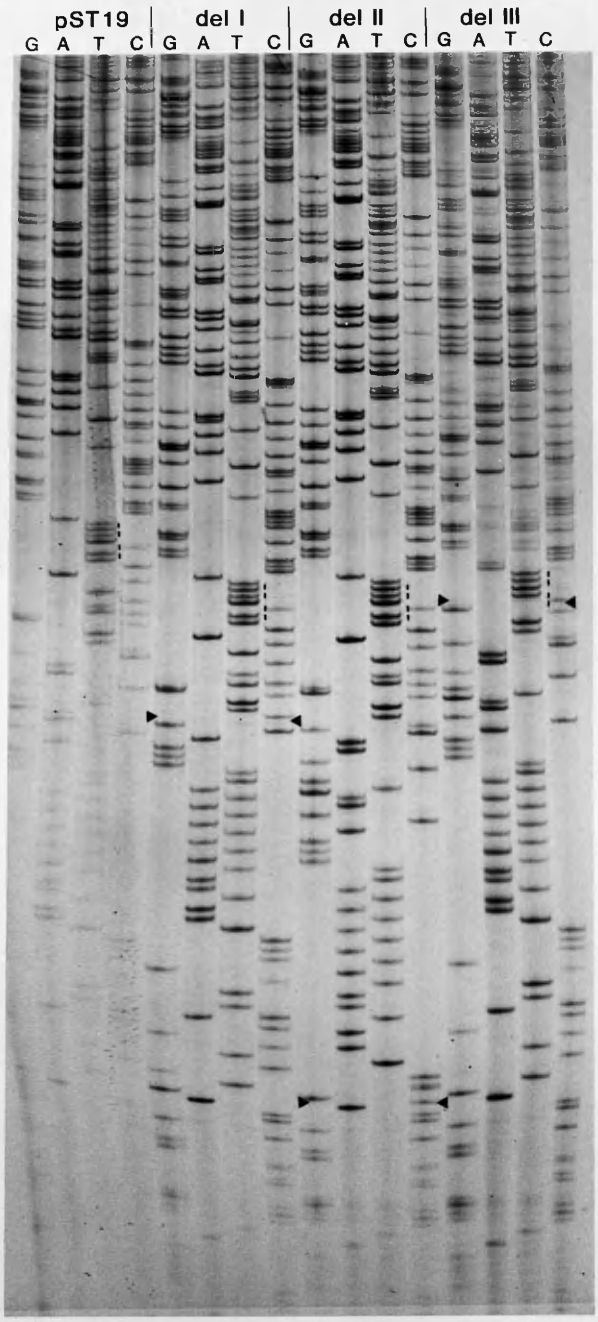

pST19
del I
del II
del III
G A T C G A T C G A T C G A T C

Figure 3.23 Dideoxy sequencing of plasmids containing 11 bp deletions within ori_S

Dideoxy sequencing reactions were performed using plasmid pST19 and three plasmids containing 11 bp deletions within ori_S as shown in Fig. 3.22 i.e. pST19 delI (del I), pST19delII (del II) and pST19delIII (del III). The sequence of the ori_S inserts read from the gel were as follows:

pST19 : CCGGCGCCAGTGCTCGCACTTCGCCCTAATAATATATATATATTGGGACG
AAGTGCGAACGCTTCGCGTTCTCACTTCTTTTACCCGGCGGCC

del I : CCGGCGCCAGTGCTCGCACTTCGCCCTAATAATATATATATATTGGGACG▼
CTTCGCGTTCTCACTTCTTTTACCCGGCGGCC

del II: CCGGCGCC▼AGCGCCCTAATAATATATATATATTGGGACGAAGTGCGAACG
CTTCGCGTTCTCACTTCTTTTACCCGGCGGCC

delIII: CCGGCGCCAGTGCTCGCACTTCGCCCTAATAATATATATATATTGGGACG
AAGTGCGAACGCTTCG▼CTTTTACCCGGCGGCC

Arrowheads mark the position of deleted sequences. The positions of broken lines beside a run of T residues illustrate the deletions in plasmids del I, del II and del III relative to pST19.

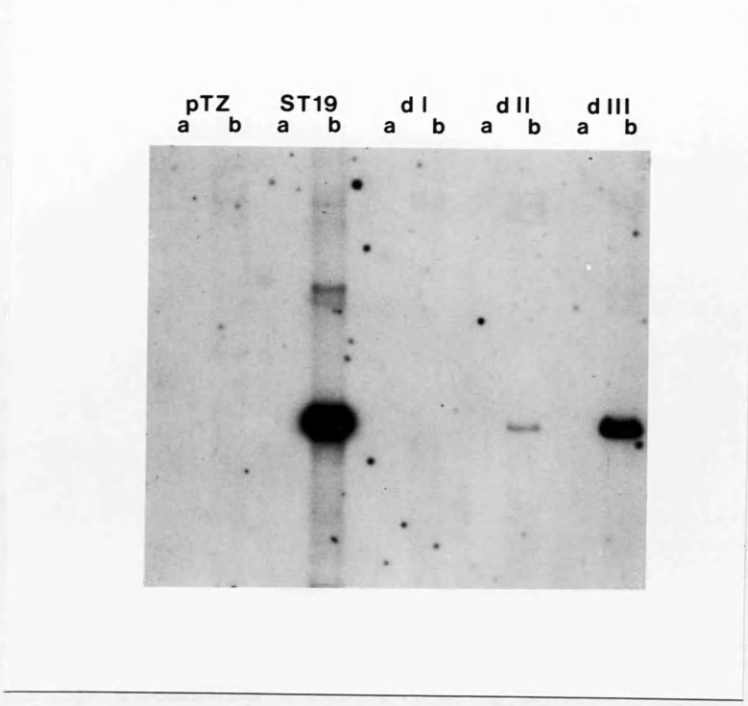


Figure 3.24 Replication of plasmids containing mutations within ori_S

BHK cells were transfected with pTZ19U (pTZ), pST19 (ST19), pST19delI (d I), pST19delII (d II) or pST19delIII (d III) as indicated and either mock-infected (tracks a) or superinfected with HSV-1 (tracks b). Samples containing 1/15 of the DNA recovered from each monolayer were digested with EcoRI plus DpnI and the fragments separated by electrophoresis, transferred to nitrocellulose and hybridised to ^{32}P-labelled pAT153. An autoradiograph of the washed filter is shown.

comparison to pST19. No pST19delI replication products were detectable {dI(b)}.

To compare activities of the mutant origins, regions of the nitrocellulose filter corresponding to the positions of the replicated plasmid molecules, detected by autoradiography, were excised and the radioactivity determined in a scintillation counter. The counts per minute for three independent experiments are shown in Table 3.1. Assigning a value of 100% to the activity of pST19, corresponding values of 46%, 44% and 36% were found for pST19delIII and 10%, 7.7% and 4.3% for pST19delII. In each experiment, pST19delI replication was not detectable by autoradiography. A comparison of autoradiographs exposed for various time intervals indicated that the activity of this plasmid was less than 0.1% the activity of pST19. pST19delI activity was not detectable after exposing the filter for five days while pST19 activity was just detectable after exposing the same filter for approximately 1/1000 of the time i.e. 7 mins.

These results therefore demonstrate that the removal of 11 bp elements from binding sites I and II causes quantitatively different effects. Disruption of site I abolishes detectable ori_S activity whereas some residual origin function remains when part of site II is deleted. Although deletion of motif III caused only a 2-fold drop in detectable ori_S activity, this element, nevertheless, appears to play some role in enabling efficient replication.

(c) binding of the UL9 DNA binding domain to deleted copies of ori_S

Gel retardation assays were performed to examine the interaction of the UL9 polypeptide with mutated copies of ori_S. The mutated ori_S inserts from plasmids pST19delI, pST19delII, pST19delIII and pST19 were excised by digestion with BamHI-SalI, end-labelled and purified. Labelled probe fragments were incubated with KB1 extract (in the absence and presence of a 10-fold excess of unlabelled oligonucleotide I) or with control extract, KR2, and the resulting complexes resolved on a polyacrylamide gel.

Retarded complexes were observed only with the KB1

Table 3.1

	Experiment 1			Experiment 2			Experiment 3		
Plasmid	A.C. (cpm)	corrected	%	A.C. (cpm)	corrected	%	A.C. (cpm)	corrected	%
control	27.9	0	-	48.5	0	-	70	0	-
pST19	1014.7	986.8	100	3354.2	3305.7	100	7673.5	7603.5	100
delI	28.7	0.8	0.13	52.2	3.7	0.1	77.5	7.5	0.09
delII	101.15	73.25	7.3	303.5	255	7.7	396	326	4.3
delIII	483.45	455.55	46	1494.2	1445.7	43.7	2803.5	2733.5	36

Table 3.1 Summary of three experiments measuring the replication activity of three mutant ori_S plasmids

Replication assays were performed as described in Fig. 3.1. using plasmids pTZ19U (control), pST19 (pST19), pST19delI (delI), pST19delII (delII) and pST19delIII (delIII). Regions of the filter corresponding to the positions of the replicated plasmid DNA were excised and the radioactivity determined in a scintillation counter. The average counts per minute (A.C. cpm) are shown for duplicate samples. These counts are corrected for background by subtracting the control value for that experiment. The wt ori_S plasmid pST19 is assigned a value of 100% activity and the corresponding values for the mutant ori_S plasmids are listed below.

The cpm given for experiment 3 were obtained from the filter shown in Fig. 3.24.

extract, and in each case, the presence of an excess of unlabelled oligonucleotide I competed for binding to the probe (Fig. 3.25). Two major retarded complexes were seen with both pST19 and pST19delIII probes {ST19(b) and dIII(b)} but only a single major complex with pST19delI and pST19delII {dI(b) and dII(b)}. These results indicate that the introduction of an 11 bp deletion at either binding site I or site II results in the loss of a major retarded complex. This confirms the earlier suggestion (section 3A) that the higher mobility weight complex represents binding to a single site and the lower mobility complex, binding to two sites within ori_S. Although the deletion of binding site I abolished detectable ori_S activity, it would appear that the mutated origin retains the ability to bind to the UL9 protein.

Protein-DNA interactions within the origin region were not significantly altered in the absence of motif III, and as with pST19, two major complexes were observed.

2. Further examination of the role of motif III

Deleting motif III resulted in a small but reproducible decrease in the replicative ability of the ori_S-containing plasmid, pST19, but had no apparent effect on the formation of protein-DNA complexes with UL9 protein. Because the sequence of motif III is very similar to sites I and II, the possibility exists that the ability of UL9 protein to recognise this binding site is masked in the presence of the other binding sites. To examine whether, in the absence of other binding sites, the UL9 protein might interact with motif III, a duplex oligonucleotide containing this sequence was therefore synthesised and used as a probe in gel retardation assays.

KB1 extract was incubated with labelled oligonucleotides containing motif III or binding site I and resolved on a polyacrylamide gel {Fig. 3.26(a)}. As previously observed, sequence-specific binding to oligonucleotide I occurred {oligo I(a)} but no retarded band was observed with motif III oligonucleotide {oligo III(a)}. This demonstrates that the failure to detect binding to motif III within <u>wt</u> ori_S is not due to interference caused

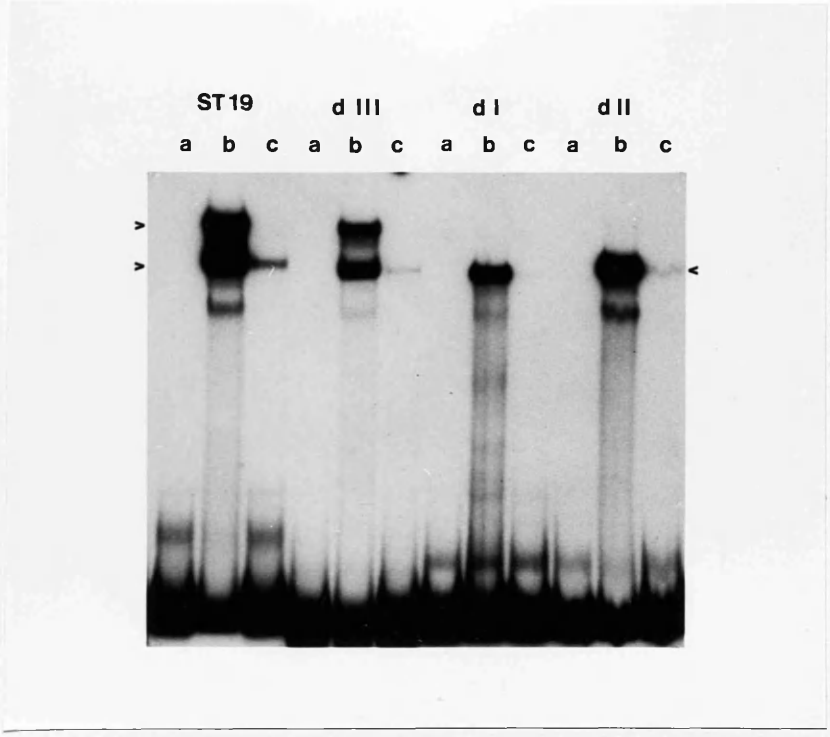


Figure 3.25 Gel retardation analysis of the binding of the UL9 fusion protein to fragments containing deletions within the ori_S region

The BamHI plus SalI inserts from pST19, pST19delI, pST19delII and pST19delIII (ST19, d I, d II and d III respectively) were ^{32}P end-labelled to comparable specific activities and incubated with control extract KR2 (tracks a) or KB1 extract either in the absence of competitor DNA (tracks b) or in the presence (tracks c) of a 10-fold molar excess of unlabelled oligonucleotide I. The positions of the major complexes, resolved in an 8% non-denaturing polyacrylamide gel are indicated by arrowheads.

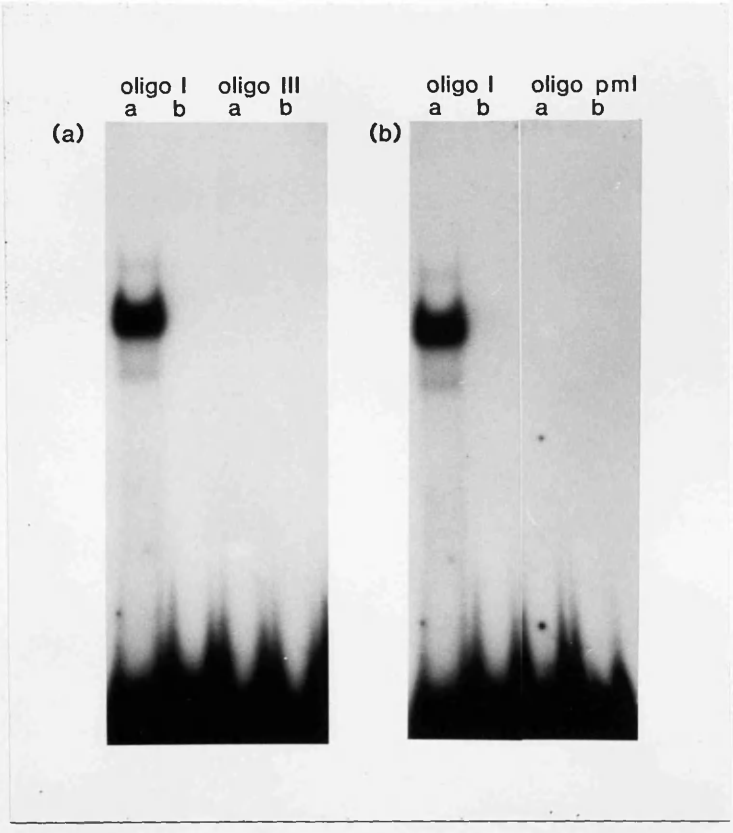

oligo I
a b
oligo III
a b
(a)
oligo I
a b
oligo pml
a b
(b)

Figure 3.26 Gel retardation analysis of the binding of the UL9 fusion protein to labelled oligonucleotides

(a) Oligonucleotides I and III (see below) were ^{32}P-labelled to comparable specific activites and incubated with KBl extract either in the absence of competitor DNA (tracks a) or in the presence of a 100-fold molar excess of unlabelled oligonucleotide I (tracks b). Complexes were analysed in a 5% non-denaturing polyacrylamide gel. Oligonucleotide III contains the motif III sequence.

Oligonucleotide III 5' GATCTAAAAGAAGTCAGAACGCGA
ATTTTCTTCAGTCTTGCGCTCTAG 5'

(b) Oligonucleotide I and pmI were ^{32}P-labelled to comparable specific activities and incubated with KBl extract as described in part (a). Oligonucleotide pmI is identical to oligonucleotide I in containing the binding site I sequence except for the substitution of a single G residue with a T residue as found at the equivalent position in motif III (shown in Fig. 3.22b and underlined below).

Oligonucleotide pmI:

5' GATCCGCGAAGCGTTCTCACTTCGTCCCA
GCGCTTCGCAAGAGTGAAGCAGGGTCTAG 5'

by UL9 binding to site I.

3. A point mutation within binding site I which abolishes the ability of UL9 to bind and an ori_S-containing plasmid to replicate

(a) effect of the point mutation on binding

As motif III and the corresponding 11 bp sequence within binding site I differ at only a single nucleotide {marked in Fig. 3.22(b)} I decided to examine the effect of introducing this single base difference from motif III into site I. A duplex oligonucleotide (oligonucleotide pmI) was synthesised which was identical to oligonucleotide I in containing the binding site I sequence except for the substitution of a single G residue with a T residue as found at the equivalent position in motif III. KB1 extract was incubated with labelled oligonucleotides I or pmI and the products analysed on a polyacrylamide gel {Fig. 3.26(b)}. No retarded bands were observed with oligonucleotide pmI {oligo pmI(a)} but, as expected, a retarded band was observed with oligonucleotide I. Hence, this point mutation within binding site I is sufficient to abolish interaction with the UL9 protein.

(b) effect on ori_S activity

As the single base change described above resulted in the inability of site I to interact with the UL9 protein, the effect of this single point mutation on ori_S activity was also investigated. Using the oligonucleotide-directed mutagenesis method outlined in 3C part 1, the point mutation was introduced into plasmid pST19 using a 20 mer oligonucleotide. The presence of the single base change within site I of the resulting plasmid, pST19pmI, was verified by DNA sequencing (Fig. 3.27). The replicative ability of plasmid pST19pmI was compared with the parental plasmid pST19, pST19delI and pST19delII.

Plasmids were transfected into BHK cells which were either mock-infected or superinfected with HSV-1 to provide

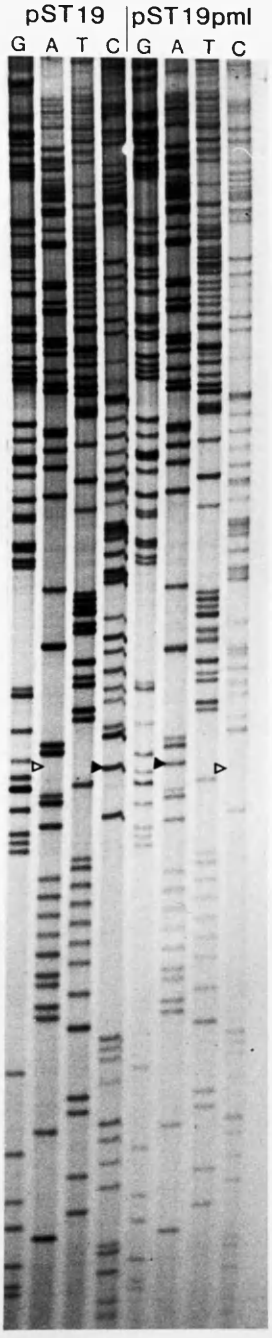

pST19
pST19pml
G A T C
G A T C

Figure 3.27 Dideoxy sequencing of plasmid pST19pmI containing a point mutation within ori_S

Using site-directed mutagenesis, a single G residue within binding site I of ori_S was substituted with a T residue (see Fig. 3.22(b) and the legend to Fig. 3.26). Individual colonies were picked and a plasmid pST19pmI carrying the desired mutation (i.e C to A, since the sequence was obtained from the complementary strand) was identified by sequencing the ori_S insert. The wt C residue in pST19, indicated by a bold arrowhead, is absent from pST19pmI (open arrowhead) and a novel A residue (bold arrowhead) appears in pST19pmI which is not found in pST19 (open arrowhead).

helper functions. Samples of DNA extracted from cells were cleaved with EcoRI and DpnI and replicated molecules detected by Southern blotting. Fig. 3.28(a) demonstrates that the introduction of the point mutation within binding site I was as effective as the 11 bp deletion in abolishing ori_S activity. No detectable replicative products were observed with pST19pmI or pST19 delI {pmI(b) and dI(b)}).

A gel retardation assay was performed to examine the interaction of the UL9 protein with pST19pmI ori_S. The mutated BamHI-SalI ori_S-containing fragment from pST19pmI was end-labelled and incubated with KR2 or KB1 extract and the products analysed on a polyacrylamide gel. As expected, the pST19pmI behaved similarily to pST19delI as only one major complex was detected {Fig. 3.28(b); pmI(b)}. These results demonstrate that a single point mutation within the UL9 protein binding site I of HSV-1 ori_S can effectively prevent protein interacting with this sequence and can also eliminate the ability of an ori_S-containing plasmid to replicate.

4. Binding of the UL9 protein to the VZV ori_S

Stow and Davison (1986) demonstrated that HSV-1 encoded functions could amplify plasmids containing VZV ori_S sequences, albeit at low efficiency (approximately 5% of that observed for HSV-1 ori_S). It was suggested that the 11 bp sequence located to the left hand side of the VZV palindrome and conserved in HSV binding site I, may play a role in origin recognition.

Based on the observation that the C-terminal 317 amino acids of the UL9 polypeptide exhibited sequence-specific DNA binding activity, the C-terminal 322 amino acids of the VZV homologue to the HSV-1 UL9 gene, gene 51, have been similiarly expressed in *E.coli* and shown to bind to specific sequences within the VZV viral origin of DNA replication using gel retardation assays (Stow *et al.*, in press). Three binding sites for the VZV gene 51 protein were identified within VZV ori_S, lying in the same orientation and on the same side of the palindrome. Each site contains an 11 bp sequence which is either identical or

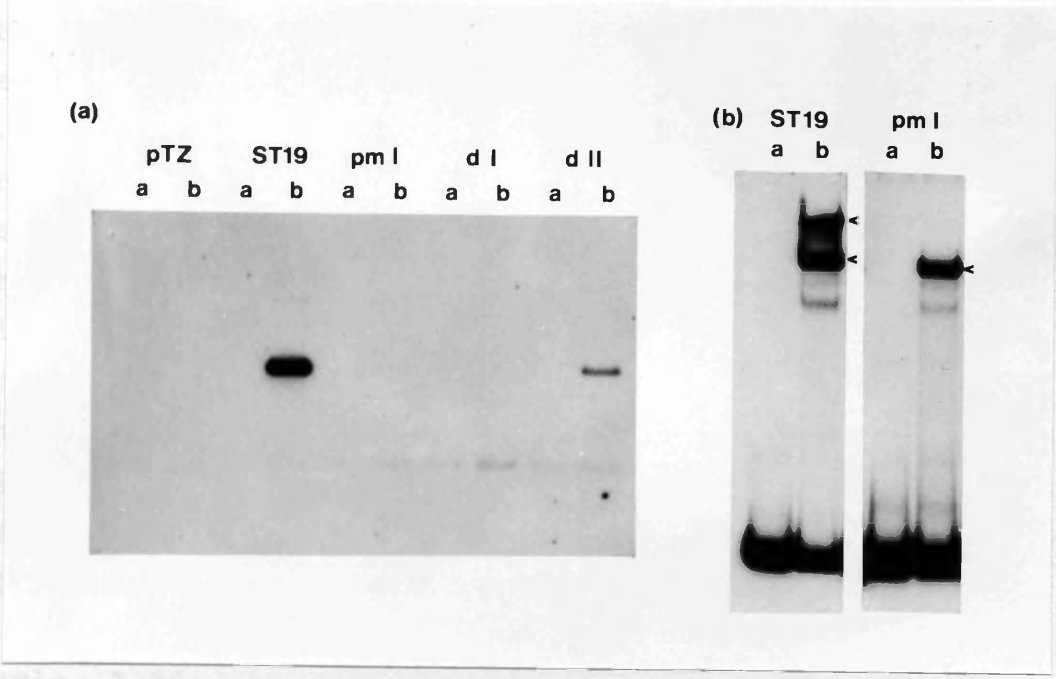


Figure 3.28 Replication and binding activity of pST19pmI

(a) BHK cells were transfected with pTZ19 (pTZ), pST19 (ST19), pST19pmI (pmI), pST19delI (d I) and pST19delII (d II) as indicated and either mock-infected (tracks a) or superinfected with HSV-1 (tracks b). Samples containing 1/15 of the DNA recovered from each monolayer were digested with EcoRI plus DpnI and the fragments separated by electrophoresis, transferred to nitrocellulose and hybridised to ^{32}P-labelled pAT153. An autoradiograph of the washed filter is shown. The faint bands visible in the lower portion of the figure represent DpnI digestion products of unreplicated input plasmid DNA molecules.

(b) Gel retardation analysis using ^{32}P end-labelled BamHI plus SalI insert fragments of plasmid pST19 and pST19pmI. Labelled DNA fragments were incubated with control extract KR2 (tracks a) or KB1 extract (tracks b)and the products separated by electrophoresis in a 8% non-denaturing polyacrylamide gel. The major complexes are indicated by arrowheads.

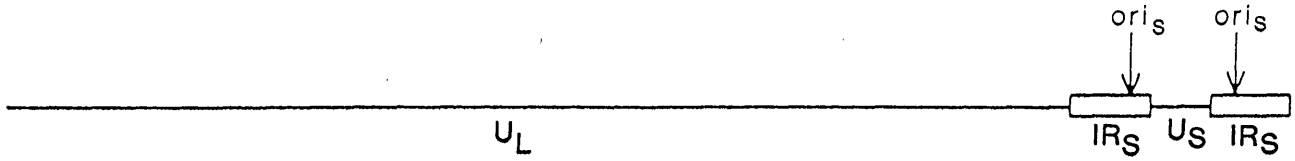
very similar to that of the 11 bp sequence in site I of HSV ori_S {see Fig. 3.29(b) and (c)}.

To compare the interaction of the two homologous proteins with the VZV origin, DNase I footprints were performed using either a HindIII or EcoRI labelled 259 bp VZV ori_S fragment from pVO2 containing the two binding sites closest to the palindrome, designated motifs A and C in Fig. 3.29. Extracts from bacteria expressing the UL9 fusion protein (KB1 extract) or the gene 51 fusion protein (KV51 extract) were incubated with the probe and treated with DNase I. The DNase I digested protein/DNA complexes were resolved on a preparative polyacrylamide gel. Two major retarded complexes were obtained with each extract similar to those seen for the protein A/UL9 fusion protein binding to HSV ori_S (see Fig. 3.25 track 2). DNA from bands corresponding to the upper and lower (i.e. lower and higher mobility) KV51 complexes , the upper KB1 complex and the free DNA probe was extracted and analysed on a denaturing gel (Fig. 3.30).

Protection of sequences in the region of both motifs A and C was detected in the upper complex formed between KV51 extract and probe labelled at the HindIII terminus. The corresponding complex formed with probe labelled at the EcoRI site showed clearer protection in the region of motif A but a weaker footprint over motif C. The patterns obtained with the upper complex formed using KB1 extract and the two probe fragments were almost indistinguishable from those formed with KV51 extract demonstrating that the two viral polypeptides are capable of binding to the same DNA sequences. Sites hypersensitive to DNase I digestion were observed within the AT-rich region of the palindrome with KV51 extract (HindIII fragment) and with KB1 (EcoRI fragment). The lower complexes formed with KV51 extract exhibited partial protection over both A and C motifs suggesting that they contain DNA molecules with the fusion protein bound to one or other site.

UL9 protein is therefore able to interact with the same binding sites in VZV ori_S as the VZV gene 51 protein. Moreover, these sites are closely related in sequence to the mapped binding sites in HSV-1 ori_S. The ability of UL9 protein to bind to VZV ori_S is consistent with the

(a)



(b)

```
KpnI
GGTACCCCGC CGATGTTTAT AACCATAATT CTCTAAACCG TTGTAGAAAA TCACAAAAAA ATTTATTCAA      70
CCATGGGGCG GCTACAAATA TTGGTATTAA GAGATTTGGC AACATCTTTT AGTGTTTTTT TAAATAAGTT
                                                  B
AAACAAGTCG AAGAACTTCA TATCTGAGGC ATGTAAACCC GTTCGCACTT CCTGGGGTGG AATGGGGTGG     140
TTTGTTCAGC TTCTTGAAGT ATAGACTCCG TACATTTGGG CAAGCGTGAA GGACCCCACC TTACCCCACC
                                                        RsaI
GGTGGGGGGG TGAAAAAGGG GGGGGGTTAA ATTGGGCGTC CGCATGTCTG TGGTGTACGC CAATCGGATA     210
CCACCCCCCC ACTTTTTCCC CCCCCCAATT TAACCCGCAG GCGTACAGAC ACCACATGCG GTTAGCCTAT
                C
CACTCTTTTG ATCTGCATTC GCACTTCCCG TTTTTTCACT GTATGGGTTT TCATGTTTTG GCATGTGTCC     280
GTGAGAAAAC TAGACGTAAG CGTGAAGGGC AAAAAAGTGA CATACCCAAA AGTACAAAAC CGTACACAGG
          A
AACCACCGTT CGCACTTTCT TTCTATATAT ATATATATAT ATATATATAT ATATAGAGAA AGAGAGAGAG     350
TTGGTGGCAA GCGTGAAAGA AAGATATATA TATATATATA TATATATATA TATATCTCTT TCTCTCTCTC

TTTCTTGTTC GCGCGTGTTC CCGCGATGTC GCGGTTTTAT GGGGTGTGGG CGGGCTTTTC ACAGAATATA     420
AAAGAACAAG CGCGCACAAG GGCGCTACAG CGCCAAAATA CCCCACACCC GCCCGAAAAG TGTCTTATAT
                                    ClaI
TATATTCCAA ATGGAGCGGC AGGCTTTTTA AAATCGAT                                        458
ATATAAGGTT TACCTCGCCG TCCGAAAAAT TTTAGCTA
```

(c)

B CGTTCGCACTT
C CATTCGCACTT
A CGTTCGCACTT

I CGTTCGCACTT

Figure 3.29 Location and sequence of the VZV ori_S

(a) The structure of the VZV genome is shown and the position of both copies of ori_S are indicated.

(b) The sequence of the KpnI plus ClaI fragment containing ori_S is shown (nucleotides 109,893-110,350 in the sequence of Davison and Scott, 1986). The palindromic sequence is marked by arrows and motifs A, B and C are boxed. The three motifs form part of the sequence of three binding sites for the VZV homologue of the UL9 protein, the gene 51 product.

(c) The motifs A, B and C are aligned and compared to the 11 bp element from binding site I (I) of HSV-1 ori_S (Fig.3.25).

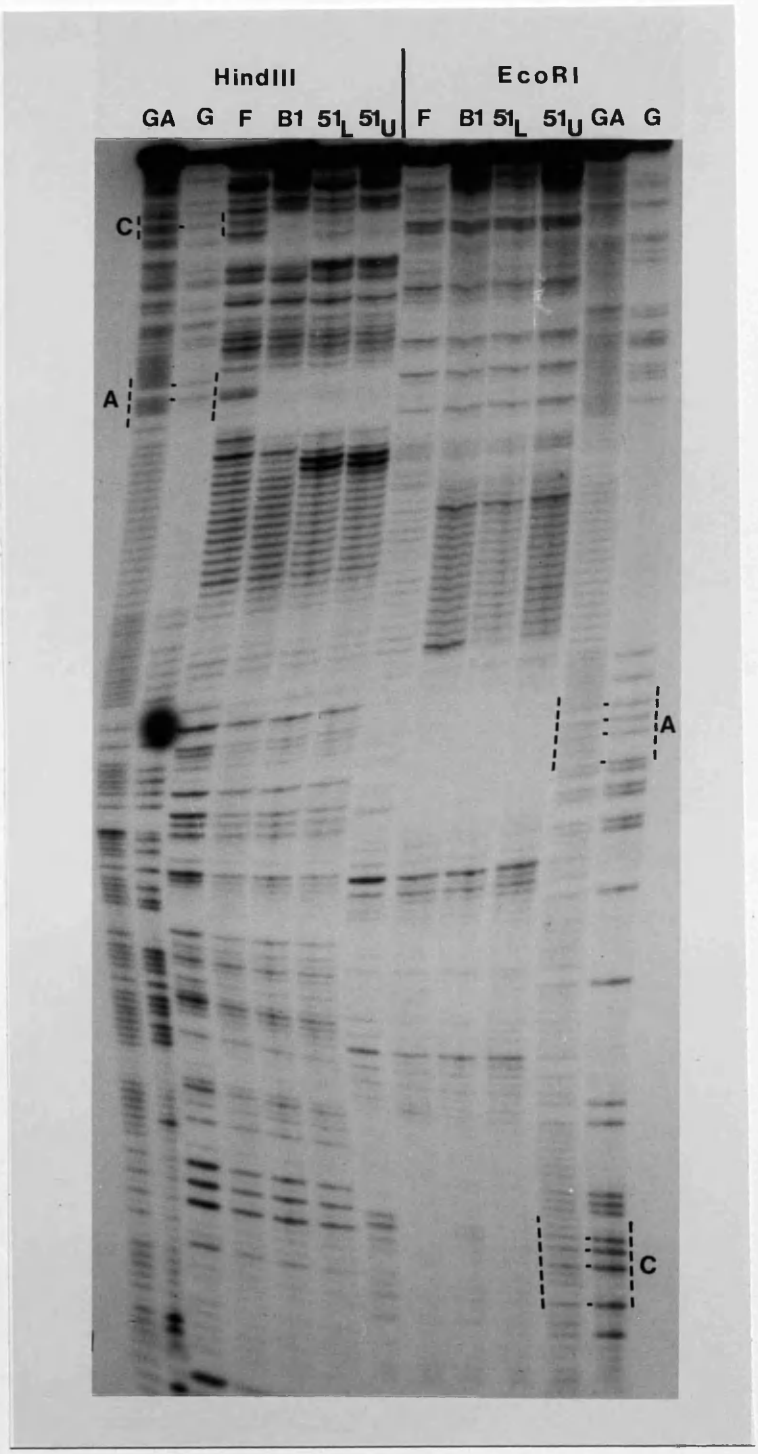

HindIII
EcoRI
GA G F B1 51L 51U F B1 51L 51U GA G
C
A
A
C

Figure 3.30 DNase I protection of VZV ori_S by HSV-1 UL9 and VZV gene 51 fusion proteins

The plasmid pVO2 ori_S insert was uniquely 3' end-labelled at either the EcoRI or HindIII terminus as indicated and allowed to form complexes with KB1 extract (containing the UL9 fusion protein) or KV51 extract (containing the VZV gene 51 fusion protein). Partial digestion with DNase I was performed prior to the resolution of complexes in a non-denaturing polyacrylamide gel. DNA was extracted from bands corresponding to free DNA (F), upper KB1 complex (B1), lower KV51 complex (51_L) and upper KV51 complex (51_U) [comparable to upper and lower complexes seen with KB1 and HSV-1 ori_S; Fig. 3.25, track 2] and analysed in a 6% denaturing gel together with markers generated by partial cleavage at G and A residues (GA) or at G residues (G). The position of sequence motifs A and C are shown and the G residues within these motifs indicated (strands labelled at the HindIII and EcoRI sites correspond to the top and bottom strands in Fig. 3.29 respectively. These sites are located adjacent to the ClaI and RsaI sites respectively).

previously observed ability of HSV-1 to activate replication of plasmids containing VZV ori_S (Stow and Davison, 1986).

CHAPTER 4: DISCUSSION

1. The HSV-1 origin binding protein

(a) Identification of the HSV-1 gene encoding the origin binding protein

A major objective of my work was to identify the HSV-1 gene product which binds specifically to HSV-1 ori_S (Elias *et al.*, 1986). Using *ts*K recombinant viruses individually expressing four of the seven HSV-1 replication genes, the UL9 gene was identified as the only replication gene whose product was required for origin binding activity. This result (Weir *et al.*, 1989) is in complete agreement with the results of Olivo *et al.* (1988) who expressed this viral protein in a baculovirus vector and assayed for binding activity by immunoprecipitation and DNase I footprinting.

The origin binding activity was also detected in nuclear extracts from cells infected with *wt* HSV-1 and HSV-2. This observation is consistent with the fact that HSV-1 and HSV-2 are closely related alphaherpesviruses and that the UL9 protein binding sites within HSV-1 ori_S are conserved in HSV-2 ori_S. Independent verification that the UL9 gene product exhibited origin binding activity was obtained using an *E.coli* expression system and an *in vitro* transcription and translation system.

The *ts* mutant, *ts*S, whose lesion maps to the UL9 gene (Matz *et al.*, 1983), exhibited a tight DNA negative phenotype at NPT yet produced similar levels of origin binding activity to *ts* mutants whose mutations map to five other essential replication genes. This indicates that the mutation does not irreversibly affect the structural DNA binding requirements of the UL9 protein. Moreover, marker rescue experiments, using fragments of the UL9 gene have shown that the mutation lies in the N-terminal 2/3 of the gene (E. C. Stow, unpublished results).

The level of origin binding activity observed in nuclear extracts from cells infected with individual DNA

negative ts mutants at NPT was significantly reduced in comparison with wt HSV-1. Although the UL9 protein is able to enter the nucleus in the absence of DNA replication (Weir et al., 1989) it is possible that it may exhibit reduced stability under these conditions. Careful examination of the replication assays performed with ts mutants and of the gel retardation assays using nuclear extract from cells infected with the ts mutants does suggest some correlation between DNA phenotype and the level of DNA binding activity i.e. ts1205, 1206 and 1234 which were slightly leaky at 38.5°C do show slightly increased levels of origin-binding activity at 38.5°C as does ts1206 at 39.5°C.

(b) Size of the UL9 protein in cells infected with wt HSV-1 or the tsK/UL9 recombinant

By developing a gel retardation assay, I was able to distinguish different mobilities of complex depending on the source of protein extract. These results are interesting to compare with other published reports which have examined UL9 activity using the same technique. The complex with the highest mobility found in wt HSV-1 infected cell extracts, complex A (e.g. Fig. 3.5), clearly contains only a portion of the intact UL9 gene product. This complex is very similar to the major complex described by Koff and Tegtmeyer (1988) using extracts from HSV-1 infected Vero cells. Crude estimates of the molecular weight of the protein moiety in complex A were around 25,000, clearly inconsistent with the binding of an intact UL9 polypeptide (molecular weight 94,000). Aside from complex A, specific lower mobility complexes were sometimes observed in nuclear extracts from wt HSV-1 infected cells. These complexes probably contain larger forms of UL9 polypeptide. The intensity of these bands was found to vary from extract to extract, probably as a result of varying levels of proteolytic activity. The small UL9 protein fragment forming complex A was however, always the predominant species.

Elias and Lehman (1988) purified UL9 from HSV-1 infected Vero cells and observed a major polypeptide of molecular weight 83,000 and two smaller fragments of 45,000

and 38,000. Considering that the method used to estimate the size of the protein fragment in complex A is very crude, it is possible that the 38,000 molecular weight fragment is equivalent to that forming complex A. Elias and Lehman obtained a very different pattern of binding for the 83,000 molecular weight protein in gel retardation assays from that which I obtained with extracts from cells infected with wt HSV-1. However, their binding pattern was similar to that seen with extract from cells infected with the tsK/UL9 recombinant which appears to express an undegraded UL9 polypeptide with molecular weight around 91,000 (J. Calder, personal communication). In both cases, a significant proportion of the labelled protein failed to enter the gel and a smeared pattern of binding was observed throughout the length of the track. These results suggest that in extracts from HSV-1 infected cells, the UL9 polypeptide may be subject to varying degrees of proteolytic cleavage. This was also demonstrated *in vitro* by adding protease to tsK/UL9 recombinant extract. A smaller protein fragment was generated which was relatively protease resistant and exhibited a similar mobility to complex A.

Whether the smaller UL9 fragments observed in this work and by Koff and Tegtmeyer (1988) are physiologically significant or whether they merely reflect proteolytic activity occuring during extraction is unknown.

The observation that extracts from cells infected with the tsK/UL9 recombinant do not contain the cleaved form of the protein i.e. do not produce complex A, suggests that the proteolytic cleavage occurs as a consequence of the virus infection. The fact that extracts from cells infected with DNA negative ts mutants produced complex A indicates that the proteolytic activity, if virus encoded, is synthesised before the onset of DNA replication (i.e. probably as an IE or E function).

Although the interaction of intact UL9 polypeptide with labelled probe fragments produced a smeared pattern in a gel retardation assay, this binding was nevertheless sequence-specific. The smearing may occur as a result of aggregation and/or dissociation of complexes during incubation of the binding reactions and gel electrophoresis. This pattern would appear to be dependent upon the UL9

sequence present, as complexes containing only proteolytic fragments of UL9 or the C-terminal 317 amino acids of the protein synthesised in the E.coli expression system produced much tighter bands. The smearing may therefore be a consequence of interactions involving the N-terminal portion of the polypeptide. Interestingly, extracts containing the C-terminal 2/3 of the UL9 polypeptide exhibited a similar binding pattern to that of the intact UL9 protein i.e. smeared binding with little formation of sharply resolved protein-DNA complexes. This suggests that sequences from the central 1/3 of the protein may be involved in protein/protein interactions. The formation of large complexes comprised of several monomers or dimers of origin binding proteins has previously been observed with the E.coli dnaA protein at oriC, the bacteriophage lambda O protein at the lambda ori and also SV40 T-ag at the SV40 core origin (section 1E).

(c) Sequence-specific binding specified by the C-terminal 1/3 of the UL9 polypeptide

Expression of fragments of the UL9 polypeptide in E.coli demonstrated that the C-terminal 317 amino acids specifies all the structural information required for sequence-specific recognition and binding. This was independently verified by transcribing and translating this fragment in vitro. These experiments exclude the possibility that sequence-specific recognition of DNA by the UL9 protein occurs in conjunction with cellular nuclear proteins.

The E.coli expression system provided a very convenient source of DNA binding activity. The bacterial extracts were simple to prepare and the DNA binding activity remained stable at -70^{o}C for at least six months. Such a system should prove useful for deletion/insertion mutagenesis of the 317 amino acid fragment to map precisely the structural requirements for DNA binding activity.

Assuming that the proteolytic DNA binding fragment of the UL9 protein seen in HSV-1 infected cell extracts lies within the molecular weight range of 25,000-30,000, this fragment probably contains only 230-270 amino acids. The

pTZ vectors could be conveniently used for introducing specific amino acid changes into the C-terminal region. The effect of these changes could be assayed by *in vitro* transcription and translation or following introduction into the *E.coli* fusion protein expression vector. As the binding activity produced by *in vitro* transcription and translation of the C-terminal 1/3 of the UL9 gene was lower than that seen with the *E.coli* extracts, it is possible that small changes in the ability of the protein to bind DNA may be difficult to detect. However, as the *in vitro* system was not fully optimised in this work, the potential of this system remains to be determined.

C-terminal DNA binding domains have previously been noted for other herpesvirus proteins for example the oriP binding protein of EBV, EBNA-1 (Rawlins *et al.*, 1988), and also the major ssDBP of HSV-1 encoded by UL29 (Leinbach and Heath, 1988) which binds in a sequence-independent manner. Following on from the work described in this thesis, the C-terminal 322 amino acids of the VZV homologue to the UL9 protein, the gene 51 product, were also shown to bind in a sequence-specific manner to VZV ori_S (Stow *et al.*, submitted for publication). Structurally distinct DNA binding domains have been identified in a variety of other proteins, often on the basis of their resistance to the action of added protease e.g. CAAT-box binding protein (van Huijsduijnen *et al.*, 1987), lambda integrase (de Vargas *et al.*, 1988), Vmw175 (Paterson and Everett, 1988; Faber and Wilcox, 1988) and glucocorticoid/progesterone steroid receptors (Tsai *et al.*, 1988).

(d) Identification of two UL9 protein binding sites within HSV ori_S

(i) using synthetic oligonucleotides

Competition experiments using synthetic oligonucleotides demonstrated the presence within HSV-1 ori_S of two binding sites for the UL9 protein. This was in contrast to a contemporaneous report of Elias *et al.* (1986) of only a single binding site (binding site I). The second site, site II, contains a sequence related to site I and is

present in an inverted orientation at the right hand end of the palindrome. Identification of this second binding site within ori_S (Weir et al., 1989) was also made independently by Elias and Lehman (1988) and Olivo et al. (1988). Additional competition experiments demonstrated that the UL9 polypeptide has approximately a 10-fold higher affinity for binding site I than site II. This result is in agreement with the work of Elias and Lehman (1988) who measured the half lives of UL9 complexes formed with site I and site II by adding an excess of specific competitor DNA at various times to reactions which had reached equilibrium. The UL9 complex with site I was approximately 10 times more stable than that with site II. The reason for the HSV ori_S containing UL9 binding sites of differing affinities is not known but possibly they may serve as a signal for determining the position within a specific DNA strand of a nicking site for initiation of rolling circle replication or confer some directionality on the synthesis of daughter DNA strands. The sequence of HSV-1 ori_L (which is a perfect palindrome) indicates that within this origin, two identical UL9 binding sites are present.

(ii) by DNase I footprint analysis

Gel retardation assays using the C-terminal fragment of the UL9 polypeptide produced two distinct complexes with ori_S DNA. DNase I footprint analysis of the lower mobility complex illustrates that this complex represents binding to sites I and II within ori_S (Fig. 3.21). Several other groups have carried out DNase I footprint analysis of UL9 binding to ori_S and in one instance methylation interference assays were used to establish which G residues are in direct contact with the protein. The results of these studies are summarised in Fig. 4.1.

In general, the protected sequences are very similar. Using HSV-1 infected cell extract, Koff and Tegtmeyer (1988) and Deb and Deb (1989) detected binding to site I alone. It is interesting to observe that the C-terminal DNA binding domain of the UL9 protein appears to protect a similar number of bases at site I as purifed

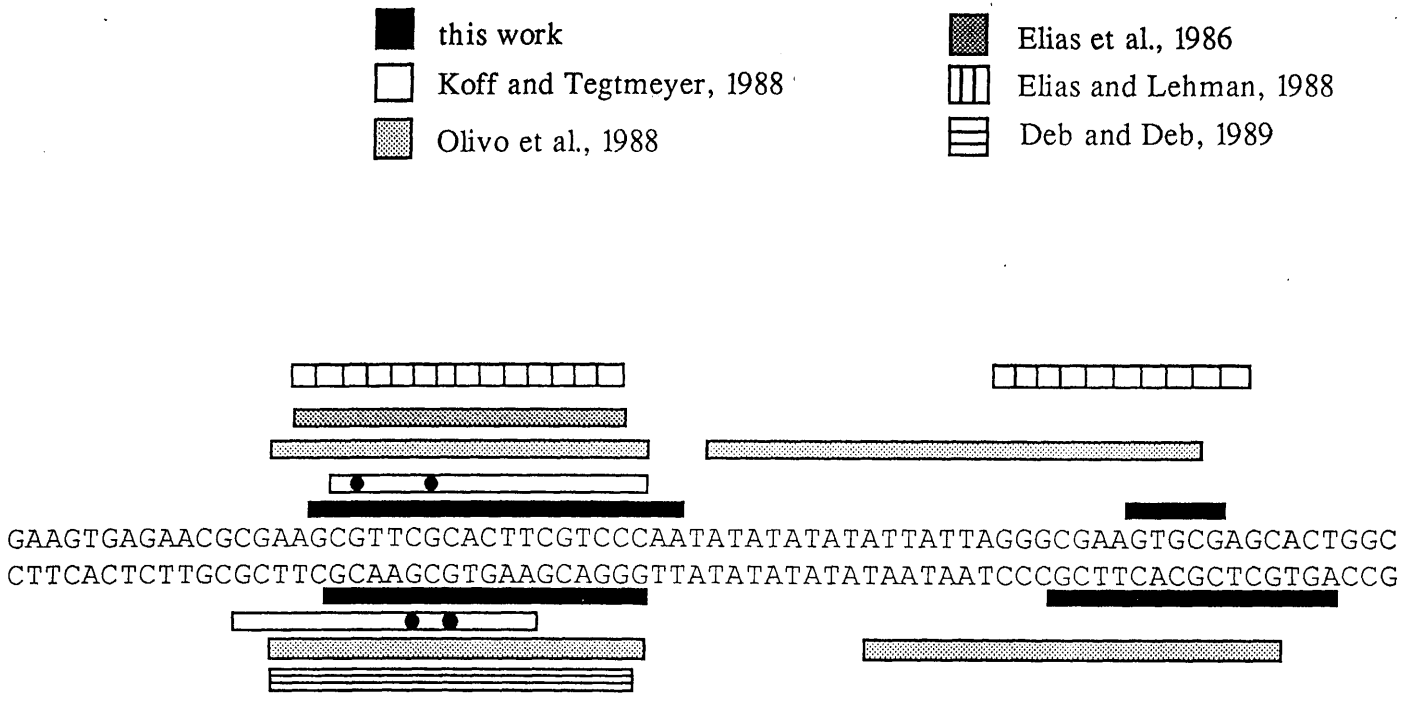

this work
Koff and Tegtmeyer, 1988
Olivo et al., 1988
Elias et al., 1986
Elias and Lehman, 1988
Deb and Deb, 1989
GAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGC
CTTCACTCTTGCGCTTCGCAAGCGTGAAGCAGGGTTATATATATATAATAATCCCGCTTCACGCTCGTGACCG

Figure 4.1 DNase I protection of the HSV-1 ori_S by UL9

The diagram shows the core HSV-1 ori_S sequence (Stow and McMonagle, 1983). The results of the DNase I footprint shown in Fig. 3.21(b) are marked as solid black bars. DNase I footprints of UL9 binding to ori_S have been performed by several other groups of workers and are summarised in the diagram and referenced in the key. Bold circles within the protected region of Koff and Tegtmeyer (1988) highlight four G residues, which by methylation interference assays, were found to contact the UL9 protein.

intact UL9 protein (Elias and Lehman, 1988; Olivo et al., 1988). Using the C-terminal DNA binding domain, weaker protection was observed at site II over a smaller region. This probably reflects the lower affinity of UL9 binding to this site. However, both Elias and Lehman (1988) and Olivo et al. (1988) using purified intact UL9 and baculovirus expressed UL9 respectively detected strong protection over a larger area. It has been suggested by Challberg and Kelly (1989) that the footprinting assays of Elias and Lehman (1988) and Olivo et al., (1988) indicate that the affinity of UL9 for the two sites when present together is equal. However, careful examination of the published data reveals that at lower UL9 protein concentrations, site I is completely protected before site II, substantiating the results of the affinity studies using synthetic oligonucleotides containing individual binding sites. The protected region identified by Olivo et al. (1988) at site II is distinctive in that it extends into and covers most of the AT-rich region of the palindrome, especially on one strand of the DNA. Elias and Lehman (1988) noted that increasing the UL9 concentration in their footprinting assays resulted in less frequent cutting in the AT-rich region and an increased intensity of some bands immediately to the left of binding site I. These alterations may be a consequence of UL9 binding to additional sites, protein/protein interactions between molecules bound to sites I and II or altered conformation in the DNA induced by binding of protein to both sites. This latter possibilty is a common feature of origin recognition proteins binding adjacent to AT-rich regions (Zahn and Blattner, 1985a; Borowiec and Hurwitz, 1988b).

Methylation interference assays by Koff and Tegtmeyer (1988) have identified four G residues within binding site I which are contacted by the UL9 protein (Fig. 4.1). The core area of interference is confined to an 8 bp sequence 5'-GTTCGCAC-3' (CAAGCGTG). This segment corresponds exactly to two inverted and overlapping repeated sequences with the consensus sequence 5'-GT(T/G)CG-3'and reads:

5'-GTTCG-3'
3'-GCGTG-5'

This inverted motif is perfectly conserved in ori_S and ori_L of all sequenced human alphaherpesviruses except for binding site II of HSV-1 and HSV-2 ori_S in which the motif is 5'-GCTCG-3'. It is interesting to note that even with the small footprint observed at site II with the E.coli C-terminal extract, the CG overlapping region between the inverted repeats is clearly protected. This observation confirms the importance of this region for binding. It is not known whether the T to C change is sufficient to account for UL9 binding to site I and II with differing affinities.

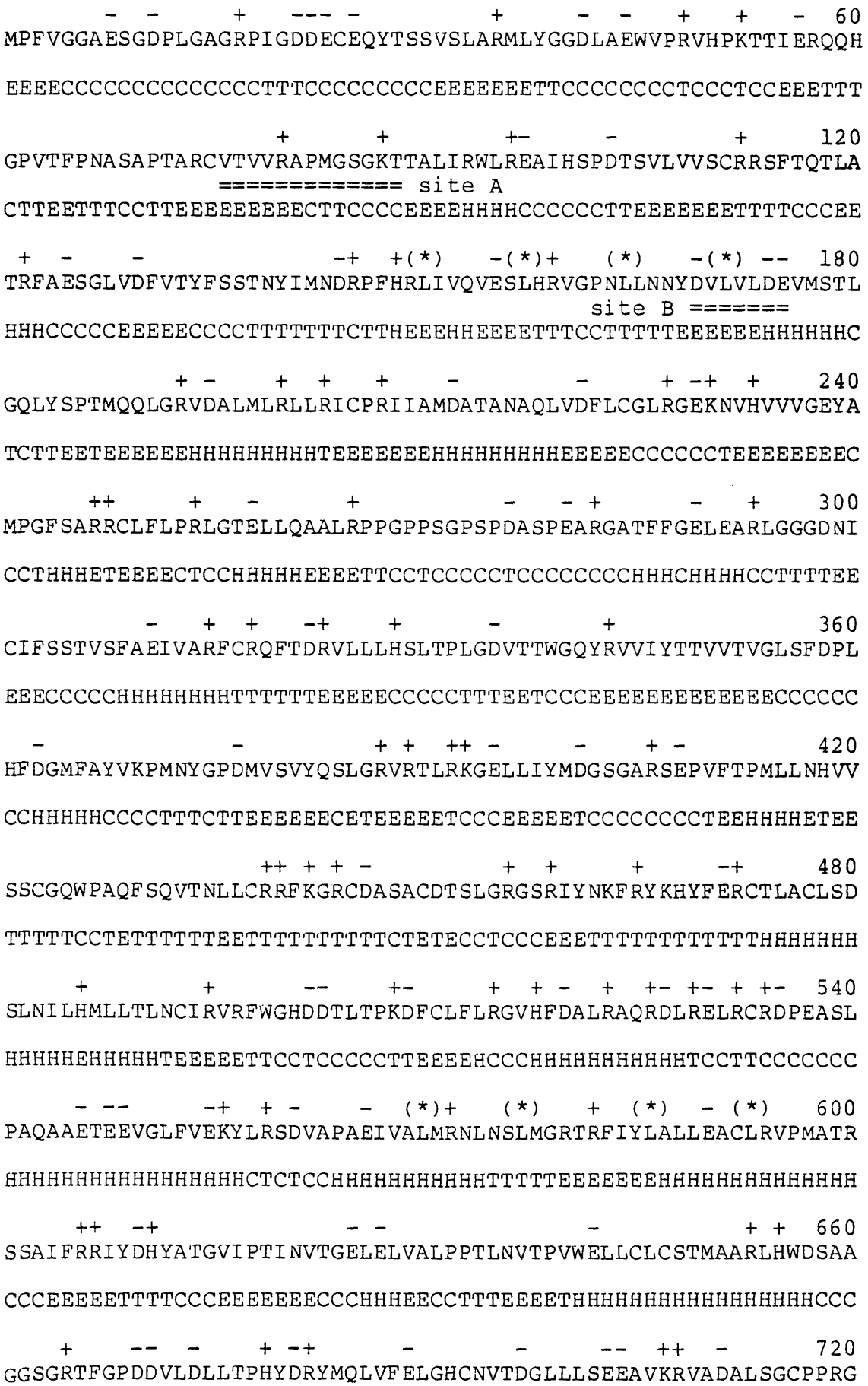
The presence of overlapping inverted DNA binding motifs within origins of DNA replication has previously been well documented for the T-ag binding site of polyoma virus. The T-ag binds to a pentanucleotide motif in a palindrome containing two direct repeats on each arm. The inverted repeats closest to the centre of the palindrome overlap by two base pairs (Trienzenberg and Folk, 1984).

The results of Koff and Tegtmeyer (1988) suggest that the UL9 protein may bind to the inverted repeat as a dimer. There is however no published evidence to suggest that this the case. Following purification of UL9 from infected cells, Elias and Lehman (1988) stated that the polypeptide exists as a monomer which tends to aggregate at low ionic strengths. However, other reports have cited unpublished sedimentation coefficients determined for purified baculovirus recombinant UL9 protein as demonstrating that the protein predominantly exists as a stable homodimer (cited in Challberg and Kelly, 1989).

2. Analysis of the predicted amino acid sequence of the UL9 gene

The sequence of the UL9 gene has been determined and the amino acid sequence of the encoded protein predicted (McGeoch et al., 1988a,b; Fig. 4.2). The UL9 gene appears to encode a large globular protein with few particularly distinctive features. Nevertheless, analysis of the predicted primary structure has revealed some interesting

Primary and Secondary Structure of the UL9 Polypeptide



```
                                                    (4)---------------
CCCCCCCCCTCEEECCCCCTTTEEEEEEHCTTTTTTTCHHHHHHHHHHHHHHETTTCCCTC

   - -           -                      + +    +        - ++ -+ 780
SVSETDHAVALFKIIWGELFGVQMAKSTQTFPGAGRVKNLTKQTIVGLLDAHHIDHSACR
-------   ----------------------(1)       (3)----------------
CCCCHHHHHHHHHHEHCCCCHHHHHTTTEEETTTCTEEEETEEEEEEHHHHHHHHHCCTTT

 ++         +++-     + + +      +  + +          -    -     -    840
THRQLYALLMAHKREFAGARFKLRVPAWGRCLRTHSSSANPNADIILEAALSELPTEAWP
------    (2)----------------------
TTHHHHHHHHHHHHHHHHHHHEEECTTTTTEETTTCCCCCTTHHHHHHHHHCCCCCHHHH

       851
MMQGAVNFST

HHHCCEEEET
```

Figure 4.2 Primary and secondary structure of the UL9 polypeptide

The predicted amino acid sequence (1-851) of the HSV-1 UL9 protein is shown in the single letter amino acid code. Secondary structure was predicted by the GARNIER program using the method of Garnier et al., (1978) and is shown below the sequence. C= random coil; E= beta sheet; H= alpha helix; T= turn. Acidic and basic residues are highlighted - and + above the amino acid sequence. Site 'A' and 'B' refer to the purine NTP-binding site identified by Gorbalenya and Koonin (1989). Underlined sequences (labelled 1-4) correspond to regions containing putative HTH motifs and leucines (indicated by *) refer to potential leucine zipper motifs.

features:

(i) the purine NTP-binding motif

McGeoch et al. (1988b) examined the predicted amino acid sequences of the HSV-1 DNA replication genes for the presence of several consensus elements associated with DNA binding, nuclear entry and nucleotide binding. In this manner, a consensus sequence derived by Walker et al. (1982) associated with ATP-binding sites was located in the UL5 gene. The presence of a variety of motifs suggested that the UL5 protein could potentially encode a helicase activity (Hodgeman, 1988) and not surprisingly, this protein has been identified as one of the polypeptides associated with a three-subunit helicase/primase complex which can utilise ATP and GTP (Crute et al., 1989). UL5 alone however does not exhibit helicase activity (Calder and Stow, submitted for publication).

More recently, Gorbalenya et al. (1989) and Gorbalenya and Koonin (1989) have carried out a systematic analysis of protein sequences containing the purine NTP-binding motif and have located a further example within the N-terminal region of the UL9 protein.

The NTP-binding motif consists of two distinct domains: an 'A` site and located adjacent on its C-terminal side, a 'B` site (Gorbalenya and Koonin, 1989). The 'A` site motif consists of:

<hydrophobic stretch>(G/A)xxGxGK(S/T)

where x represents any amino acid and the hydrophobic stretch normally contains at least two hydrophobic residues out of five. From X-ray crystallography data, the GxG flexible loop structure is thought to bind the phosphate group of ATP or GTP. The 'B` site motif consists of:

<hydrophobic stretch>D(E/D)

The hydrophobic stretch should contain at least three hydrophobic residues out of five. Again, X-ray data have suggested that the invariant D residue chelates Mg^{2+}.

Site 'A` of the UL9 gene motif is clearly located between residues 77-89 (Fig. 4.2; site A) where three out of five residues are hydrophobic in the hydrophobic stretch. Site B is located between residues 169-175 (Fig. 4.2; site

B) where four hydrophobic residues out of five are found in the hydrophobic stretch.

X-ray crystallography data have also shown that both motifs are usually folded in a beta sheet-turn-alpha helix structural unit with the N-terminal hyrophobic beta strands being the most highly conserved. To look for such structures in the purine NTP-binding site of UL9, a computer prediction of the potential secondary structure of the UL9 protein was performed using the GARNIER program which makes use of the algorithms of Garnier et al. (1978). The program determines the liklihood of each residue being in one of four secondary structures; H (alpha helix), E (extended chain/beta sheet), T (reverse turn) or C (random coil). The predictions are about 40% accurate for small globular proteins but of questionable application to larger globular proteins where little structural information is known. The output of the GARNIER prediction for the UL9 protein is shown in Fig. 4.2. Not surprisingly, the hydrophobic stretches of site A and site B are predicted to fold as beta sheets. There is also a predicted turn in site A at the first invarient G (residue 85).

The study by Gorbalenya and Koonin (1989) of viral and cellular proteins containing such NTP binding motifs allowed the delineation of four large superfamilies of proteins based on criteria such as statistically significant sequence similarities and the presence of distinct highly conserved sequences in addition to the 'A` and 'B` motifs of the NTP binding pattern. The UL5 protein was assigned to superfamily 1 and UL9 to superfamily 2. Proteins from each of these two families contain seven conserved regions which span most of the polypeptide chain. As both families contain proteins known or thought to encode helicase activity, this would suggest some intrinsic helicase activity associated with the UL9 protein. Unpublished experiments from M. Challberg's laboratory and our own (J. Calder personal communication) have used model substrates containing single and double-stranded regions and detected a helicase activity associated with UL9 which is ATP dependent.

This raises the interesting possibility that the UL9 protein, in addition to recognising and binding

specifically to ori_S and ori_L, can locally unwind a short stretch of DNA at the origin in an analogous manner to SV40 T-ag. The SV40 T-ag carries out the dual role of opening strands of duplex DNA at the origin and at the replication forks (Stahl et al., 1986; Wold et al., 1987). In HSV, these functions may be carried out by separate proteins; UL9 at the origin, and the UL5/UL8/UL52 helicase/primase complex at the replication forks. Alternatively, the relatively large helicase/primase complex may require a 'melted' region of DNA produced by the action of the UL9 protein at the origin in order to make its initial attachment. A similar situation has been previously described for the dnaB helicase of E.coli (section 1E). There is no convincing evidence as yet, that the UL9 protein binds or hydrolyses ATP or is capable of unwinding dsDNA at the HSV origin.

As described in part 1(a) marker rescue experiments have indicated that the mutation in tsS (UL9 mutant) is located in the N-terminal 2/3 of the protein. It is interesting to speculate that the mutation may in fact disrupt the helicase activity which would suggest an essential role in replication for this activity. As the sequence-specific recognition and binding of UL9 are specified by the C-terminal 1/3 of the polypeptide, the helicase function is clearly not required for these activities.

(ii) potential DNA binding motifs

Analysis of information derived from published sequences of DNA binding proteins and X-ray crystallography has allowed the identification and characterisation of conserved structural motifs by which many proteins recognise DNA or dimerise (Anderson et al., 1981; Fairall et al., 1986; Landschulz et al., 1988; Johnson and McKnight, 1989). These motifs have mainly been identified in prokaryotic and eukaryotic transcription regulatory proteins and products of cellular oncogenes which bind as dimers to their DNA binding sites. Whether one should expect to find such motifs within an HSV replication protein is questionable. However, analysis of the amino acid sequence of UL9, with a view to the identification of DNA binding motifs, has produced some

interesting observations:

(a) helix-turn-helix (HTH) motif

The HTH motif was initially characterised through analysis of the DNA binding domains of three prokaryotic regulatory proteins (Anderson et al., 1981; Pabo and Lewis, 1982; Steitz et al., 1982). The essential structural components of the binding domain consist of two short alpha helices separated by a beta turn located within a 22 amino acid stretch. The carboxy terminal helix, helix 3, interacts with the major groove of the DNA and is locked in place by hydrophobic interactions with helix 2 which is positioned over helix 3, relative to the DNA.

Brennan and Matthews (1989) have compiled a statistical procedure for identifying putative HTH motifs. The putative amino acid sequence is compared with a 'master' set of prealigned sequences, 22 amino acids long, taken from proteins known, or highly likely to contain, HTH motifs. The sequence of interest is given a probability score by evaluating the correspondence between every possible 22 amino acid segment of the protein and the 'master' set. By inspection of the 'master' set sequence alone, four common characteristics for such motifs have been proposed:

(1) residue 9 of the 22 amino acid segment should be a glycine
(2) residues 4 and 15 should not be charged
(3) residues 3-8 and 15-20 should be proline free (being within alpha helices)
(4) residue 5 should not be a beta-branched residue (being wedged between the two helices)

Manually scanning the amino acid sequence of the UL9 protein identified a 22 amino acid segment at positions 731-752 (1; Fig. 4.3) which closely fits these criteria except for a proline at residue 20. The statistical scoring method did not produce a significant score for this segment.

A computer program compiled by Dr. A. Bailey in this department, using the scoring method of Dodd and Egan (1987) (which is similar to Brennan and Matthews (1989) but based on a larger 'master' set of HTH proteins) was used to

search every 22 amino acid segment of the UL9 protein. The top four scoring regions are marked in Fig. 4.3; position 731 - score: 841; position 793 - score: 771; position 765 - score: 464; position 706 - score: 305. A genuine HTH motif normally scored 1100 and above while random protein sequences scored less than 600. Hence putative motifs at positions 3 and 4 can probably be disregarded. Although the scores of putative motifs at positions 1 and 2 are not very significant, it is interesting that these two segments lie within the DNA binding domain. No distinctive HTH secondary structure was identified in each of these segments. It will be interesting to see if future fine mapping of the minimal domain required for DNA binding correlates with the requirement for these two putative HTH segments.

(b) leucine zipper motif

The leucine zipper DNA binding motif, first described by Landschulz et al. (1988) consists of two distinct parts; a structural region involved in dimerisation of the protein and an adjacent N-terminal DNA binding domain, rich in basic amino acids. The structural region contains a repeat of 4-5 leucine residues separated by seven amino acid residues within a sequence that can form a stable alpha helix with the leucine residues present on one face of the protein molecule. The protein monomers are thought to bind in a parallel configuration as a result of interactions between the leucine containing helices. The dimerisation leads to a unique positioning of basic regions from the two subunits that is critical for sequence specific binding (Landschulz et al., 1988).

A repeat of four leucine residues can be found between residues 150 and 170 of the UL9 protein (Fig. 4.2). It is unlikely that this is significant for DNA binding for several reasons. This region does not have an N-terminal 30 amino acid basic stretch and the leucines themselves do not appear to be part of a stable alpha helix. The leucine repeat lies outwith the DNA binding domain and overlaps with the proposed B site of the helicase domain.

A second leucine repeat is also found within the DNA binding domain of the protein at positions 570-593 (Fig. 4.2) but again it is doubtful whether this region is

significant as there is no adjoining basic region and there are actually 8 residues between the second and third leucines L2 and L3. It has recently been shown that the product of the FOS oncogene is incapable of sequence specific interaction with DNA unless it is complexed with the product of the JUN oncogene, via their respective leucine zippers, suggesting that the zipper regions may promote the formation of heterodimeric DNA binding complexes (Distal et al., 1987; Kouzarides and Ziff, 1988; Sassone-Corsi et al., 1988). The possibility that following the formation of an open region at the origin by the UL9 helicase, the leucine repeat, located in the N-terminal region, is involved in protein-protein interactions with other replication proteins, is open to investigation. Alternatively, the leucine repeats may be involved in dimerisation of the UL9 protein (see section (d)ii).

A third DNA binding motif, the zinc finger, has been identified in some eukaryotic regulatory proteins. A classic example of this motif is found in the eukaryotic transcription factor TFIIIA where a sequential and ordered occurrence of cysteine and histidine residues is repeated nine times in the amino acid sequence ($Cys-N_2-Cys-N_{12}-His-N_3-His$; Rhodes and Klug, 1986). This motif is not present in the UL9 amino acid sequence.

(iii) location of a UL9 binding site within the UL9 open reading frame

Having characterised the UL9 recognition sites within ori_S, a computer search of the HSV-1 sequence for additional possible UL9 binding sites was conducted using the FIND program. Using the consensus sequence of Koff and Tegtmeyer (1988), YGYTCGCACT (Y=pyrimidine), the search located seven 10 bp sequences within the HSV-1 genome. Six of these sequences were located within previously identified UL9 binding sites; two in each copy of ori_S and two in ori_L. The seventh sequence was located within the coding sequence of the UL9 gene, close to the N-terminal end of the polypeptide which differed in only 2 out of the 11 bases of the 11 bp conserved sequence identified by Stow and Davison (1986). The mismatches lie outwith the 8 bp UL9 recognition

sequence (GYTCGCAC) proposed by Koff and Tegtmeyer (1988; Fig. 4.3).

To investigate whether this element was recognised by the UL9 protein, plasmid pX8 (section 3B) was digested with PvuII and BamHI, yielding a 132 bp fragment containing the putative binding site. The fragment was end-labelled and incubated with control bacterial extract KR2 or KB1 extract which contains the UL9 DNA binding domain fusion protein and the products analysed on a polyacrylamide gel (Fig. 4.3; as indicated). A single complex was obtained with KB1 extract alone {KB1(a)} which was competed in the presence of specific competitor DNA {KB1(b)}. The signal intensity of this complex was much less than previously seen with KB1 extract using probes containing HSV-1 ori_S binding sites. The results are consistent with this fragment containing a low affinity binding site for UL9 protein.

The location within coding regions of binding sites for the encoded protein has previously been described for two other origin binding proteins; E.coli dnaA (section 1E) and the EBV EBNA-1 protein (section 1D). In the case of EBNA-1, the coding sequence binding site is the lowest affinity site detected in the genome and it is thought that this site might faciliate as a negative feedback control for the expression of EBNA-1 i.e. occupation of this site only occurs when the protein concentration has increased beyond that necessary to saturate two higher affinity sites located at the latent phase origin of replication thereby regulating amplification of episomal EBV genomes (Jones et al., 1989). It is doubtful whether this particular situation is applicable to HSV, as there is no detectable replication of the HSV genome in latently infected cells. However, once the UL9 protein has bound to all available higher affinity sites at ori_S during lytic infection, the protein might halt UL9 gene transcription by binding to this third site located 80 bp from the cap site.

The presence of this binding site, assuming that it allows repression of transcription and has a lower affinity than the ori_S binding sites, may provide some explanation for the failure to detect origin binding activity in cells co-transfected with plasmids pGX58 and pJR3 (IE-3 and IE-1 gene) and pGX125 (UL8 + UL9 gene).

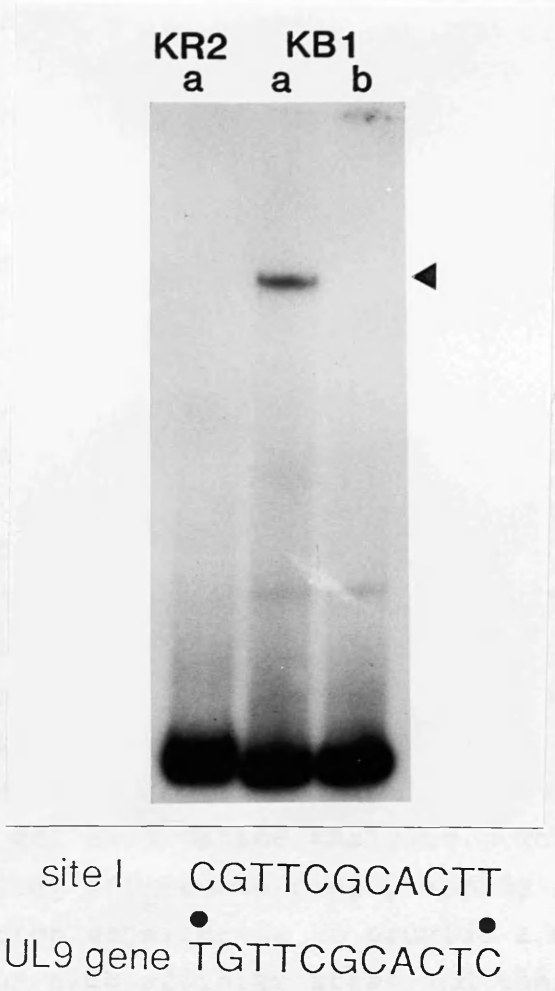


Figure 4.3 Gel retardation analysis of the UL9 fusion protein binding to a fragment from the UL9 gene

Plasmid pX8 which contains the N-terminal 2/3 of the UL9 gene was digested with PvuII plus BamHI yielding a 132 bp fragment containing a putative UL9 binding site, indicated below the autoradiograph (UL9 gene). The fragment was ^{32}P end-labelled and incubated with control extract KR2 or KB1 extract either in the absence of competitor DNA (tracks a) or in the presence of a 20-fold excess of oligonucleotide I (track b). The products were separated in an 8% polyacrylamide gel. A retarded complex is indicated by an arrowhead.

When HSV DNA was transfected into cells, some origin binding activity was detected (Fig. 3.12). The hypothetical model predicts that any UL9 protein produced during transfection will bind to six higher affinity sites in the HSV genome before repressing transcription by binding to the UL9 gene itself. Moreover, the expression of the other replication proteins following transfection will probably allow further amplification of the number of origins. Thus, significant amounts of the UL9 protein may accumulate before repression occurs. In contrast, in cells co-transfected with the three plasmids there is a copy of ori_S in plasmid pGX58 but because the other replication genes are absent, it cannot be amplified. The availability of fewer high affinity binding sites might therefore result in rapid repression of UL9 gene transcription as the protein binds to the lower affinity site within its own gene. As a consequence, insufficient UL9 protein may be synthesised to be detected by gel retardation analysis. This model could be tested by using ori_S-containing plasmids as carrier DNA in co-transfection experiments to provide a much higher concentration of high affinity sites and thereby reduce any binding to the UL9 gene itself.

The key argument against a repressive role for UL9 is that relatively high levels of protein can be expressed in the baculovirus expression system (Olivo et al., 1988; N. D. Stow, unpublished results). Perhaps in insect cells the transcriptional machinery is not inhibited by UL9 binding. In addition, the tsK/UL9 recombinant expresses relative high levels of UL9 protein in the absence of DNA replication. Six UL9 protein binding sites are available within the HSV genome but it is unlikely that this small number would be sufficient to prevent rapid repression by the UL9 polypeptide. Possibly significant is the high efficiency of transcription directed by the IE-3 promoter in this construct in comparison to the lower presumed activity of the UL9 promoter.

3. The role of UL9 and its recognition sites in initiation of HSV DNA synthesis

The site-directed mutagenesis experiments described in section 3C demonstrate that a functional binding site I is essential for ori_S activity, whereas the binding of UL9 protein to site II is not an absolute requirement. The requirement for site I is in agreement with the deletion analysis of Lockshon and Galloway (1988) and also the results of Deb and Deb (1989). The latter workers showed that a 6 bp deletion affecting this site was inactive in both UL9 binding and replication. The observation that a single point mutation within site I also abolished both replicative ability and the interaction of UL9 at this site strongly suggests that the abolition of ori_S activity is directly related to the failure of UL9 protein to bind to this site and not to any physical alterations in the spacing of the other sequence elements within ori_S. The position of the inactivating point mutation within site I corresponds to the second of two bases within the UL9 protein recognition sequence at which the inverted pentanucleotides overlap (Fig. 4.3 Koff and Tegtmeyer, 1988). In addition, the G residue within the wt sequence which was replaced by a T residue represents one of the essential contact residues for the UL9 protein identified by methylation interference assays (Koff and Tegtmeyer, 1988). These results demonstrate that this base plays a key role in the UL9 protein interaction with ori_S.

Conflicting results have previously been reported concerning the role of site II in HSV ori_S activity. Deb and Doelberg (1988) reported that a mutated ori_S which lacked the entire binding site II region functioned as efficiently in a plasmid amplification assay as wt ori_S {Fig. 3.22(a)}. In contrast, in defining the minimal sequence requirement for the almost identical ori_S of HSV-2, Lockshon and Galloway (1988) found that deletion of part of binding site II abolished detectable origin activity.

Results presented in this work demonstrate that although the lack of a functional binding site II causes a significant reduction in plasmid amplification, the presence of two UL9 binding sites is not an absolute requirement for ori_S activity. This result is supported by previous work of Stow and Davison (1986) which demonstrated that HSV-encoded helper functions were able to activate the ori_S of VZV with

approximately 5% the efficiency of HSV-1 ori_S. The VZV origin contains sequences homologous to UL9 binding site I on one side of the palindrome only. Furthermore, DNase I footprint assays showed that the sequence-specific DNA binding domains of both the UL9 protein and its VZV counterpart interacted with these predicted sequences within VZV ori_S (Fig. 3.30).

The reasons for the apparent differences in importance of binding site II in the three pieces of work (Lockshon and Galloway, 1988; Deb and Doelberg, 1988; this thesis) are not known but may include differences in virus strains and experimental protocols.

There is nevertheless a correlation between the observation that copies of ori_S lacking site I or site II exhibit undetectable or residual origin activity respectively and measurements of the affinity of the UL9 protein for these sites. As described earlier, the affinity of the UL9 protein is approximately 10-fold higher for site I than for site II (Elias and Lehman, 1988; this work). The UL9 protein would be expected to bind more tightly to an origin lacking site II than to one lacking site I, which may account for the residual function of the origin deleted for site II sequences. Additionally, DNA binding studies (results of M.Challberg, cited in Challberg and Kelly, 1989) suggest that deleting site II reduces the affinity of UL9 protein for site I which may in part account for the reduction in activity with the site II deletion mutant.

Although the intrinsic affinity of the UL9 protein for the two binding sites differs (as demonstrated using individual oligonucleotides containing site I and site II), the initial binding of UL9 to the higher affinity site I may induce a conformational change in the ori_S DNA so as to increase the affinity of UL9 for site II. Evidence for such cooperativity is provided by the work of Lockshon and Galloway (1988) in which a series of mutant origins were constructed in which n copies of the AT dinucleotide were inserted into the centre of the AT-rich region of the palindrome. As n increases from 0 to 8, replication first decreases to a minimum at n=3, then rises to a maximum at n=5 or 6 (corresponding to an insertion of approximately one helical turn of DNA) then decreases again. It is possible

that this oscillation in activity corresponds to a requirement for the UL9 protein molecules bound to the two sites, to be spatially orientated in an appropriate manner. This arrangement may be critical to accommodate essential UL9-UL9 interactions.

Attempts to define the left hand boundary of a minimal HSV ori_S indicated a requirement for an approximately 20 bp region to the left of site I (Fig. 3.22; Lockshon and Galloway, 1988). As this region contains an almost perfect inverted repeat of site I (motif III; Fig. 3.22) it was suggested that this region might represent another binding site for the UL9 protein (Lockshon and Galloway, 1988). Gel retardation assays show that the UL9 protein does not interact with this sequence (this work) which is consistent with the DNase I footprint assays summarised in Fig. 4.1. The possibility that binding to this region had not been previously detected because of the presence of neighbouring site I was also excluded by using oligonucleotides containing motif III alone.

Although motif III does not bind the UL9 protein, its deletion nevertheless causes a reproducible reduction in ori_S activity of 2-3 fold. In contrast, Lockshon and Galloway (1988) showed that an HSV-2 ori_S deletion mutant (pS207) with an end-point located within motif III was almost completely inactive in replication assays (Fig. 3.22). These data suggests the possibility that the sequence TAAAAG which abuts motif III, and is present in both the core origin sequence of Lockshon and Galloway (1988) and pST19pmI, but missing from pS207, may have an important function in ori_S activity. It is interesting to note that the presence of motif III and the TAAAAG element confer a very strong bias toward purines on one strand of the DNA (16 out of 1 bases). Such sequences are thought to be inherently unstable and as previously described in section 1E, a sequence of this type within the SV40 core origin (early palindrome) is, in fact, the first region to be opened by T-ag. The early palindrome region is located next to the T-ag binding site which in turn is adjacent to the AT-rich element which allows T-ag to extend the replication bubble first initiated within the early palindrome. The ability of T-ag to 'melt' the early palindrome is not energy

dependent and constitutes a third function of the polypeptide in DNA replication, distinct from its ability to recognise the pentanucleotide motif and its energy dependent helicase activity.

The experiments on HSV raise the interesting possibility that the TAAAAG element forms an essential part of an element to the left of site I which is unwound by the UL9 helicase activity. It is also interesting to note that at increased UL9 protein concentrations in footprinting assays, Elias and Lehman (1988) observed DNase hypersensitivity immediately to the left of site I which suggests that this region may contain single-stranded DNA or is at least made more susceptible to DNase I. The fact that the TAAA sequence is brought closer to the AT-rich centre of ori_S by deleting motif III may explain the decrease in ori_S activity.

To date, no HSV variant which lacks both copies of ori_S has been reported. This may indicate that virus replication requires at least one functional cis-acting ori_S, or that attempts to inactivate the origin may have some deleterious effect on the expression of an essential trans-acting gene product (Hubenthal-Voss et al., 1987; Hubenthal-Voss and Roizman, 1988). The observation that ori_S function can be inactivated by a single point mutation may go some way to resolving this question. It should now be feasible to introduce a point mutation into both copies of ori_S within the viral genome without affecting the expression and function of any essential trans-acting product specified by this region (the coding potential of this region remains controversial; see section 1A, part 4). In contrast, previous attempts to inactivate the genomic copies of ori_S have employed large deletions and insertions which may have inactivated such a function.

It would appear that the sequence-specific recognition of the HSV-1 origin is a very early event in the initiation of replication. The UL9 protein had previously been recognised as being essential for DNA synthesis, and the work presented here demonstrates that its interactions with specific sequences in the origins plays a key role. Following binding, the UL9 protein probably participates in unwinding the viral DNA and/or assembling a replicative

complex at the HSV origin of replication.

Future experiments will doubtless aim to investigate these reactions further. Important will be:

(i) defining the region of the protein responsible for sequence-specific binding

(ii) defining regions of the protein required for helicase activity and for dimerisation

(iii) a detailed analysis of the perturbation to DNA caused by UL9 binding to the origin (e.g. melting and unwinding)

(iv) analysis of the interactions of the UL9/ori complex with other components of the replicative machinery

The initiation of HSV DNA synthesis however will only be fully understood following the development of an *in vitro* system capable of faithful initiation. This development will be essential for the complete elucidation of the sequence of events which takes place at an HSV origin of replication following initial interaction with the UL9 protein. Given that all the viral proteins required for replication have now been overexpressed to high levels in both insect and mammalian cells, such a system is now a realistic possibilty.

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Binding of the herpes simplex virus type 1 UL9 gene product to an origin of viral DNA replication

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ABSTRACT

The binding of a herpes simplex virus type 1 (HSV-1) encoded polypeptide to a viral origin of DNA replication has been studied by using a gel retardation assay. Incubation of nuclear extract from HSV-1 infected cells with a labelled origin-containing fragment resulted in the formation of a specific retarded complex, the migration of which was further reduced in the presence of an antibody reactive with the UL9 gene product. Introduction of an additional copy of the UL9 gene, under the control of an immediate early (IE) promoter, conferred the ability to express origin binding activity at the non-permissive temperature upon an HSV-1 ts mutant blocked at the IE stage of infection. Endogenous or exogenous proteolytic activity revealed the presence of a relatively protease-resistant domain which retained sequence-specific DNA binding activity. The C-terminal 317 amino acids of the UL9 gene expressed as a fusion protein in Escherichia coli also bound to the origin. Our results demonstrate that the UL9 gene product binds to a viral origin and that sequence specific recognition and binding are specified by the C-terminal 37% of the polypeptide.

INTRODUCTION

The herpes simplex virus type 1 (HSV-1) genome is a linear double-stranded DNA of approximately 152 kb. Replication of this genome requires the presence of cis-acting elements which function as origins of DNA replication and of virus-encoded trans-acting proteins which perform essential roles in DNA synthesis.

The complete sequence of the HSV-1 genome has been determined and open reading frames capable of encoding 70 distinct polypeptides identified (1). Two approaches have indicated that the products of seven of these genes (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) are involved in viral DNA

replication. Firstly, genetic analyses of HSV-1 mutants have shown that seven complementation groups containing mutants which exhibit defects in DNA synthesis map to these genes (2-10). Secondly, Challberg and his colleagues have demonstrated, using a plasmid amplification assay for HSV-1 origin-dependent DNA replication, that the products of these seven genes are both necessary and sufficient for DNA synthesis (11-13). Three of these seven DNA replication genes encode previously well-characterized proteins; the major single-strand-specific DNA binding protein is encoded by UL29 (3,14), the viral DNA polymerase by UL30 (14,15), and a 65,000 molecular weight protein which binds sequence-independently to double stranded DNA by UL42 (16,17). The products of the other four genes (UL5, UL8, UL9 and UL52) have not been so well characterized and are presumed to be present in much lower abundance in infected cells.

The viral origins of DNA replication are specified by two distinct but related sequences. One (ori_L) lies close to the centre of the long unique (U_L) region whilst the other (ori_S) is present within the inverted repeats, TR_S and IR_S (18-21 and Figure 1a). DNA fragments of 100 bp or less which specify a functional ori_S have been identified (22-24) and include a 45 bp near perfect palindrome with a central A+T-rich region. Although the sequence specifying ori_L contains a much longer (144 bp) palindrome there is high sequence similarity between the two origins covering the region of the ori_S palindrome and approximately 40 bp to one side of it.

Using a DNase I footprinting assay Elias and his colleagues (25,26) have demonstrated the presence of two specific binding sites for a virus-induced polypeptide within ori_S. Site I consists of an 18 bp region which overlaps the left end of the ori_S palindrome (Figure 1b). Lower affinity binding occurs to site II which is present in the opposite orientation to site I and is identical in 15 of the corresponding 18 positions. Two copies of a sequence which differs in only one position from that of site I are similarly located within ori_L and are also assumed to act as binding sites. Elias and Lehman (26) showed that the origin-binding

protein (OBP) had a molecular weight of 83,000, but the viral gene encoding it was not identified. It seemed very likely however that OBP would be the product of one of the four genes essential for HSV-1 DNA synthesis to which a function had not yet been ascribed. We therefore developed a gel retardation assay to facilitate the testing of the UL5, UL8, UL9 and UL52 gene products for origin-binding activity. Our results confirm and extend the very recent report by Olivo et al. (27) that the UL9 gene specifies OBP.

MATERIALS AND METHODS

Cells and viruses: Baby hamster kidney 21 clone 13 (BHK) cells (28) were grown in Eagle's medium supplemented with 10% tryptose phosphate broth and 10% foetal calf serum. Extracts were prepared from cell monolayers (10^7 cells/90 mm plastic Petri dish) infected with 5-10 p.f.u./cell and incubated for 8 h at 37°C or 38.5°C. The viruses used were wild type (wt) HSV-1 (Glasgow strain 17, syn^+), the HSV-1 temperature-sensitive mutant tsK (29) and recombinant viruses tsK/UL5, tsK/UL8, tsK/UL9 and tsK/UL52. The recombinant viruses contain additional copies of the early genes UL5, UL8, UL9 and UL52 inserted under the control of the immediate early (IE) gene 3 promoter within the thymidine kinase gene of tsK. They were constructed essentially as described by Hummel et al. (30).

Preparation of nuclear extracts: In initial experiments nuclear extracts were prepared by the method of Piette et al. (31) from 3 infected cell monolayers. Proteins eluted from nuclei with buffer B containing 600 mM NaCl were precipitated with $(NH_4)_2SO_4$ and resuspended in 100 µl buffer B containing 100 mM NaCl. Subsequently it was noted that a procedure essentially as described by Dignam et al. (32) and modified by Preston et al. (33) gave greater reproducibility in terms of yield and activity. The nuclear pellet from 3 cell monolayers was eluted with 100 µl buffer C (20 mM Hepes pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT) containing 600 mM NaCl and after centrifugation the supernatant extract was flash frozen and stored at -70°C. Protein concentrations were determined by using a Bio-Rad Protein Assay kit.

Expression in Escherichia coli: Fusion proteins containing parts of the UL9 polypeptide were synthesised using the plasmid vector system pRIT2T (supplied by Pharmacia; ref. 34). Two additional plasmids were constructed such that the SmaI, BamHI, SalI and PstI cloning sites (Figure 6a) were positioned in the 2 other reading frames. EcoRI cleaved pRIT2T DNA was treated with mung bean nuclease to remove precisely the single-stranded overhangs and the plasmid recircularised either in the absence or presence of an 8 bp EcoRI linker (GGAATTCC). Resulting plasmids pRIT2T-4 and pRIT2T+4 respectively contain deletions and insertions of 4 bp and their identities were confirmed by DNA sequencing. DNA fragments were inserted into the appropriate plasmid as described in Figure 6 and the constructs used to transform E. coli strain K12ΔH1Δtrp (35) to ampicillin resistance. Bacteria containing the desired plasmids were isolated and propagated at 28°C. To induce synthesis of the fusion protein bacteria were grown to an A630 value of 0.4 in 50 ml L broth and rapidly shifted to 42°C for 75 min. The cells were harvested by centrifugation, washed twice with 10mM Tris-HCl pH 7.5, 1mM EDTA and resuspended in 0.5 ml buffer C containing 600 mM KCl. The suspension was extensively sonicated and centrifuged at 11,600 g for 10 min. The resulting supernatant was used as extract in the binding assays.

DNA fragments and oligonucleotides: The 100 bp BamHI/SalI ori_S-containing fragment from plasmid pS19 (22) was recloned between the corresponding sites of pTZ19U (36) and used as a source of the HSV-1 ori_S sequence (pS19 fragment). Plasmid pBY3 contains an unrelated but similarly sized HaeIII fragment of HSV-1 DNA originating from within BamHI y (37) and cloned into the SmaI site of pTZ19U. Oligonucleotides were synthesised using a model 8600 Biosearch DNA synthesiser and purified on a 15% acrylamide DNA sequencing gel. Duplex oligonucleotides were formed by mixing equivalent masses of the two complementary strands, heating to 95°C and allowing to cool slowly to room temperature. The duplex oligonucleotides were end-labelled and blunt-ended using the filling-in reactions of the Klenow fragment of DNA polymerase I (38). The following duplex oligonucleotides were used:

oligonucleotide I 5' GATCCGCGAAGCGTTCGCACTTCGTCCCA
GCGCTTCGCAAGCGTGAAGCAGGGTCTAG 5'

oligonucleotide II 5' GATCTGGGGCGAAGTGCGAGCACTTCGCG
ACCCCGCTTCACGCTCGTGAAGCGCCTAG 5'

oligonucleotide X 5' GATCGGATATGCTAATTAAATACAT
GCCTATACGATTAATTTATGTG 5'

Oligonucleotides I and II respectively contain the sequences of binding sites I and II within HSV-1 ori_S (see Figure 1 and ref. 26). Oligonucleotide X, containing an unrelated DNA sequence, was kindly provided by Dr A. Bailey.

Gel retardation analysis (39): Probe fragments, 3' end-labelled with [^{32}P]deoxyribonucleoside triphosphates, were eluted from 8% non-denaturing acrylamide gels, extracted sequentially with phenol and chloroform and precipitated with ethanol. Either 1 ng labelled oligonucleotide or 5 ng of labelled 100 bp ori_S fragment were incubated at 25^{o}C with 2 µg sonicated calf thymus DNA and 5 µg extract in a 20 µl reaction mix containing 1 x buffer C plus 100 mM NaCl. After 20 min 5 µl loading buffer (25% glycerol, 0.05% bromophenol blue in 1 x TBE [90 mM Tris-borate, 2.5 mM EDTA]) were added and the samples loaded onto 5% polyacrylamide gels (55:1, acrylamide:N,N'-methylene bisacrylamide) containing 1 x TBE. Gels were run in 1 x TBE at 25 mA for 1.5-2 h, fixed in 10% acetic acid, dried and exposed for autoradiography.

Antisera: Rabbit antisera raised against decapeptides from the C-termini of the UL5, UL8, UL9 and UL52 gene products and reactive with the corresponding polypeptides (27) were kindly provided by Dr M.D. Challberg. 1 µl of antiserum was added to binding reactions.

RESULTS

Detection of ori_S binding activity in nuclear extracts of cells infected with wt HSV-1. The sequence of part of the 100 bp HSV-1 DNA insert of plasmid pS19, which specifies functional ori_S activity (22), is shown in Figure 1b together with the two binding sites for a viral polypeptide mapped by Elias and Lehman (26). Double-stranded oligonucleotides containing the sequences of binding sites I and II (oligonucleotide I and oligonuclectide

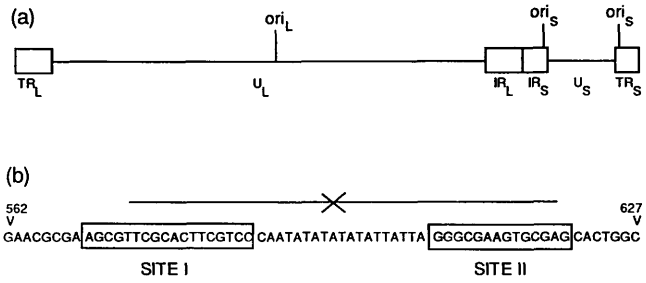

Figure 1. (a) Structure of the HSV-1 genome showing the locations of the origins of DNA replication. (b) DNA sequence from the ori$_S$ region (22). The 45 bp palindrome is indicated by arrows and the two binding sites identified by Elias & Lehman (26) are boxed.

II respectively) were synthesised and used with the 100 bp pS19 fragment in a gel retardation assay for the presence of proteins binding to these sites.

In initial experiments proteins eluted with 600 mM NaCl from nuclei of mock-infected and HSV-1 infected cells were precipitated with $(NH_4)_2SO_4$ and redissolved in buffer B plus 100 mM NaCl (31). These extracts were incubated with end-labelled pS19 fragments in the presence of sonicated calf thymus DNA and sequence specific competitor DNAs. The reaction products were analysed by electrophoresis through 5% non-denaturing polyacrylamide gels.

Figure 2 shows the results of a typical experiment. In the absence of added competitor DNA two retarded bands (A and B) representing protein-DNA complexes were detected (track 1). To verify the specificity of binding, 100-fold molar excesses of unlabelled ori$_S$ fragment (track 2), oligonucleotides I and II (tracks 3 and 4) and the 100 bp insert of plasmid pBY3 (containing unrelated HSV-1 DNA sequences [track 5]) were added to the assay. The ori$_S$ fragment and oligonucleotides I and II competed efficiently for binding resulting in a great decrease in the signal intensity of bands A and B. In contrast the pBY3 fragment did not compete. Complexes A and B therefore result from site specific binding to DNA sequences present within oligonucleotides I and II, and probably represent the same activity as previously detected by DNase footprinting (26). No

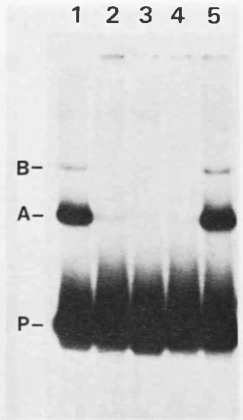

Figure 2. Gel retardation analysis of extracts of wt HSV-1 infected cells. Nuclear extract was incubated with ^{32}P end-labelled pS19 fragment either in the absence of competitor DNA (track 1) or in the presence of 100-fold molar excesses of unlabelled pS19 fragment (track 2), oligonucleotide I (track 3), oligonucleotide II (track 4) or pBY3 fragment (track 5). The products were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. A and B indicate specific retarded complexes and P the free probe.

similar activity was detected in extracts from mock-infected cells (e.g. Figure 3a, track 1).

The product of the UL9 gene interacts with binding site I. The recombinant viruses tsK/UL5, tsK/UL8, tsK/UL9 and tsK/UL52 contain additional copies of the early genes UL5, UL8, UL9 and UL52 inserted into the genome of the HSV-1 temperature-sensitive mutant tsK under the control of the immediate early (IE) gene 3 promoter. At the non-permissive temperature (38.5^{o}C) tsK accumulates IE polypeptides but fails to induce the expression of early and late genes (29,40). The 4 recombinant viruses exhibit polypeptide profiles at 38.5^{o}C indistinguishable from tsK with the exception that each induces a single additional polypeptide corresponding to the product of the inserted gene (J.M. Calder and N.D. Stow, manuscript in preparation).

Proteins were eluted with 600 mM NaCl from the nuclei of mock-infected cells and from cells infected at the non-permissive temperature with wt HSV-1, tsK or the four recombinant viruses. The extracts were tested in a gel

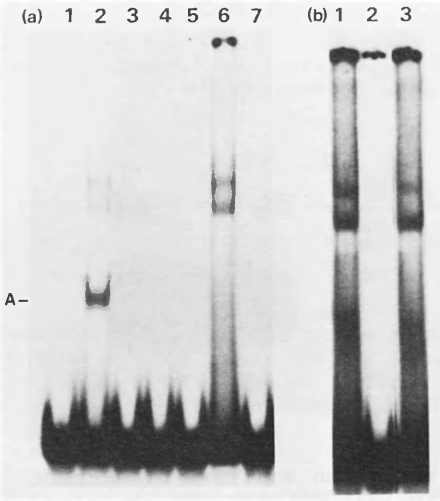

Figure 3. Gel retardation analysis of extracts from cells infected with recombinant viruses. Nuclear extract was prepared from cells incubated at 38.5°C and reacted with ^{32}P-labelled oligonucleotide I. (a) track 1, mock-infected cells; tracks 2-7, cells infected with wt HSV-1, tsK, tsK/UL5, tsK/UL8, tsK/UL9 or tsK/UL52 respectively. "A" indicates the major complex detected with wt HSV-1 extract. (b) Nuclear extract from cells infected with tsK/UL9 was incubated in the absence of competitor DNA (track 1) or in the presence of 100-fold molar excesses of oligonucleotide I (track 2) or oligonucleotide X (track 3).

retardation assay using ^{32}P-labelled oligonucleotide I as probe (Figure 3a). As expected, extract from cells infected with wt HSV-1 generated a major complex (A) and some minor bands (track 2). The only other extract which caused significant retardation of the probe fragment was from cells infected with recombinant virus tsK/UL9 (track 6). Although the pattern of retardation observed with extracts from cells infected with this virus varied from assay to assay the presence of material which failed to enter the gel and of a smear extending down most of the track were consistently observed. In addition, whereas somewhat diffuse bands migrating more slowly than complex A were usually seen, bands co-migrating with complex A were absent.

Figure 3b demonstrates that the pattern of retarded DNA probe represents sequence specific binding. Addition of a 100-fold excess of unlabelled oligonucleotide I but not

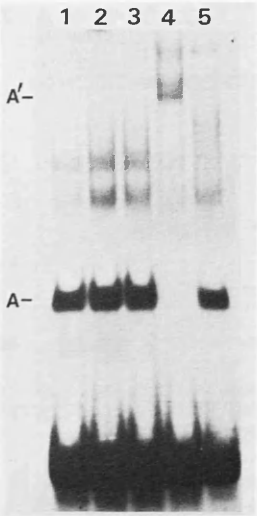

Figure 4. Effect of antisera on the wt HSV-1 complex. Nuclear extract from cells infected with wt HSV-1 was incubated with ^{32}P-labelled oligonucleotide I either in the absence of added antiserum (track 1) or in the presence of antisera reactive with the UL5, UL8, UL9 or UL52 gene products (tracks 2, 3, 4 and 5 respectively). The products were analysed as described in Figure 2. A and A' indicate the major complexes formed in the absence of antiserum or in the presence of antiserum against the UL9 gene product respectively.

oligonucleotide X (which is a similar size but of unrelated DNA sequence) efficiently competed for binding.

Because the tsK/UL9 recombinant differs from tsK only in its ability to express the UL9 gene product at the non-permissive temperature the data indicate that this polypeptide participates in the formation of a complex with binding site I.

The UL9 gene product is present in complex A formed with extracts from cells infected with wt HSV-1. Nuclear extract from cells infected with wt HSV-1 was incubated with ^{32}P-labelled oligonucleotide I either in the absence of added antibody or in the presence of antibodies reactive with the UL5, UL8, UL9 or UL52 polypeptides and the products analysed on a non-denaturing polyacrylamide gel. Figure 4 shows that the antibodies which react with UL5, UL8 or UL52 had little effect

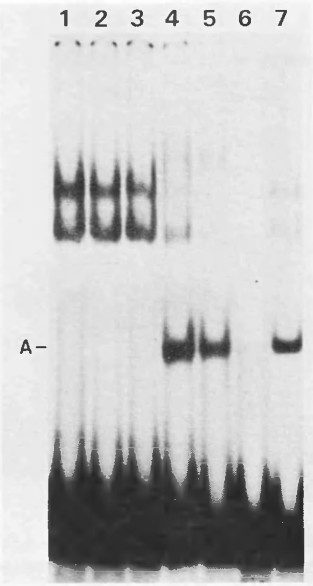

Figure 5. Effect of proteinase K on the tsK/UL9 complex. Nuclear extract from cells infected with recombinant tsK/UL9 was incubated with ^{32}P-labelled oligonucleotide I either in the absence of proteinase K (track 1) or in the presence of 1, 10, 100, 1000 or 2000 ng proteinase K (tracks 1-6 respectively). Wt HSV-1 extract was assayed in parallel in the absence of proteinase (track 7). The products were analysed as described in Figure 2. "A" indicates the major complex formed with wt extract.

on the pattern of retarded fragments. In contrast addition of antibody to the UL9 polypeptide resulted in the loss of the major retarded complex, A, and its replacement by a more slowly migrating form, A', indicative of the additional presence of antibody molecules in the complexes. Therefore at least a part of the UL9 polypeptide is contained within complex A, and since the antibody was raised against a C-terminal oligopeptide (27) this region of the protein must be present.

Effect of protease digestion on the binding activity from cells infected with the tsK/UL9 recombinant. Given that the major retarded complex from cells infected with wt HSV-1 reacted with an antibody against the UL9 polypeptide it was rather surprising that the pattern of retarded fragments observed using extract

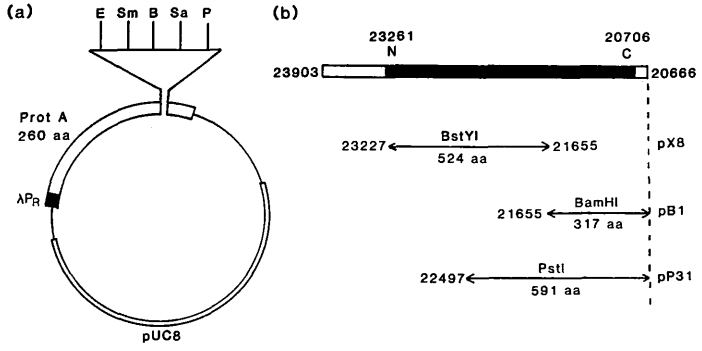

Figure 6. Expression of UL9 gene fragments in E. coli. (a) Structure of plasmid pRIT2T. pRIT2T-4 and pRIT2T+4 are essentially similar except that in pRIT2T-4 the EcoRI site is replaced by an ApaI site. The positions of the phage lambda promoter, P_R, and coding sequences for the N-terminal region of the Staphylococcus aureus A protein are indicated. (b) Fragments inserted into vectors. The upper line shows the SstI fragment containing the UL9 gene which was initially cloned into the EcoRI site of pUC8. The N- and C-terminal regions of the coding sequences (black bar) are indicated. Plasmids pX8, pB1 and pP31 contain the indicated fragments inserted in frame and in the appropriate orientation into pRIT2T-4, pRIT2T-4 and pRIT2T respectively. The C-terminal ends of the pB1 and pP31 inserts lie within the pUC8 polylinker. The nucleotide numbering is from McGeoch et al. (1).

from cells infected with the tsK/UL9 recombinant was very different. One possible explanation is that complex A contains only a fragment of the UL9 polypeptide.

Our attempts to size complex A using pore-gradient polyacrylamide gels (41) had suggested a molecular weight of approximately 45,000, clearly inconsistent with the binding of an intact UL9 polypeptide (molecular weight 94,000) to the labelled oligonucleotide (data not shown). We therefore examined the effect of proteinase K addition to binding reactions containing tsK/UL9 nuclear extract and labelled oligonucleotide I. The results (Figure 5) show that with increasing amount of added proteinase K there is a reduction in the amount of slowly migrating complexes and a concomitant appearance of a faster migrating species which exhibits a mobility very similar to that of complex A. No band of this mobility was detected when extracts from mock-infected cells or

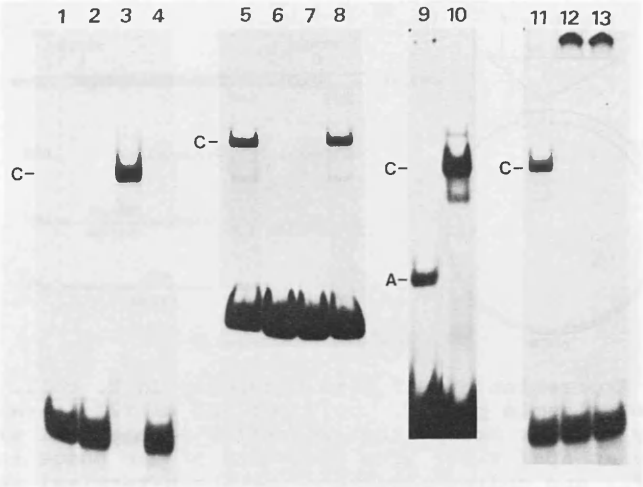

Figure 7. Gel retardation analysis of the pBl encoded fusion protein. Binding reactions were performed without extract (track 1) or with extracts from untransfomed bacteria (track 2), bacteria transformed with pRIT2T (track 4) or pBl (tracks 3, 5-8, 10-13), or from wt HSV-1 infected BHK cells (track 9). The probe fragments were ^{32}P-labelled oligonucleotide I (tracks 1-4, 9-13) and pS19 fragment (tracks 5-8). Unlabelled oligonucleotide I (track 6), oligonucleotide II (track 7) and oligonucleotide X (track 8) were added as competitors. Antisera reactive with UL9 and UL52 respectively were added to the reactions shown in tracks 12 and 13. C and A indicate the major complexes from bacteria containing pBl and from wt HSV-1 infected cells.

cells infected with tsK were similarly treated with proteinase K (data not shown).

The data thus suggest that the addition of proteinase K to extracts containing the UL9 polypeptide results in the cleavage of the polypeptide and the generation of a relatively protease-resistant domain which retains DNA binding activity.

Expression of the DNA binding domain of UL9 in Escherichia coli. Three DNA fragments representing approximately the N-terminal 2/3 and C-terminal 2/3 and 1/3 of the UL9 coding sequences were cloned in the correct reading frame into the vector pRIT2T or an appropriate frameshift derivative (see Figure 6 and Materials and Methods). The constructs were introduced into E. coli strain K12ΔH1Δtrp, extracts prepared from heat induced bacteria, and tested for binding activity using ^{32}P-labelled

oligonucleotide I. No binding activity was detected in extracts from the host bacteria alone or following the introduction of the vector pRIT2T (Figure 7, tracks 2 and 4). Of the three hybrid plasmids pB1 (track 3) and pP31 but not pX8 encoded binding activity. The activity encoded by pB1 also binds to the 100 bp ori_S-containing fragment and is efficiently competed by the addition of unlabelled oligonucleotides I and II but not oligonucleotide X (tracks 5-8). The C- terminal 317 amino acids of the U9 polypeptide therefore contain all the amino acid sequences required for sequence-specific binding to sites I and II within ori_S.

Figure 7 also shows that the fusion protein encoded by pB1 (predicted molecular weight 63,000) forms a complex which exhibits a considerably lower mobility than complex A formed using extract from cells infected with wt HSV-1 (tracks 9 and 10), in agreement with complex A containing only a proteolytic fragment of the UL9 polypeptide. The mobility of the pB1 complex was further reduced by the addition of antibodies reactive with the UL9 or UL52 polypeptides to the binding reaction (tracks 11-13). The ability of both these antibodies to bind to the DNA protein complex results from the presence of the protein A moiety in the pB1 fusion protein, and verifies that the hybrid protein is responsible for the observed binding.

DISCUSSION

The initial objective of the experiments presented in this paper was to identify the HSV-1 gene product which recognises the two specific binding sites within HSV-1 ori_S characterised by Elias and Lehman (26). The expression of binding activity in E. coli indicates that the only viral polypeptide essential for this activity is encoded by the UL9 gene and very strongly suggests that this gene product is directly responsible for sequence specific binding. Our identification of the UL9 polypeptide as an origin-binding protein is in complete agreement with the recent results of Olivo et al. (27) who expressed this viral protein in a baculovirus expression system and assayed for binding by an immunoprecipitation assay and DNase footprinting.

In addition to expressing binding activity in E. coli we also detected binding using nuclear extracts from cells infected with wt HSV-1 or the recombinant virus tsK/UL9. Competition experiments with synthetic oligonucleotides demonstrated that in each instance the binding target contained sequences homologous to binding sites I and II initially characterised by Elias and Lehman (26). The baculovirus-expressed UL9 polypeptide protected similar DNA sequences in DNase footprinting experiments (27).

By employing a gel retardation assay we were able to distinguish different sizes of complex formed by the addition of extracts from various sources. These results are interesting to compare with other recent reports which examined origin-binding activity by the same technique. The rapidly migrating complex A detected with extracts from cells infected with wt HSV-1 clearly contains only a portion of the UL9 gene product. This complex appears to be similar to the major complex described recently by Koff and Tegtmeyer (42). In contrast Elias and Lehman (26) purified origin binding activity from virus infected cells and observed a major (presumably intact) polypeptide of molecular weight 83,000 and two smaller components. This preparation gave a very different pattern, more closely resembling that obtained with tsk/UL9 extracts, in a gel retardation assay. This suggests that in extracts of cells infected with wt HSV-1 the UL9 polypeptide may be subjected to varying extents of proteolytic cleavage. We do not know whether the small forms observed by ourselves and by Koff and Tegtmeyer (42) are of any physiological significance or whether they merely reflect proteolytic activity occurring during the isolation procedure. The observation that extracts made in parallel from cells infected with the tsk/UL9 recombinant do not contain the cleaved form of the polypeptide responsible for the formation of complex A suggests that the proteolytic activity occurs as a consequence of infection proceeding from the IE to early and late stages, and could thus possibly be virus encoded.

Although interaction of intact UL9 polypeptide with labelled probe fragments produces a smeared pattern in a gel retardation assay this nevertheless represents sequence-specific

binding (Figure 3 and ref. 26). The smearing may occur because of aggregation and/or dissociation of complexes during incubation of the binding reactions and gel electrophoresis. This behaviour is not so noticeable in complexes containg only proteolytic fragments of UL9 or fragments synthesized in E. coli where much "tighter" bands are observed. The smearing may therefore be a consequence of interactions involving the N-terminal region of the UL9 polypeptide.

By expressing a fragment of the UL9 polypeptide in E. coli we have demonstrated that the C-terminal 317 amino acids specify all the structural information required for sequence-specific recognition and binding. A similar situation has previously been noted for another herpesvirus sequence-specific DNA binding protein, EBNA-1 of Epstein-Barr virus, the C-terminal domain of which is able to bind to its target recognition sequence (43). In addition, discrete domains of several other proteins are known to be responsible for sequence-specific binding to DNA and these are often relatively resistant to the action of added protease (41, 44-48).

It is likely that sequence-specific recognition of the HSV-1 origins of DNA replication is a very early event in initiation of HSV-1 DNA synthesis. Following binding of the UL9 polypeptide to the origins, regions of the protein not involved in direct interaction with DNA probably participate in other functions which may include interacting with other replication proteins, or unwinding or nicking DNA to facilitate the initiation of DNA synthesis. Expression systems for the UL9 polypeptide such as those described in this paper should prove useful in the study of these functions.

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