ANALYSIS OF THE DNA BINDING DOMAIN OF THE HERPES SIMPLEX VIRUS TYPE 1 UL9 PROTEIN

By

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Margaret Isabel Arbuckle (February 1993).

SUMMARY

The UL9 gene is one of seven genes which map within the long unique region of the HSV-1 genome and are both necessary and sufficient for viral origin-dependent DNA replication in transfected tissue culture cells. The UL9 gene encodes a polypepetide of 851 amino acids which binds specifically to the HSV-1 replication origins ori_S and ori_L. From DNase I footprinting and gel retardation analyses, two binding sites for the origin binding protein (OBP) have been identified within both of these origins. Previous work (Weir et al., 1989) demonstrated that the sequence-specific recognition and binding activities resided within the C-terminal 317 amino acids of the UL9 protein.

My work has involved a mutational analysis of the UL9 DNA binding domain in an attempt to define the regions involved in interaction with its target sequence.

Using an E.coli expression system, a series of C-terminal deletion (with 16 - 62 amino acids deleted) and small in-frame insertion mutants of the DNA binding domain were constructed and expressed as fusions linked to the N-terminal one third of the Staphylococcus aureus protein A. This system enabled easy detection of mutant proteins in Western blot assays by virtue of interaction of the Fc portions of the antibody conjugates with the protein A moieties. Fusions proteins were tested for sequence-specific DNA binding activity using gel retardation assays. Protein extracts were incubated with radio-labelled oligonucleotides containing ori_S binding sites I or II or a 100 bp fragment containing a functional ori_S (i.e. both sites) at 22°C or 37°C. Retarded complexes were resolved by electrophoresis through non-denaturing polyacrylamide gels. Analysis of C-terminal truncations demonstrated that the C-terminal 33 amino acids of UL9 are dispensable for binding and that essential residues lie between amino acids 801 and 818. Analysis of a set of ten mutants containing insertions of four amino acids at various positions identified different regions of the UL9 DNA binding

domain in which insertions either had little effect upon (insertions at amino acids 691, 708, 719, and 838) or abolished (insertions at amino acids 581, 591, 652 and 668) sequence-specific DNA binding. Two mutants with insertions at amino acids 630 and 799 which were intermediate in their binding activity were also shown to exhibit temperature- or sequence-specific effects.

These results suggest that the UL9 DNA binding domain contains at least two distinct regions which are important for sequence-specific DNA binding.

Selected mutations were introduced into the full length UL9 open reading frame to investigate whether the alterations had any effect on the replicative function of the intact protein. The resulting mutant UL9 proteins were individually expressed under the control of the strong polyhedrin promoter in recombinant baculoviruses. This expression system was chosen because it allows mutant HSV-1 DNA replication proteins to be both overexpressed (facilitating biochemical studies) and screened for ability to participate in origin-dependent DNA replication (Stow, 1992).

I confirmed that multiple infection of Spodoptera frugiperda insect cells with seven recombinant baculoviruses expressing the seven essential HSV-1 replication proteins can facilitate replication of a co-transfected HSV-1 ori_S-containing plasmid. By substituting the wt UL9 recombinant baculovirus with each of the mutant UL9 viruses, the effect of the alterations on UL9 function in origin-dependent DNA replication was investigated.

Each of the mutations which had been previously shown to abolish DNA binding activity also prevented replication of the ori_S-containing plasmid suggesting that interaction of UL9 with the origin is essential for the process of DNA replication. Of the mutant proteins which retained their DNA binding activity, one failed to promote replication of the origin containing plasmid while the others showed wt or diminished levels of activity. These results suggest that the C-terminal domain of the UL9 protein may have essential functions in

DNA replication in addition to its origin-binding activity.

ABBREVIATIONS

AcNPV	Autographa californica Nuclear Polyhedrosis Virus
AP	alkaline phosphatase
APS	ammonium persulphate
ATP	adenosine-5'-triphosphate
bp	base pairs
BPB	bromophenol blue
BSA	bovine serum albumin
Ci	Curie
CLB	cell lysis buffer
cm	centimetres
CMV	cytomegalovirus
cpm	counts per minute
C-terminal	carboxy terminal
DBP	DNA binding protein
DDAB	dimethyl diaoctadecylammonium bromide
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DOPE	dioleoyl phosphatidyl ethanolamine
ds	double stranded
DTT	dithiothreitol
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dNTP	2'-deoxynucleoside 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
E	early (gene)
EBV	Epstein-Barr virus
E.coli	Escherichia coli
EDTA	sodium ethylenediamine tetra-acetic acid
EM	electron microscopy
EtBr	ethidium bromide
Fc	crystallisable fragment (immunoglobulin)
g	grams
h	hour(s)

PCNA proliferating cell nuclear antigen

HCMV	human cytomegalovirus
HeBs	hepes buffered saline
HHV6	human herpesvirus 6
HHV7	human herpesvirus 7
HRP	horseradish peroxidase
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
IE	immediate early (gene)
IEC	immediate early complex
Ig	immunoglobulin
IgG	immunoglobulin G
IR	internal repeat
kDa	kilodalton
Kb	kilobase
l	litre
LTR	long terminal repeat
M	molar
mA	milliamp(s)
mDBP	major DNA binding protein
MI	mock infected
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
moi	multiplicity of infection
mol.wt.	molecular weight
Mr	relative molecular mass
mRNA	messenger ribonucleic acid
NPT	non permissive temperature
N-terminal	amino terminal
OBP	origin binding protein
OD	optical density
ORF	open reading frame
ori	origin of replication
ori_L	long region origin of replication
ori_S	short region origin of replication
^{32}P	phosphorous-32 radio-isotope
PAGE	polyacrylamide electrophoresis
pfu	plaque forming units
PK	protein kinase

PMSF	phenylmethylsulphonyl fluoride
PRV	pseudorabies virus
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RR	ribonucleotide reductase
RT	room temperature
s	second(s)
^{35}S	sulphur-35 radio-isotope
SDS	sodium dodecyl sulphate
ss	single-stranded
SSB	single-stranded DNA binding
Staph A	Staphylococcus aureus
SV40	simian virus 40
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethylethylene diamine
TK	thymidine kinase
TR	terminal repeat
Tris	tris(hydroxymethyl) aminomethane
ts	temperature sensitive
Tween-20	polyoxethylene sorbitan monolaurate
U	unique
U_L	long unique region
U_S	short unique region
UV	ultra violet
V	volts
Vmw	viral protein of molecular weight
vol	volume
VP	viral polypeptide
v/v	volume per volume
VZV	varicella-zoster virus
w	weight
wt	wild type
w/v	weight per volume
uCi	microcurie(s)
ug	microgram(s)
ul	microlitre(s)
uM	micromolar

Nucleotide bases

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

Amino acids

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

CHAPTER 1: INTRODUCTION

The work presented in this thesis is concerned with the interaction of the herpes simplex virus type 1 origin binding protein UL9, with the viral origins of DNA replication and includes a mutagenic analysis of the C-terminal DNA binding domain. The following introduction briefly describes the virus and its life cycle and reviews our understanding of herpes simplex virus DNA replication. A brief comparison with other origin binding protein/DNA interactions during initiation of DNA replication is also included.

1.1 THE HERPESVIRUSES

1.1.1 The Family herpesviridae

The members of the family herpesviridae form a large group of DNA containing viruses which have been isolated from a wide range of higher eukaryotic hosts (Roizman et al., 1992).

The family is characterised on the basis of distinct morphological properties of the virion; all members possess a large double-stranded DNA genome present within an enveloped particle, 150-200 nm in diameter. The virus particle is composed of four distinct elements, namely the core, the capsid, the tegument and the envelope. The core contains the genomic DNA (Booy et al., 1991). It is surrounded by an icosohedral shaped capsid assembled from 162 capsomeres of which 150 are hexomeric and 12 are pentameric prisms (Wildy et al., 1960; Schrag et al., 1989). The tegument is described as a layer of amorphous, proteinaceous material between the core and the outer lipid envelope. The envelope is the outermost structure of the virus and consists of a lipid bilayer derived from host cell nuclear and plasma membranes from which protrude numerous viral-encoded glycoprotein spikes of approximately 8 nm in length (Wildy and Watson., 1962; Spear and Roizman., 1972).

In addition to having common morphological

characteristics, the herpesviruses share the ability to establish and maintain a latent state in their infected hosts. The wide occurrence of herpesviruses in vertebrates and their high degree of species specificity suggest that they have evolved in close association with their hosts. Historically they have been classified into three sub-families, referred to as alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae on the basis of their biological properties such as host range, reproductive cycle, cytopathology and characteristics of latent infection, rather than their genetic properties (Roizman, 1982; Mathews, 1982).

Alphaherpesvirinae

Many Members of this subfamily are neurotropic and exhibit a wide *in vitro* host range. The reproductive cycle is short, usually less then 24 hours in cell culture, and results in the destruction of the susceptible cell. Latent infections are frequently established in the ganglia. Examples within this group include herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2, respectively), varicella-zoster virus (VZV) and pseudorabies virus (PRV).

Betaherpesvirinae

This group exhibits a restricted host range and a longer reproductive cycle. Infection frequently results in an enlargement of the cells, and the establishment of latency may occur in a variety of tissues including secretory glands, kidneys and lymphoreticular cells. Human cytomegalovirus (HCMV) is a member of this sub-family.

Gammaherpesvirinae

This sub-family also has a narrow host range which is usually restricted to B or T lymphocytes. The length of reproductive cycle and cytopathology are variable and latent infections are frequently established in lymphoid tissue. This family includes Epstein-Barr virus (EBV).

Comparisons of the predicted amino acid sequences of many herpesvirus proteins have shown that genetic relationships between individual viruses exist which are in general agreement with the biological classification. However some exceptions do exist e.g. human herpesvirus 6 (HHV6). Originally, HHV6 was assigned to the Gammaherpesvirinae on the basis of its tropism for lymphocytes. However, on the basis of sequence homology and gene organisation, HHV6 is related to HCMV and more properly belongs to the Betaherpesvirinae. The existence of certain "core" genes common to all the subfamilies suggests that viruses belonging to these subfamilies share a common evolutionary origin (Reviewed by McGeoch, 1992).

The recent determination of the complete DNA sequence of channel catfish virus, which had previously been classified as a alphaherpesvirus, and which is morphologically indistinguishable from other herpesviruses (Wolf and Darlington, 1971), revealed only very distant relationships to any other herpesvirus (Davison, 1992). This may indicate a wider evolutionary divergence amongst herpesviruses than previously suspected or possibly a separate evolutionary origin for this virus.

1.1.2 The Pathogenicity of HSV-1

To date, seven herpesviruses have been described which are known to infect humans, namely HSV-1, HSV-2, VZV, EBV, HCMV, HHV-6 and HHV-7.

Herpes simplex virus (HSV) is an unusual virus in that it can cause a variety of clinical diseases. These diseases can be divided into two forms; those due to a primary infection and those resulting from reactivation of the latent virus.

In humans, infection with HSV-1 typically occurs early in life and the virus is transmitted by close physical contact. The most common clinical form of infection is associated with cold-sore lesions of the

infections of the eye (herpes keratoconjunctivitis). The genital region is also a common site for primary infection by HSV taking the form of vesicular eruptions. HSV-2 infection is largely associated with these genital lesions although in an increasing number of cases they have been shown to be as a result of an HSV-1 infection. HSV-2 infections are usually sexually transmitted but severe generalised infections can also occur at birth as a result of a genital infection of the mother (Whitley et al., 1985).

Despite recovery from a primary infection, HSV can establish a long term latent infection in the neuronal cells of the sensory ganglia and brain (Stevens and Cook., 1971; Baringer and Swoveland., 1973; Cook et al., 1974; Fraser et al., 1981). Several factors such as trauma, fatigue and exposure to UV light have been associated with reactivation of the virus and re-occurrence of cutaneous lesions. Reactivation can occur sporadically throughout life.

1.1.3 HSV-1 Latency

A major characteristic feature of HSV-1 infection is the ability of the virus to establish its genetic information in a non-infectious, non-replicating latent state in the neuronal cells of the sensory ganglia and brain (Stevens and Cook, 1971; Baringer and Swoveland, 1973; Cook et al., 1974; reviewed by Hill, 1985). It is this property which plays an important role in its pathogenesis.

Using hybridisation techniques, Puga et al, (1978) first detected HSV DNA in the sensory ganglia of mice which had been latently infected with HSV-1. The precise state of the viral DNA in latently infected cells is not clear. The genome does not appear to be present as a linear molecule as terminal fragments have never been detected (Rock and Fraser, 1983; Efstathiou et al., 1986), and integration of the viral DNA into the host genome does not occur (Rock and Fraser, 1983; Mellerick and Fraser, 1987). It is therefore thought that the viral

genome exists extrachromosomally as a circle or less likely a concatemer.

Limited transcription of the latent HSV-1 genome results in the accumulation of latency-associated transcripts (LATS) in the nuclei of latently infected cells (Stevens et al., 1987; Rock et al., 1987; Spivak and Fraser., 1987; Steiner et al., 1988; Wagner et al., 1988a). Three LATs of 2, 1.5 and 1.45 kb in length which are transcribed from within the inverted repeat sequences flanking the U_L region have been identified (Spivak and Fraser, 1987; Wagner et al., 1988a). These transcripts partially overlap the immediate early gene 1 (IE-1) mRNA, but are transcribed from the opposite strand (Spivak and Fraser., 1987; Stevens et al., 1987). Open reading frames within the LATs have been proposed (Perry and McGeoch, 1988; Weschler et al., 1988). However, neither unique polyadenylated cytoplasmic LAT messages nor encoded polypeptides have been detected (Wagner et al., 1988a;b).

The isolation of an HSV-1/HSV-2 recombinant which was deficient in LAT expression but was still able to establish a latent infection in mice suggested that LATs do not play an essential role in the establishment of latency (Javier et al., 1988). Using a rabbit eye model, the same mutant was shown to possess an impaired ability to re-activate in vivo (Hill et al., 1990). A similar situation was observed with the deletion mutant 1704 (Maclean and Brown, 1987). Although the mutant was able to establish a latent infection in mice, re-activation of the virus from explanted ganglia was found to be slower than that of the wt virus (Steiner et al., 1989). Taken together, these studies suggest that the LATs, although not necessary for latency establishment, may have a role in mediating the frequency of viral reactivation.

Both in vivo and in vitro models have been used to study the viral requirements for the establishment, maintenance and reactivation of a latent HSV-1 infection (Wigdahl et al., 1982; Shiraki and Rapp., 1986; Russell

and Preston., 1986). As yet, no gene products have been shown to be specifically required for establishment and maintenance (Polvino-Bodnar et al., 1987; Meigneir et al., 1988; Russell et al., 1987). However, the immediate early protein Vmw110 (Russell et al., 1987; Harris et al., 1989; Clements and Stow, 1989), the viral thymidine kinase (Coen et al., 1989; Efstathiou et al., 1989; Tenser et al., 1989) and the HSV ribonucleotide reductase (RR) (Jacobsen et al., 1989) appear to be important for reactivation in various assays.

1.1.4 The HSV-1 Genome

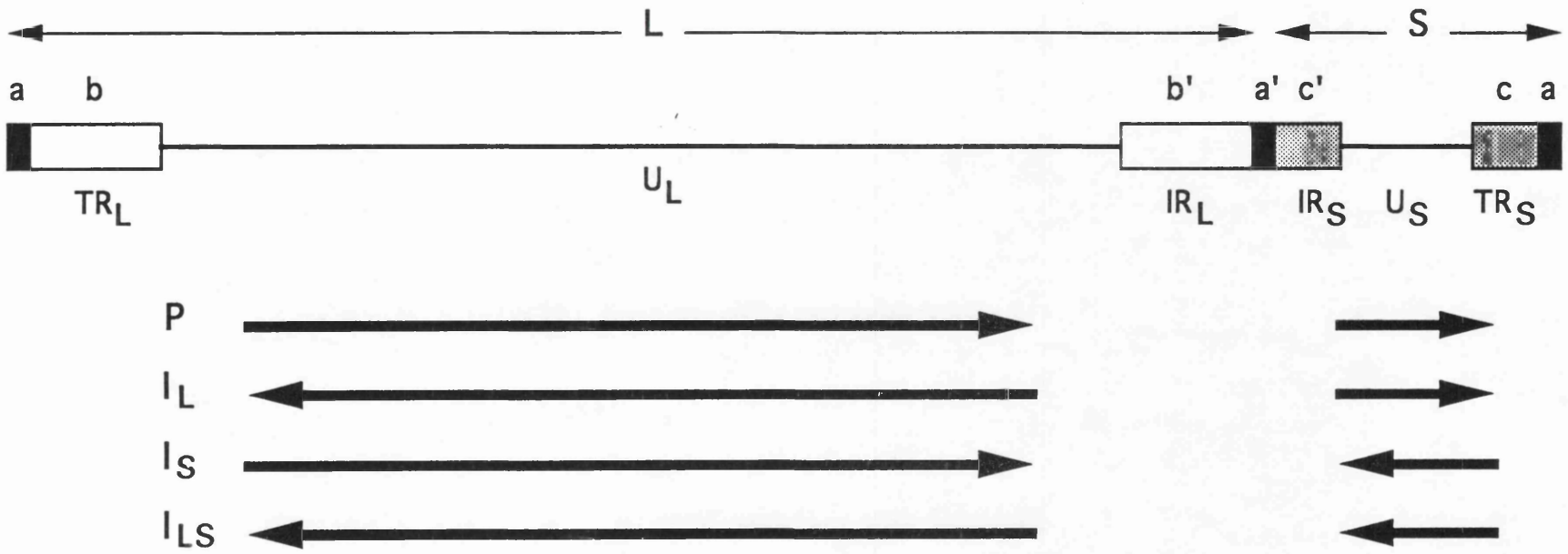
(a) Structure of the HSV-1 Genome

The HSV-1 genome is a large, linear ds molecule containing approximately 152 kb of DNA with a molecular weight of approximately $10x10^7$ kDa (Becker et al., 1968; Kieff et al., 1971). Electron microscopic studies and restriction endonuclease mapping have shown that the genome consists of two covalently linked components, referred to as the Long (L) and Short (S) segments, (**Figure 1.**). Both segments contain unique sequences, U_L (unique, long) and U_S (unique, short) which are flanked by large terminal and internal inverted repeats; TR_L, IR_L (terminal and internal repeat, long) and TR_S, IR_S (terminal and internal repeat, short). The sequences of the L and S inverted repeats are distinct from one another (Sheldrick and Berthelot, 1974; 1975; Hayward et al., 1975). A direct repeat of about 400 bp, termed the "a" sequence is present at each end of the genome (Davison and Wilkie, 1981). A single copy of this sequence is found at the S terminus, one or more at the L terminus and a variable number are located, in an inverted orientation, at the junction between the L and S segments (Roizman et al., 1979). A single residue 3′ overhang occurs at both genome termini (Davison and Wilkie, 1981; Mocarski and Roizman, 1982a;b).

The two unique regions can invert relative to one another and preparations of HSV-1 DNA from plaque

Figure 1. Organisation of the HSV-1 Genome

The viral genome which comprises of two covalently linked segments L and S is shown. Each segment contains unique sequences U_L and U_S (solid lines) flanked by terminal and internal inverted repeat elements TR_L and IR_L, TR_S and IR_S respectively (open and shaded boxes). A direct repeat, termed the "a" sequence is present at each end of the genome and also in inverted orientation at the L - S junction. Inversion of the L and S components occurs and preparations of viral DNA contain an equimolar mixture of four isomeric molecules (orientation of segments indicated by arrows). These have been designated P (prototype), I_S (inversion of S), I_L (inversion of L) and I_{LS} (inversion of both L and S).


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

purified virus have been found to contain an equimolar mixture of four isomeric forms, differing from one another in the relative orientation of U_L with respect to U_S (**Figure 1.**). These isomers heve been designated P (prototype), I_S (inversion of S), I_L (inversion of L) and I_{LS} (inversion of both L and S), (Hayward et el., 1975; Delius and Clements, 1976; Wilkie and Cortini, 1976; Roizman., 1979).

The prototype arrangement of the genome was chosen for mapping purposes (Roizman, 1979; McGeoch et al., 1988). The internal recombination events which create all four isomers appear to be mediated by the "a" sequence (Mocarski and Roizman, 1982a;b). There is also evidence to suggest that all four isomers are able to take part in the production of infectious progeny (Davison and Wilkie, 1983a) and participate in latent infections (Efstathiou et al., 1986). The isolation of viable HSV-1 mutants with genomes frozen in each of the four isomeric forms suggests that inversion is not essential for viral replication (Jenkins and Roizman, 1986).

The complete genome of HSV-1 strain 17 syn+ has been sequenced (McGeoch et al., 1985; 1986; 1988a; Rixon and McGeoch, 1985; Perry and McGeoch, 1988) and contains 152,260 bp, (small variations in length occur due to differences in copy number of the "a" sequence and other tandemly repeated sequences), with a mean G+C% base composition of 68.3%. Three other human herpesviruses have also been completely sequenced; VZV, EBV and HCMV. DNA hybridisation studies have shown that as well as having similar gross structures, HSV-1 and HSV-2 genome sequences are related and closely co-linear. The parts of U_S and U_L of the HSV-2 strain HG52 which have been sequenced exhibit a 70 - 80% nucleotide identity within coding sequences with corresponding HSV-1 genes (Davison and Wilkie, 1981; 1983; Swain and Galloway, 1983; Swain et al., 1985; Lockshon and Galloway, 1986; Draper et al., 1986; McGeoch et al., 1987; Worrad and Caradonna, 1988).

(b) Genetic Content of HSV-1

DNA sequence analysis identified a high percentage of the HSV-1 genome as having protein coding potential. A total of 72 open reading frames, with the potential to encode 70 distinct polypeptides were initially identified within the L and S regions of the genome (**Figure 2**). Fifty six genes, UL1 - UL56, were located within the U_L region and twelve, US1 - US12, within U_S. HSV-1 contains one spliced gene, UL15 whose intron contains the UL16 and UL17 ORFs. Immediate-early genes 1 and 3 (IE-1 and IE-3) were mapped to the R_L and R_S repeat sequences, and are therefore represented twice (McGeoch et al., 1985; 1988a). A second ORF, encoding the protein ICP34.5, within R_L was first described in HSV-1 strain F (Chou and Roizman, 1986; 1990) and has since been confirmed in HSV-1 strain 17 (Dolan et al., 1992).

Recent data has also revealed a distinct transcriptional unit mapping within the UL26 gene. The product of its ORF, UL26.5 corresponds to the C-terminal 329 amino acids of the UL26 protein (Liu and Roizman, 1991). Barker and Roizman, (1992) and Barnett et al., (1992) have also reported the existence of an additional gene, designated UL49.5 between the UL49 and UL50 ORFs.

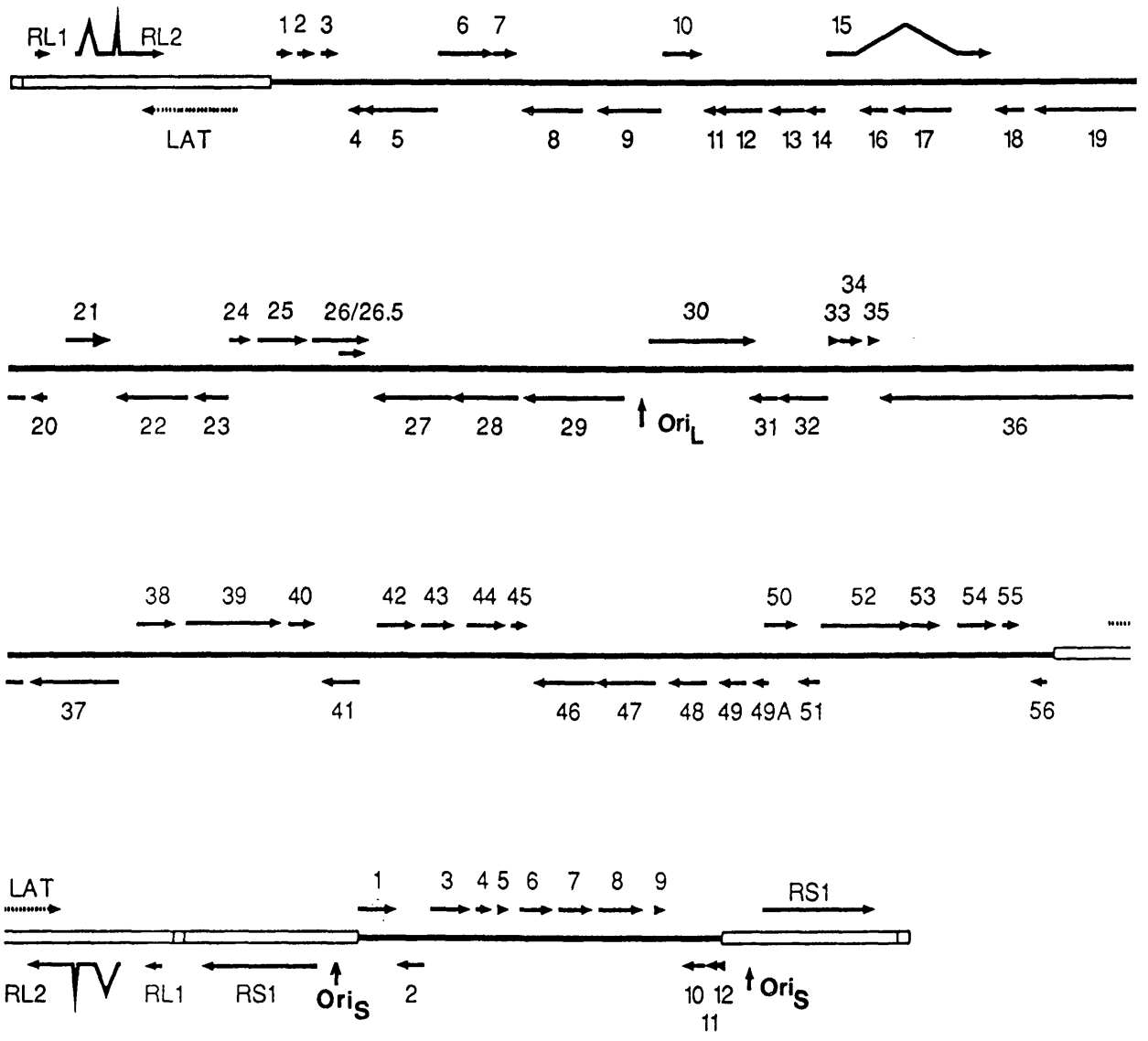
Of the genes identified, the functions of approximately one third remain unknown. Functions of the remaining gene products include roles in transcriptional regulation, DNA replication and virion structure and assembly. Eleven out of twelve genes present in the U_S region and at least nine of the U_L genes have been shown to be non-essential for viral growth in tissue culture (Umene, 1986; Weber et al., 1987; Longdecker and Roizman, 1986; 1987; Baines and Roizman, 1991; MacLean et al., 1991).

1.1.5 HSV-1 Structural Proteins

Around thirty different virion structural polypeptides have been detected, the majority of which are encoded by HSV-1 late genes (Spear and Roizman, 1972; Heine et al., 1974; Marsden et al., 1976; reviewed by Dargan., 1986). These proteins comprise of glycoproteins,

Figure 2. Genetic Content of the HSV-1 Genome

The viral genome is represented as four successive lines of 40 kb with unique sequences shown as solid lines and major repeat elements as open boxes. The location and orientation of proposed functional open reading frames are shown by solid arrows and LAT transcripts as arrowed, dotted lines. Genes are numbered UL1 - UL56 and US1 - US12 plus RL1 (encoding ICP 34.5), RL2 (IE-1) and RS1 (IE-3). The origins of DNA replication (ori_S and ori_L) are also indicated. This diagram was kindly provided by Dr. D.J. McGeoch.


RL1
RL2
1 2 3
6 7
10
15
LAT
4 5
8
9
11 12
13 14
16 17
18
19
21
24 25
26/26.5
30
34
33 35
20
22
23
27
28
29
Ori$_L$
31 32
36
38
39
40
42 43
44 45
50
52
53
54 55
37
41
46
47
48
49
49A
51
56
LAT
1
3 4 5 6 7 8 9
RS1
RL2
RL1
RS1
Ori$_S$
2
10 12
11
Ori$_S$

tegument proteins and capsid proteins.

Glycoproteins

The HSV-1 genome encodes at least nine antigenically distinct glycoproteins: gB (encoded by UL27), gC (UL44), gD (US6), gE (US8), gG (US4), gH (UL22), gI (US7), gK (UL53) and gL (UL1) (Spear, 1976; Marsden et al., 1978; 1984; Bauke and Spear, 1979; Buckmaster et al., 1984; Roizman et al., 1984; Gompels and Minson, 1986; Frame et al., 1986; Longnecker et al., 1987; Johnson and Feenstra, 1987; McGeoch et al., 1985; 1987; 1988a; Hutchinson et al., 1992). These glycoproteins comprise the major virus-specific components of the virus envelope and virus-infected cell membranes (Spear, 1976; Roizman et al., 1984).

The roles of gB, gD, gC and gH in the initial stages of infection are described later in the text. gC, gE and gI are also believed to have functions which may modulate the immune response to infection (Eisenberg et al., 1987; Johnson and Feenstra, 1987; Dublin et al., 1990); gC functioning as a complement receptor and the gE/gI complex as an Fc receptor.

Tegument Proteins

The tegument is the least well defined component of the HSV-1 virion and appears as an amorphous region present between the capsid and envelope (Spear and Roizman, 1972).

Tegument proteins are defined as those not found associated with viral capsids or envelopes which are released from the virion by non-ionic detergents (Roizman and Furlong, 1974; Spear, 1980; Lemaster and Roizman, 1980). It is uncertain how many virus encoded proteins constitute this region and how they are structurally oriented. However, major components of the tegument include the protein involved in transactivation of IE gene expression, Vmw65 (UL48), (Post et al., 1981; Batterson and Roizman, 1983; Campbell et al., 1984), and the 81/82 kDa products of the UL47 gene (McLean et al.,

1990) which have been implicated in the modulation of Vmw65 activity (McKnight et al., 1987).

Capsid Proteins

Among the approximately thirty structural proteins which constitute the HSV-1 virion, only seven are components of the nucleocapsid.

Capsids isolated from HSV infected cells are either "full", (containing viral DNA) or "empty", (lacking viral DNA). Initial experiments identified four proteins, VP5, VP19c, VP23 and VP24 as components of empty viral capsids from the nuclei of infected cells (Gibson and Roizman, 1972). A fifth protein p12, with a molecular weight of 12 kDa, was subsequently identified (Heilman et al., 1979; Cohen et al., 1980). Two other proteins, unique to full capsids, VP21 and VP22a, have also been identified.

VP5 (ICP5) is referred to as the major capsid protein and is the product of the UL19 gene (Davison and Scott, 1986b; McGeoch et al., 1988a). It forms the hexameric and possibly the pentameric capsomeres of the capsid (Schrag et al., 1989). VP19c is a DNA binding protein identified by direct amino acid sequencing of purified capsid proteins as the product of the UL38 gene (Rixon et al., 1990). A similar approach demonstrated that VP23 is encoded by the UL18 gene.

VP22a and VP21 are components of the core region of full capsids. Preston et al, (1983) proposed that the VP22a protein was encoded by the UL26 gene since a mutant with a ts lesion within the gene was defective in VP22a processing. This mutant also failed to package its DNA into capsids at the NPT therefore implicating the protein in viral DNA encapsidation. McNabb and Courtney, (1992) have recently reported that the p12 protein may be encoded by UL35. The two proteins, VP24 and VP21, have recently been suggested to be the products of the UL26 gene (Davison et al., 1992).

1.2 HSV-1 LYTIC INFECTION

1.2.1 Initial Stages of Infection

At present, the pathway by which HSV-1 gains entry into its host is not completely clear. A model for entry of HSV-1 has been proposed which involves a series of interactions between the viral envelope and the cell plasma membrane which trigger membrane fusion, nucleocapsid penetration and virion disassembly (Sarmiento et al., 1979; Little et al., 1981; Weller et al., 1983; Gompels and Minson, 1986; McGeoch and Davison, 1986b; Ligas and Johnson, 1988; Highlander et al., 1987; 1988; Johnson and Ligas, 1988; Desai et al., 1988; Fuller et al., 1989; Buckmaster et al., 1984; Fuller and Spear, 1987; Cai et al., 1988; Herold et al., 1991).

The initial attachment of HSV-1 to cells has been shown to involve the binding of viral envelope glycoproteins to heparan sulphate proteoglycans on the cell surface (WuDunn and Spear, 1989; Lycke et al., 1991). Evidence supporting this proposal came from observations that digestion of cell surface heparan sulphate can reduce HSV binding to cells (WuDunn and Spear, 1989) and that certain aminoglycosides can block the binding of the virus to its cell receptor (Langeland et al., 1987; 1988).

The cellular receptor for basic fibroblast growth factor (bFGF) was recently proposed to act as a viral entry point (Kaner et al., 1990). This seems unlikely, however, as a report by Muggeridge et al, (1992) demonstrated that bFGF receptor was not required for viral infection in most cell types.

Adsorption of HSV-1 to the cell surface is thought to be mediated by virally encoded glycoproteins which protrude from the viral envelope. It is possible that a non-glycoprotein component may also be involved in viral attachment as adsorption can take place in the absence of normal glycosylation (Campadelli-Fiumi et al., 1982; Spivak et al., 1982; Svennerholm et al., 1982; Kuhn et al., 1988). Initial non-specific adsorption may be followed by specific irreversible binding to the cell surface (Hochberg and Becker, 1968; Rosenthal et al.,

1984).

Three HSV-1 envelope glycoproteins have been shown to be essential for viral infectivity: gB (Sarmiento et al., 1979; Little et al., 1981), gD (Ligas and Johnson, 1988) and gH (Weller et al., 1983; McGeoch and Davison, 1986; Gompels and Minson, 1986; Desia et al., 1988). Studies utilising monoclonal antibodies, liposomes containing virion proteins and ts mutants, implicate these proteins in the processes of viral attachment, fusion of the viral envelope with the cell and virion entry (Deluca et al., 1982; Bzik et al., 1984; Gompels and Minson, 1986; Highlander et al., 1987; Fuller and Spear, 1987; Cai et al., 1988; Desai et al., 1988; Fuller et al., 1989; Fuller and Lee, 1992; Herold et al., 1991; Kuhn et al., 1990).

Following penetration of the host cell membrane, the nucleocapsid is transported through the cytoplasm to the nucleus where it is uncoated and the DNA is released into the nucleus via nuclear pores (Batterson and Roizman, 1983). The nuclear entry process is thought to require the participation of a viral protein as studies with a HSV-1 ts mutant whose mutation was later mapped to the UL36 gene (McGeoch et al., 1988), identified a block in the release of viral DNA and an accumulation of capsids at the nuclear pores (Batterson et al., 1983). It is possible however that the mutant UL36 protein may prevent normal activity of cellular uncoating proteins.

1.2.2 Effect of HSV-1 Infection on Host Cell Macromolecular Synthesis

Infection of cells with HSV-1 results in a rapid decrease in host cell macromolecular synthesis, degradation of cellular mRNAs and induction of viral gene expression (reviewed by Fenwick., 1984; Roizman and Roane, 1964; Nishioka and Silverstein, 1977; 1978; Schek and Bachenheimer, 1985; Kwong and Frenkel, 1987). The host cell "shut-off" activity can be divided in two distinct phases.

The "early shut-off" phase is mediated by one or more

virion components and does not require viral gene expression (Fenwick et al., 1979; Hill et al., 1985). It involves a reduction in synthesis of the majority of host cell polypeptides (Sydiskis and Roizman, 1967; 1968) arising from the disaggregation of polyribosomes and the degradation of host mRNA (Nishioka and Silverstein, 1977; 1978; Shek and Bachenheimer, 1985).

Studies using viable HSV-1 mutants defective in inducing the virion host cell shut-off (vhs), (Read and Frenkel, 1983) showed that the virion-mediated shut-off function involved the HSV-1 UL41 gene (Kwong et al., 1988). This involvement was confirmed when insertion of the UL41 gene from HSV-2 strain G, which exhibits a strong shut-off function, into the HSV-1 strain 17, which is less efficient in early shut-off, was found to restore vhs function (Fenwick and Everett, 1990). In addition, HSV-2 strain HG52, which exhibits poor shut-off has been shown to encode a truncated UL41 product (Everett and Fenwick, 1990).

UL41 gene mutants defective in vhs also produce more stable viral mRNAs and host mRNAs have significantly longer half-lives than in wt infections (Read and Frenkel, 1983; Kwong and Frenkel, 1987; Oroskar and Read, 1987; 1989; Strom and Frenkel, 1987). The vhs polypeptide appears to provide a non-specific mRNA degradation function which may play a role in regulating the abundance and patterns of accumulation of both viral and cellular mRNAs (Kwong and Frenkel, 1987; Oroskar and Read, 1987). It has been proposed that the vhs product may be a RNase or may interact with a cellular factor to de-stabilise both host and viral mRNA (Kwong and Frenkel, 1989).

The "delayed shut-off" phase requires the expression of viral genes (Fenwick and Clark, 1982; Read and Frenkel, 1983) and involves further reduction in the levels of host protein synthesis and enhanced degradation of host mRNA.

In contrast to the shut-off described above, the synthesis of some cellular polypeptides including

cellular stress and heat-shock proteins is in fact stimulated during HSV-1 infection. (LaThangue et al., 1984; Patel et al., 1986; Kemp et al., 1986).

1.2.3 Control of HSV-1 Gene Expression

During lytic infection of tissue culture cells by HSV-1 viral genes are transcribed by the host RNA polymerase II enzyme (Alwire et al., 1974; Preston and Newton, 1976; Costanzo et al., 1977) in a temporally regulated fashion. Three distinct phases based on the kinetics and characteristics of their expression can be identified (Honess and Roizman, 1974; Clements et al., 1977; Jones and Roizman, 1979).

The first set of genes to be transcribed are the immediate early (IE) or alpha genes. The IE transcripts are produced in the absence of de novo protein synthesis (Honess and Roizman, 1975; Preston et al., 1978) and functional IE gene products are essential for the subsequent induction of early viral gene expression which takes place prior to the onset of viral DNA replication (Honess and Roizman, 1974; Clements et al., 1977; Wagner, 1985). The IE and E gene products are in turn required for the expression of true late proteins (some late proteins are expressed before DNA replication) and viral DNA synthesis (Honess and Roizman, 1974; Jones and Roizman, 1979; Clements et al., 1977).

(a) Immediate Early Genes

The HSV-1 genome posseses five IE genes: IE-1, IE-2 (UL54), IE-3, IE-4 (US1) and IE-5 (US12), which encode the polypeptides Vmw 110, Vmw63, Vmw175, Vmw68 and Vmw12 respectively. All the IE genes, with the exception of IE-2, have promoters located within the repeat regions of the genome. IE-1 and IE-3 are located wholely within the R_L and R_S repeats respectively and are therefore represented twice (Watson et al., 1979; Anderson et al., 1980; Mackem and Roizman, 1980 Marsden et al., 1982; Rixon et al., 1982). The IE-4 and IE-5 genes have identical promoters and 5′ untranslated leader sequences

present in the R_S region, but their coding sequences differ and are located at opposite ends of the U_S region (Watson et al., 1981). The IE-2 gene maps entirely within the U_L sequence (Whitton et al., 1983).

Studies of the IE promoter regions have revealed the presence of multiple cis-acting elements upstream of the transcriptional initiation start site, which are recognised by both viral and cellular trans-acting proteins (Mackem and Roizman, 1980; 1981; Murchie and McGeoch, 1982; Whitten et al., 1983; Whitton and Clements, 1984a). Two distinct components are contained within these promoters. A "TATA" box sequence, located about 20-30 nucleotides upstream of the mRNA start site is necessary for basal and induced levels of transcription (Mackem and Roizman, 1982a;b;c; Cordingly et al., 1983; Preston et al., 1984; Kristie and Roizman, 1984; Bzik and Preston, 1986). Further upstream, a separate domain containing multiple cis-acting elements confers enhancer functions and responsivity to the regulation of IE expression by Vmw65.

The upstream regulatory regions contain "GC-rich" elements (GC-boxes), which function as binding sites for the transcriptional activator Sp1 (Jones and Tjian., 1985; Briggs et al., 1986), in addition to a consensus sequence, TAATGARAT (where R is a purine), which is present in one to three copies, in either orientation (Mackem and Roizman, 1982c; Whitton et al., 1983; Whitton and Clements, 1984; Preston et al., 1984; Gaffney et al., 1985).

The specific induction of IE gene transcription is mediated via the TAATGARAT element by the tegument protein Vmw65 (VP16), encoded by the UL48 gene (Mackem and Roizman, 1982b; Kristie and Roizman, 1984; Campbell et al., 1984; Dalrymple et al., 1985; Gaffney et al., 1985; Bzik and Preston, 1986; O'Hare and Hayward, 1987a). Vmw65 alone is unable to bind directly to DNA (Marsden et al., 1987), but its transactivation activity has been proposed to arise from a physical interaction with the cellular transcription protein Oct-1 and one or more

other cellular factors. These together form an immediate early complex (IEC) which binds specifically to the TAATGARAT element (McKnight et al., 1987; Preston et al., 1988; O'Hare and Goding, 1988; Gerster and Roeder, 1988; Kristie et al., 1989; Ace et al., 1988). Chemical modification interference analysis has demonstrated that Oct-1 binds to the octamer consensus sequence ATGCAAAT, overlapping the TAATGARAT element (ATGCAATGARAT), (Pruijn et al., 1986; Fletcher et al., 1987; O'Hare and Goding, 1988).

Mutational analyses of Vmw65 have identified a highly acidic domain at the C-terminus which functions as a potent transcriptional activating region (Triezenberg et al., 1988a; Sadowski et al., 1988; Cousens et al., 1989), and it has been proposed that Vmw65 interacts with Oct-1 via the residual N-terminal portion (Triezenberg et al., 1988a; Werstuck and Capone, 1989a;b).

In addition to Vmw65, the IE proteins Vmw110 and Vmw175 also appear to be involved in the regulation of IE gene expression (O'Hare and Hayward, 1985a;b; 1987; Gelman and Silverstein, 1987a;b).

(b) Immediate Early Gene Products

Four of the IE gene products, Vmw175, Vmw110, Vmw68 and Vmw63 are phosphorylated nucleoproteins. Vmw12, in constrast, is cytoplasmic and unphosphorylated (Pereira et al., 1977; Marsden et al., 1978; 1982; Fenwick and Walker, 1979; Hay and Hay, 1980; Wilcox et al., 1980; Ackerman et al., 1984). Studies with ts and deletion mutant viruses in the five IE genes have indicated that only Vmw175 and Vmw63 are absolutely essential for viral growth in tissue culture cells (McCarthy et al., 1989; Post and Roizman, 1981; Sacks et al., 1985; Sears et al., 1985; Longnecker and Roizman, 1986; Stow and Stow, 1986; 1989).

Vmw175

Initial studies with HSV-1 ts mutants demonstrated that a lesion in the IE-3 gene of the tsK mutant resulted

in the over-expression of IE gene products at the NPT. In addition, neither early nor late genes were expressed, with the exception of the large subunit of the ribonucleotide reductase protein. These results demonstrated that Vmw175 plays a role in the stimulation of early and late gene promoters in addition to the repression of IE gene expression (Marsden et al., 1976; Preston et al., 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1980).

The stimulation of an HSV-1 E gene promoter by Vmw175 was subsequently demonstrated using transfection assays in which the presence of a plasmid expressing Vmw175 resulted in a marked increase in the activity of an E gene promoter linker to a reporter gene (Everett, 1984b; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a). Repression of an IE promoter/CAT construct by Vmw175 has also been shown in similar experiments (O'Hare and Hayward, 1985b; Deluca and Schaffer, 1985; Gelman and Silverstein, 1986).

Vmw175 is the only IE protein known to bind specifically to a conserved DNA sequence, 5'-ATGCTG-3'. The identification of such a site within the IE-3 (Vmw175) promoter (Muller, 1987) led to the proposal that the binding of the protein to this site, which corresponds to the mRNA cap site, represses expression. Whether Vmw175 represses the other IE promoters in a similar fashion is unclear as IE-1 is the only other IE gene to contain the binding site consensus within its promoter (Beard et al., 1986; Faber and Wilcox, 1986; Kristie and Roizman, 1986; Muller, 1987; Patterson and Everett, 1988). Even so, a subsequent study reported that although mutation of the Vmw175 binding site within the IE-1 promoter reduced repression of the IE-1 promoter in vitro, it failed to have the same effect on Vmw110 expression during normal HSV-1 infection (Everett and Orr, 1991).

In addition to recognising the ATCGTC motif, Vmw175 is also able to bind to other sequences (Kristie and Roizman, 1986; Michael et al., 1988), but as yet, no

specific sequence responsive to Vmw175 or indeed any other viral IE protein has been identified in either early or late promoters (Everett, 1984a; Coen et al., 1986; Michael et al., 1988; Kattar-Cooley and Wilcox, 1989)

Vmw110

The effect of Vmw110 on gene promoter activity has been investigated using transient expression assays similar to those used for the study of Vmw175. The results show that Vmw110 is a potent activator of gene expression from all three classes of virus promoters in addition to a number of cellular gene promoters (Everett, 1984; O'Hare and Hayward, 1985a;b; Quilan and Knipe, 1985; Gelman and Silverstein, 1985; Mavromara-Nazos et al., 1986). This potent transactivation effect has been shown to take place with Vmw110 acting independently or in association with Vmw175 (Everett, 1984b; 1986; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a; Deluca and Schaffer, 1985; Gelman and Silverstein, 1986). However, in the context of the viral genome, Vmw110 is not sufficient to transactivate early gene transcription since a functional Vmw175 is also required (Deluca et al., 1985).

Vmw110 has also been shown to be the only HSV-1 gene product required to reactivate HSV-2 from latently infected cells in vitro (Russell et al., 1987b; Harris et al., 1989).

Vmw63

Mutant viruses containing ts lesions within the IE-2 gene have been isolated which over-produce an abberant form of Vmw63 and the Vmw175 protein at the NPT (Sacks et al., 1985). Although the ts mutants exhibited normal levels of viral DNA synthesis and E gene expression, the production of many late gene products was restricted (Rice and Knipe, 1988), suggesting that Vmw63 has a role in the regulation of late gene expression.

Activation of E gene promoters by Vmw63, in concert

with Vmw110 and Vmw175 has been reported and there is also evidence that the protein can act as a trans-repressor of transcription in combination with Vmw175 and Vmw110 (Sekulovich et al., 1988; Everett, 1986; Rice and Knipe, 1988). Vmw63 is known to act at a post-transcriptional level (McLauchlan et al., 1992).

Vmw68

Characterisation of an HSV-1 deletion mutant revealed that Vmw68 is only essential for viral growth in certain cell types, suggesting that an analogous function may be present in other cell types (Post and Roizman, 1981). As the impaired growth correlated with a deficiency in late gene expression, a possible role for Vmw68 in late gene regulation has been suggested (Sears et al., 1985).

Vmw12

Vmw12 is the least characterised of the IE proteins. The function of this protein has not been defined but it is non-essential for viral growth in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

(c) Early Genes

Early gene expression is dependent upon promoter trans-activation by IE proteins (Honess and Roizman, 1974; Clements et al., 1977; Wagner, 1985; Zhang and Wagner, 1987; Weinheimer and McKnight, 1987). In comparison to IE genes, E genes appear to show a greater variation in the kinetics of their expression and as a result, have been divided into two subgroups, early ($beta_1$) and delayed-early ($beta_2$).

The HSV-1 UL39 gene, which encodes the large subunit of the ribonuclotide reductase enzyme has been traditionally classified as an early gene product but its expression under IE conditions and the presence of a TAATGARAT element in its promoter suggest that it should more correctly be considered as an IE gene. (Roizman and

Batterson, 1985; Wymer et al., 1989).

The glycoprotein, gD, is expressed as an early protein but is not maximally synthesised until after the onset of viral DNA replication (Gibson and Spear, 1983), a property typical of delayed early genes (Roizman and Batterson, 1985; Wagner, 1985). This increased expression may simply reflect an increased template copy number (Everett and Johnson, 1986b).

Mutational analysis of the promoter regulatory regions of many HSV-1 early genes has failed to detect any specific sequences involved in their transactivation by Vmw110 and Vmw175. Linker scanning mutagenesis of the HSV-1 TK gene has identified important elements such as a "TATA" box upstream of the transcriptional start site, GA- and GC-rich regions and a "CCAATT" box (McKnight et al., 1985; Everett, 1983; Eisenberg et al., 1985; El kareh et al., 1985). Binding of various cellular transcriptional factors such as the "CCAATT" box binding factor and Sp1 to such elements has been demonstrated (Jones et al., 1985; Graves et al., 1986). It has therefore been suggested that the transactivation of early gene expression by the IE gene products may be mediated indirectly through their interactions with certain cellular transcription factors (Everett, 1984b; Eisenberg et al., 1985; Coen et al., 1986).

Although many HSV-1 promoters contain homologies to "TATA", "CCAATT" and GC-rich elements, a great deal of diversity between promoters of genes in the same temporal class has been observed (Mackem and Roizman, 1982c; Wagner, 1985). This may explain why differential levels of expression are exhibited by genes belonging to the same class (Honess and Roizman, 1974; O'Hare and Hayward, 1985a; Harris-Hamilton and Bachenheimer, 1985).

(d) Late Genes

In addition to requiring functional IE and E gene products (Watson and Clements, 1980), experiments with DNA synthesis inhibitors and DNA-negative ts mutants demonstrated that late gene expression also shows a

dependency on viral DNA synthesis (Honess and Roizman, 1974; Holland et al 1980; Conley et al., 1981; Pederson et al., 1981).

Late genes exhibit variations in their kinetics of expression and are sub-divided into two groups; leaky-late ($gamma_1$) and true-late ($gamma_2$). Leaky-late genes (eg. UL19 encoding the major capsid protein), are expressed at low levels before the initiation of DNA synthesis, while true-late gene products (eg UL47 which encodes a tegument protein, McLean et al., 1990) are undetectable in the absence of viral DNA synthesis (Roizman and Batterson, 1985; Wagner, 1985; Johnson et al., 1986).

Like the promoters of early genes, no sequence specific elements for transactivation by viral gene products have been identified in late promoters (Everett, 1984b).

Late gene promoters lack the upstream regulatory regions which are present in IE and E promoters. Detailed mutational analysis of the promoter regions of two HSV-1 late genes, US11 and UL44, demonstrated that the presence of a "TATA" box and a mRNA transcriptional start site, in combination with a cis-acting HSV-1 origin of replication was sufficient for full expression (Johnson and Everett, 1986a; Homa, 1986). Indeed, deletion of the regulatory regions of the gD gene leaving the "TATA" box and mRNA cap site intact resulted in conversion of the gene from early to late regulation (Johnson and Everett, 1986b).

It is unclear what role viral DNA replication plays in late gene expression. An increased template copy number must contribute towards an increase in late gene expression but this is unlikely to be the only explanation as mRNA accumulation during transfection experiments is far greater (Johnson and Everett, 1986a). An alternative explanation may be that late gene expression is activated as a result of structural changes arising during the replication process (Johnson and Everett, 1986a; Mavromara-Nazos and Roizman, 1987).

1.2.4 HSV-1 DNA Replication: Properties of Replicating DNA

Analysis of parental viral DNA by restriction enzyme digestion suggested that following entry of the genome into the nucleus, it is rapidly circularised (Jacob and Roizman, 1977; Poffenberg and Roizman, 1985) probably by direct ligation of the terminal "a" sequences (Davison and Wilkie, 1983; Poffenberg et al., 1983). This process may be facilitated by the presence of complementary 3' single base extensions at the L and S termini (Mocarski and Roizman, 1982a;b., Davison and Rixon, 1985). Either a host cell enzyme or a component of the virion is thought to carry out the ligation as de novo protein synthesis is not required (Poffenberg and Roizman, 1985).

Analysis of newly synthesised, radio-labelled, viral DNA, demonstrated that it sedimented more rapidly than unit length genomes (Jacob and Roizman, 1977). In addition, enzyme digestion patterns of the replicative intermediates indicated that they lacked terminal fragments leading to the proposal that the DNA is present in an "endless" state, either as circular monomers or large concatemers (Jacob et al., 1979; Jongeneel and Bachenheimer, 1981).

The rate of sedimentation in neutral velocity gradients varies amongst the replicative intermediates. Those appearing early in infection, around 2-3 hpi, sediment up to twice as fast as unit length viral genomes while those appearing later in infection, around 4-6 hpi, sediment at least 100x faster (Jacob and Roizman, 1977; Ben-Porat and Tokazewski, 1977). The earlier intermediates are thought to arise during an initial template amplification stage which produces circular monomers (Ben-Porat and Tokazewski, 1977).

Electron microscopic studies of the latter intermediates have described their appearance as "large tangled masses" (Ben-Porat and Rixon, 1979). This observation, along with their high sedimentation rate and lack of genomic termini suggest that during later stages of infection viral DNA replication occurs by a rolling-

circle mechanism generating concatemers consisting of tandem head-to-tail repeats of the viral genome (Jacob et al., 1979). Further evidence for this mechanism has been provided by an in vitro HSV-1 DNA replication system in which the seven replication proteins purified from HSV-infected cells were used to reconstitute viral DNA synthesis on a circular template with a pre-formed replication fork. Electron microscopy and alkaline agarose gel electrophoresis demonstrated that DNA replication in this system occurs via a rolling circle-type mechanism (Rabkin and Hanlon, 1990).

It is, however, possible that the rapidly sedimenting DNA synthesised late in infection may arise by other mechanisms. For example newly synthesised circular daughter molecules may remain linked after each round of replication (Jongeneel and Bachenheimer, 1981), or homologous recombination may occur between replicating genomes. It is known that HSV-1 exhibits a high frequency of recombination during infection (Schaffer et al., 1974; Honess et al., 1980; Weber et al., 1988).

During DNA replication, the L and S segments of the viral genome invert relative to one another producing four different isomers which are functionally equivalent and are present in equimolar amounts. Although the presence of the viral "a" sequence is thought to be essential for segment inversion, inversion has also been observed between inverted copies of other sequences in different regions of the genome (Pogue-Geile et al., 1985; Jenkins et al., 1985; Varmuza and Smiley, 1985; Weber et al., 1987; 1988). The only viral proteins required for homologous recombination between replicating DNA molecules are the seven proteins directly required for viral DNA synthesis (see Section 1.3; Weber et al., 1988). It is possible that replicating viral DNA is a highly recombinogenic substrate for host enzymes.

1.2.5 HSV-1 DNA Processing and Assembly of Virions

Following replication of the HSV genome, the large concatemeric DNA molecules must be processed into unit

length genomic molecules, probably by site-specific cleavage, before they can be packaged into viral capsids.

The HSV "a" sequence, present at both termini of the viral genome has been shown to contain the cis-acting elements required for site specific cleavage and packaging (Vlazny and Frenkel, 1981; Mocarski and Roizman, 1982; Stow et al., 1983; Spaete and Mocarski, 1985). Insertion of an additional "a" sequence within the viral genome resulted in the production of a novel terminus at that position (Smiley et al., 1981; Mocarski and Roizman, 1982a;b; Varmuza and Smiley, 1985), and plasmid DNA containing an "a" sequence along with an HSV-1 origin of replication could be packaged and propagated as a defective genome in the presence of a helper virus (Stow et al., 1983; 1986; Deiss and Frenkel, 1986).

The structure of the HSV-1 "a" sequence is illustrated in **Figure 3.**

A direct repeat of 17-21 bp (DR_1) is present at each end of the "a" sequence with a further two direct repeats DR_2 and DR_4, varying in copy number, located internally. Two unique regions, U_b and U_c, proximal to the repeat sequences b (R_L) and c (R_S), lie between the DR1 and internal repeat regions (Davison and Wilkie, 1981; Mocarski and Roizman, 1981).

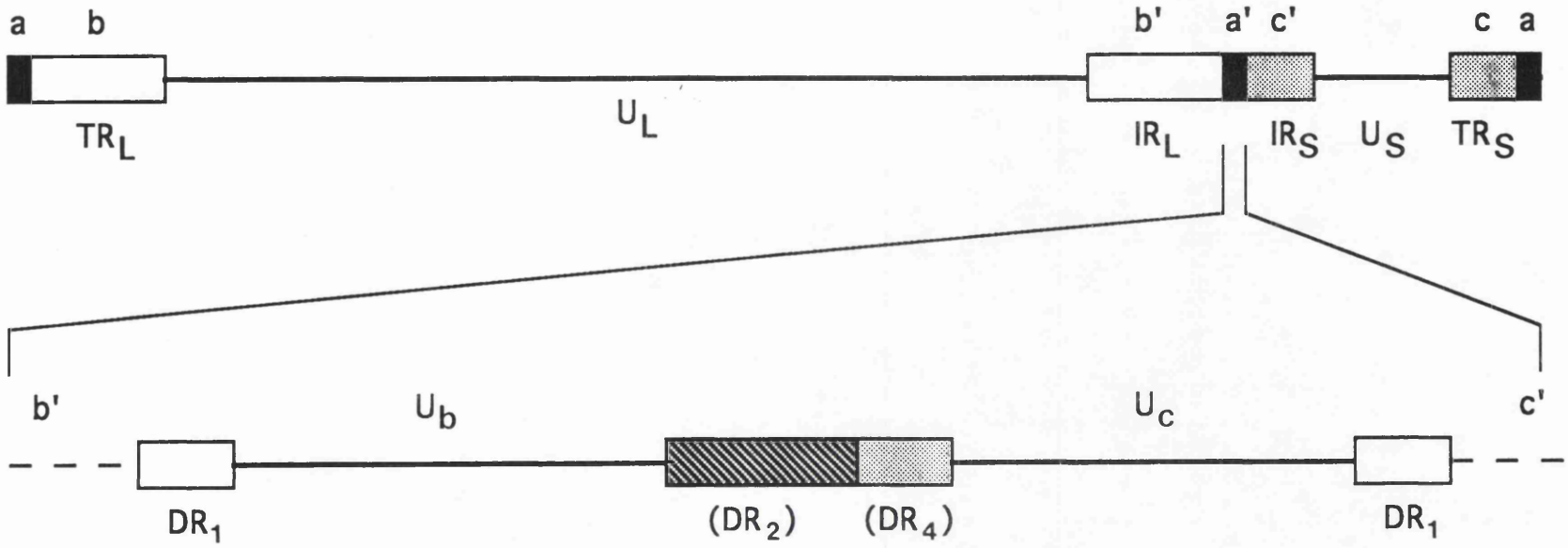
Although the nucleotide sequence of the "a" element varies between different virus strains, two highly conserved regions termed pac-1 and pac-2 are present in the Ub and Uc regions respectively and both appear to be essential for the cleavage/packaging process (Varmuza and Smiley, 1985; Deiss et al., 1986).

The DNA cleavage and packaging events are closely coupled and although the exact nature of events is not known, several models have been proposed (Ladin et al., 1980; Vlazny et al., 1982; Deiss and Frenkel, 1986; Addison, 1986).

The DR_1 elements at the L and S termini of the viral genome, unlike their internal counterparts, are incomplete in sequence. Circularisation of the linear genome however results in the generation of a novel joint

Figure 3. Organisation of the HSV-1 "a" Sequence

The organisation of the "a" sequence from the L - S junction (a') is shown. A direct repeat of 17 - 21 bp DR_1, (represented by open boxes) is present at each end of the sequence. Two separate repeat elements, DR_2 and DR_4, are located internally. DR_2 (stripped box) consists of 1 - 22 copies of a 12 bp direct repeat and DR_4 (shaded box) represents a directly repeated 37 bp element present in 0 - 3 copies. Two unique regions U_b and U_c (solid lines), proximal to the IR_L and IR_S repeat sequences (b' and c'), lie between DR_1 and DR_2/DR_4.


a
b
b'
a'
c'
c
a
TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$
b'
U$_b$
U$_c$
c'
DR$_1$
(DR$_2$)
(DR$_4$)
DR$_1$

fragment, in which tandem "a" sequences share a DR1 element. Nasseri and Mocarski (1988) have shown that a 179 bp fragment from across this a-a junction contains the cleavage signal. It has been proposed that during replication a putative cleavage/packaging protein complex recognises and cleaves an a-a junction within the concatemeric molecules of viral DNA only when it occurs in a particular orientation. Cleavage at the next similarly orientated junction will therefore generate a unit length genome with an "a" sequence at both termini. Cleavage of the viral DNA at an "a" sequence is thought to initiate the packaging of unit genome lengths by enabling insertion of a free terminus into a pre-formed capsid. The DNA may then be continuously packaged into this structure until the next identically orientated "a" sequence is recognised and cleaved.

Once a full length genome has been packaged, the capsid aquires an envelope by budding through the nuclear membrane which is a rich source of virally encoded glycoproteins (Roizman and Furlong, 1974). From the nucleus the virus particles are then transported to the cytoplasmic membrane via the golgi apparatus and are released from the cell as mature virions.

Studies on DNA encapsidation have been aided by the isolation and characterisation of ts mutants which replicate their DNA but are defective in its processing and packaging. ts lesions in such mutants have been mapped to within the UL6, UL12, UL26, UL28 and UL32 genes suggesting that encapsidation is dependent upon several viral gene products (Weller et al., 1983; 1987; 1990; Preston et al., 1983; Matz et al., 1983; Sacks and Schaffer, 1987; Rixon et al., 1988; Anderson et al., 1990). It is however unclear what particular role these proteins play in viral DNA processing and packaging.

1.3 ELEMENTS REQUIRED FOR HSV-1 DNA REPLICATION

1.3.1 Elements Required in cis

The presence of specific viral DNA replication

origins was first inferred from early electron microscopy studies of replicating HSV-1 DNA which revealed the presence of replication bubbles at particular regions within the genome (Friedman et al., 1977). Indirect evidence for the existence of two viral replication origins was obtained from studies with defective genomes generated during the serial passage of viral stocks at a high moi (Frenkel et al., 1975).

Defective viral genomes, consisting of tandemly repeated copies of small segments of viral DNA were classified into two groups, class I and class II, according to the origin of these sequences (Frenkel et al., 1976; 1980; Schroder et al., 1975; 1976; Graham et al., 1978; Locker and Frenkel, 1979).

Class I defective genomes were found to contain segments from the S region (Frenkel et al., 1975; 1976; Locker and Frenkel, 1979; Kaerner et al., 1979; 1981) while class II contained segments from near the centre of U_L (Schroder et al., 1975; Kaerner et al., 1979; Frenkel et al., 1980; Locker et al., 1982; Spaete and Frenkel, 1982). These observations thus indicated the existence of separate replication origins within the L and S segments (Vlazny and Frenkel, 1981). The two origins were subsequently referred to as ori_S and ori_L.

(a) Analysis of Ori_S

Fragments of DNA from the S region of the wt HSV-1 genome were cloned into plasmids and analysed in transfection assays either in the absence or presence of superinfecting wt helper virus (Stow, 1982). Fragments containing functional viral replication origins were identified by their ability to enable amplification of the linked plasmid vector sequence when helper functions were provided by the superinfecting virus. An origin was identified within a 995 bp fragment which mapped entirely within the repeat unit R_S. Deletion analysis of this ori_S-containing fragment defined a 90 bp region containing all the necessary cis-acting signals required for the initiation of HSV-1 DNA synthesis (Stow, 1982;

Stow and McMonagle, 1983). Because of its location within the viral R_S region, the HSV-1 genome contains two identical copies of ori_S.

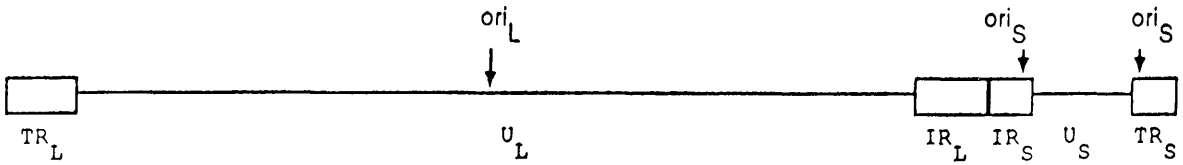
Ori_S lies between the 5' ends of two divergently transcribed IE genes (IE-3 in R_S and US1 or US12 at either side of U_S). Although this region has been proposed to contain a transcribed ORF, the status and possible role in DNA replication of this transcript remains unclear (Hubenthal-Voss et al., 1987; Hubenthal-Voss and Roizman, 1988).

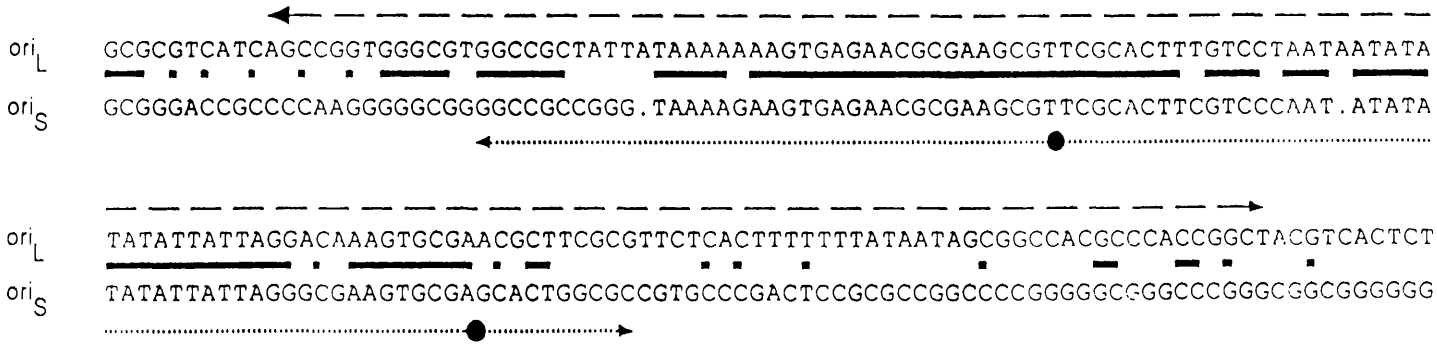
The 90 bp core ori_S sequence contains an almost perfect 45 bp palindrome with an 18 bp tract containing only A and T residues at its centre (Stow and McMonagle, 1983), (**Figure 4.**). Deletion mapping of the highly homologous HSV-2 ori_S (Lockshon and Galloway, 1986) defined a 75 bp minimal origin also containing a palindrome. The importance of this palindromic sequence in both HSV-1 and HSV-2 was demonstrated when disruption of the region with insertions and deletions resulted in a loss of origin activity (Stow, 1985; Lockshon and Galloway, 1988). Although a conflicting report by Deb and Doelberg, (1988), suggested that the entire right arm of the palindrome was dispensable for origin function, subsequent studies investigating the role of protein binding sites within the origin provided no support for this assertion (Weir and Stow, 1990).

Plasmid amplification studies with ori_S-containing fragments have shown that regions flanking the palindrome enhance DNA replication (Stow, 1982; Stow and McMonagle, 1983; Lockshon and Galloway, 1988; Wong and Schaffer, 1991). Indeed in one study deletion of the flanking sequences containing IE gene transcriptional regulatory elements resulted in an 80 fold reduction in DNA replication (Wong and Schaffer, 1991). As a result, it has been proposed that the HSV-1 ori_S region, like other eukaryotic origins, may consist of two functional components; a core region and an auxiliary component containing enhancer or promoter sequences (DePamphilis, 1988). Binding of transcription factors to the auxiliary

Figure 4. Comparison of Ori_L and Ori_S Sequences

The positions of the HSV-1 origins of replication (ori_L and ori_S) within the viral genome are shown schematically in the upper part of the figure. Below, the sequences of ori_L and ori_S have been aligned and the solid bars represent conserved bases. Above the ori_L sequence the limits of the 144 bp palindrome are indicated by the dashed arrows. The dotted lines below the ori_S sequence show the limits of the minimal ori_S as defined by Stow and McMonagle, (1983) and the closed circles indicate the limits of the ori_S palindrome.


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


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

sequences may directly enhance DNA synthesis (Wong and Schaffer, 1991) or, as observed at the E.coli origin, transcription initiated near ori_S may facilitate duplex melting (Kornberg, 1988).

The ori_S of VZV shares some homology with HSV-1 ori_S. It too contains a palindrome of similar length although the central AT-rich tract is longer. To the left of the VZV palindrome lies an 11 bp sequence which is identical to a segment on the left arm of the HSV-1 palindrome. The observation that HSV-1 trans-acting functions can amplify plasmids containing the VZV origin suggested that the 11 bp conserved region may be part of a sequence involved in origin recognition (Stow and Davison, 1986), (See also Section 1.3.2d.

(b) Analysis of Ori_L

Analysis of sequences comprising the HSV-1 ori_L region has proved to be difficult mainly because of the tendency of this region to suffer deletions when cloned into bacterial hosts (Spaete and Frenkel, 1982; Gray and Kaerner, 1984; Weller et al., 1985). The region was first sequenced from virion DNA (Gray and Kaerner, 1984; Quinn and McGeoch, 1985). Weller et al, (1985), subsequently cloned a 425 bp fragment containing the deletion prone sequences into a yeast vector and showed that it exhibited origin function in a plasmid amplification assay when HSV-1 helper functions were supplied in trans. DNA sequence analysis of the ori_L region revealed the presence of a 144 bp perfect palindrome with striking homology (85%) to the region covering the ori_S palindrome and extending approximately 40 bp to one side (Quinn and McGeoch, 1985; Weller et al., 1985), (**Figure 4.**). The presence of a long perfect palindromic sequence probably accounts for the instability of ori_L in bacterial plasmids (Collins, 1980). The essential role of the palindromic sequence in origin function was again demonstrated when deletions from the centre resulted in loss of activity (Weller et al., 1985). In the sequence of Gray and Kaerner, (1984), two tandem copies of the

It should be noted that in EHV-1, the ori_L is not located in a equivalent position to its HSV-1 counterpart and also that VZV lacks an ori_L altogether.

long palindrome were present within the ori_L region.

The ori_L sequence lies within an untranscribed region of the HSV-1 genome between two divergently transcribed genes, UL29 and UL30. Interestingly, these genes encode the major DNA binding protein and the DNA polymerase respectively, both of which are essential for HSV-1 DNA replication.

Ori_L of HSV-2 has also been cloned and sequenced. A 136 bp almost perfect palindrome is present in this region which exhibits 88% identity to HSV-1 ori_L (Lockshon and Galloway, 1986).

The significance of three origins of replication within the HSV-1 genome is unclear. Deletion mutants containing only a single copy of ori_S have been isolated and are unimpaired in their growth in tissue culture (Longnecker and Roizman, 1986). The isolation of a viable deletion mutant lacking 150 bp of ori_L demonstrates that this origin is dispensable for viral replication (Polvino-Bodnar et al., 1987). Indeed VZV does not posses an equivalent of ori_L, only two copies of ori_S (Davison and Scott, 1986; Stow and Davison, 1986). Finally, viruses lacking ori_L but containing two partially defective copies of ori_S have been constructed which exhibit a significant growth defect (Challberg, 1991). An analogous mutant virus containing the wt ori_L however showed no defect. Ori_S and ori_L therefore appear to be functionally equivalent, and wt growth seems to require only two (or perhaps even one) functional origin.

1.3.2 Trans-acting Functions Essential for HSV-1 DNA Synthesis

An important initial approach to the identification of genes encoding trans-acting functions required for HSV-1 DNA synthesis was the isolation and characterisation of mutants exhibiting defects in DNA synthesis (Schaffer et al., 1987). Analysis of ts and null mutants defective in DNA synthesis lead to the identification of seven distinct genes which encode products essential for HSV-1 DNA replication.

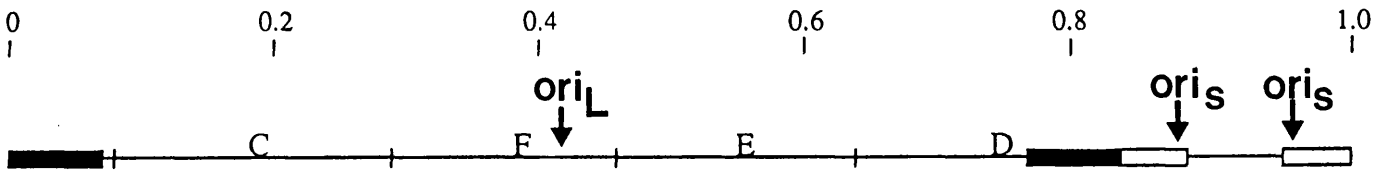
A separate approach, using a transient complementation assay involved testing the ability of cloned HSV-1 genomic fragments to amplify a co-transfected HSV-1 origin-containing plasmid (Challberg, 1986; Wu et al., 1988). Initially a set of five plasmids covering most of the HSV-1 genome was shown to support replication of an origin-containing plasmid. Subsequently a combination of sub-cloning, deletion analysis and restriction enzyme digestion of these fragments, in conjunction with HSV-1 sequence data (McGeoch et al., 1988) led to the identification of seven HSV-1 genes, UL5, UL8, UL9, UL29, UL30, UL42 and UL52, which were both necessary and sufficient for origin dependent plasmid amplification (Wu et al., 1988). The locations of the seven genes within the HSV-1 genome are shown in **Figure 5.** The IE genes IE-1, IE-2 and IE-3 were also identified as being important for DNA replication in this assay. Although none of the IE genes was absolutely essential, various combinations were necessary for plasmid amplification. This presumably reflects their role in turning on the other seven genes which belong to the E class. (Wu et al., 1988; Heilbronn and Zur Hausen, 1989).

Detailed mapping of the lesions within the DNA negative ts mutants described above demonstrated that the mutations lay within the same seven genes identified in the transient transfection assay (Dixon and Schaffer, 1980; Purifoy and Powell, 1981, Littler et al., 1983; Coen et al., 1984; Weller et al., 1987; Zhu and Weller, 1988; Marchetti et al., 1988).

The identical results obtained from the two approaches suggest that all the HSV-1 genes essential for viral DNA synthesis have been identified. Indeed the extent of plasmid amplification demonstrated in transfection assays using individual genes was only slightly lower than that observed when fragments representing most of the viral genome were used (Challberg, 1986). It remains possible that other functions which are not considered to play a significant role in DNA replication in tissue culture may actually

Figure 5. Location of DNA Replication Functions on the Herpes Simplex Virus Genome

The HSV-1 genome is shown schematically with the unique regions drawn as thin lines and the inverted repeats flanking the long and short unique regions as closed and open boxes respectively. The positions and orientation of the ORFs encoding the seven proteins essential for viral DNA replication are shown by arrows and the predicted molecular size (in kilodaltons) of each protein indicated below. The origins of replication ori_S and ori_L are also indicated, (adapted from Olivo and Challberg, 1989, Functional analysis of the herpes simplex virus gene products involved in DNA replication. In: Herpesvirus transcription and its regulation, Ed. E.K.Wagner.).



	UL5	UL8	UL9	dbp	pol	UL42	UL52
ORF Size (kdal)	99	80	94	130	136	51	114

play an essential role *in vivo* (Field and Wildy, 1978; Cameron *et al.*, 1988; Goldstein and Weller, 1988a;b).

(a) DNA Polymerase Holoenzyme (encoded by genes UL30 and UL42)

A novel DNA polymerase activity biochemically distinct from the host DNA polymerase was first identified in HSV-1 infected cell extracts by Keir and Gold, (1963). Genetic analysis of *ts* and drug resistant mutants demonstrated that this polymerase activity was virally encoded and essential for viral DNA synthesis (Hay and Subak-Sharpe, 1976; Purifoy *et al.*, 1977; Honess and Watson, 1977; Knipe *et al.*, 1979; Dixon and Schaffer, 1980; Purifoy and Powell, 1981; Coen *et al.*, 1982; 1984).

The DNA polymerase activity was mapped to the UL30 gene using marker rescue analysis (Chartrand *et al.*, 1980; Coen *et al.*,1984) and the ORF predicted to encode a polypeptide of approximately 136 kDa (Gibbs *et al.*, 1985; Quinn and McGeoch *et al.*, 1985). purification of the polymerase demonstrated the presence of a polypeptide of 140 kDa (Powell and Purifoy, 1977; Knopf, 1979; O'Donnell *et al.*, 1987a).

The production of viral DNA polymerase activity from the UL30 gene using *in vitro* transcription and translation, (Dorsky and Crumpacker, 1988), yeast expression systems (Haffey *et al.*, 1988) and recombinant baculovirus expression (Marcy *et al.*, 1990) indicated that the UL30 protein is sufficient for catalysis.

Biochemical studies of the viral polymerase demonstrated that the UL30 protein is associated with several distinct functions. In addition to the DNA polymerase activity, the protein also exhibits an intrinsic proof-reading 3'-5' exonuclease activity (Knopf, 1979; Ostrander and Cheng, 1980, Marcy *et al.*, 1990), and a 5'-3' exonuclease/RNaseH activity which specifically degrades RNA/DNA hybrids or duplex DNA in the 5'-3' direction (Crute and Lehman, 1989; Marcy *et al.*, 1990). This is likely to be analogous to the 5'-3' exonuclease function of *E.coli* DNA pol I required during

DNA repair and removal of RNA primers (Kornberg, 1980).

Several mutants have been isolated which exhibit resistance to a variety of anti-viral drugs and have alterations mapping within the UL30 gene (Hay and Subak-Sharpe, 1976; Crumpacker et al., 1980; Coen et al., 1982; Honess et al., 1984). These drugs, e.g. acyclovir, aphidicolin, phosphonoacetic acid and phosphonoformic acid, exert their effects by mimicking and/or competing with natural dNTPs or pyrophosphate. Mapping of the mutations localised them to four highly conserved regions within the C-terminal half of the DNA polymerase protein proposed to constitute a nucleotide binding site (Gibbs et al., 1985; 1987; Larder et al., 1987; Tsurumi et al., 1987; Wong et al., 1988).

Many DNA dependent DNA polymerases exist as a complex with one or more non-covalently linked proteins, the presence of which is required to increase the efficiency or modify the activity of the enzyme. The HSV-2 DNA polymerase co-purifies with a 50-60 kDa polypeptide (Powell and Purifoy, 1977; Vaughan et al., 1985). An equivalent protein has also been identified which co-purifies with the HSV-1 DNA polymerase and has been shown to be the product of gene UL42.

The UL42 gene encodes a 65 kDa phospho-protein which was originally observed as a double-stranded DNA binding activity in HSV-1 infected cells (Bayliss et al., 1975; Marsden et al., Parris et al., 1988; Gallo et al., 1988).

Antisera generated against the UL42 gene product were shown to precipitate not only the UL42 protein but also the UL30 polypeptide from HSV-1 infected cells indicating the presence of a complex between the two (Gallo et al., 1988; Parris et al., 1988). The proposal that the DNA polymerase holoenzyme exists as a heterodimer consisting of subunits encoded by UL30 and UL42 has been substantiated by results obtained from sedimentation and filtration analysis of highly purified preparations of the enzyme (Gottlieb et al., 1990).

Although the UL42 protein is not required for the catalytic activity of the DNA polymerase, it does appear

to have a 4 - 10 fold specific stimulatory effect on polymerase activity. This effect can be blocked by the addition of a 65 kDa DNA binding protein (DBP) monoclonal antibody suggesting a specific role for the protein as an accessory subunit of the DNA polymerase (Gallo et al., 1989). The UL42 gene product has been proposed to accomplish the stimulatory effect by enhancing the DNA polymerase's affinity for primer termini thereby increasing its processivity (Gottlieb et al., 1990; Hernandez and Lehman, 1990). Indeed a comparison of the catalytic properties of baculovirus-expressed UL30/UL42 protein complex with that of the individual subunits demonstrated that the inclusion of the UL42 protein results in processive polymerisation and the generation of full length products on a singly primed M13 ss-DNA template. In constrast, UL30 alone synthesises only short products using this template.

The region of the UL30 gene product which interacts with the UL42 protein has been mapped using in vitro transcription/translation and immuno-precipitation experiments to the C-terminal 208 amino acids. This region is distinct from those involved in substrate binding (Digard and Coen, 1990).

It is not known whether the uncomplexed UL42 protein has a specific role in viral DNA synthesis, and the significance of its ds-DNA binding activity during replication is still unclear. Like other DNA polymerase accessory proteins the UL42 gene product may function as a tight "clamp" to hold the UL30 enzyme and its DNA template together thereby reducing the possibility of dissociation (Mace and Alberts, 1984; Huber et al., 1987a;b).

(b) Major ss DNA-Binding Protein (encoded by gene UL29)

The major single-stranded DNA-binding protein (mDBP) was first recognised as an abundant major viral-induced protein of 130 kDa in HSV-1 infected cells (Honess and Roizman, 1973; Powell and Courtney, 1975). Analysis of ts mutants later determined that this protein was the

product of the UL29 gene and that it was essential for viral DNA replication (Conley et al., 1981; Weller et al., 1983; Dixon et al., 1983; Godowski and Knipe, 1983; 1985; 1986; Leinbach et al., 1984, Holland et al., 1984).

mDBP binds preferentially and cooperatively to ss-DNA templates with no detectable sequence specificity (Bayliss et al., Powell and Purifoy, 1976; Knipe et al., 1982; Ruyechan, 1983; Ruyechan and Weir, 1984). This observation suggested that the function of mDBP could be analogous to that of the E.coli ss-DNA binding protein (SSB) which binds to and stabilises regions of ss-DNA at the replication fork (Powell et al., 1981; Ruyechan, 1983; Ruyechan and Weir, 1984; Lee and Knipe, 1983). Powell et al, (1981) also reported that mDBP can reduce the melting temperature of parental duplex DNA in vitro thus suggesting a role in facilitating strand separation.

Analysis of UL29 ts mutants was carried out in an attempt to map the functional domains of the mDBP. Several mutants were found to specify products which were either defective in ss-DNA binding activity or defective in their localisation to the cell nucleus (Lee and Knipe, 1983; Leinbach, 1984; Quinlan et al., 1984; Gao et al., 1988; Gao and Knipe, 1989). Two regions of UL29 have been implicated in binding to ss DNA (Knipe, 1989; Leinbach and Heath, 1988; Gao et al., 1988; Wang and Hall, 1990). Both regions are conserved in the VZV and EBV mDBP homologues (Wang and Heath, 1990). The smaller region contains a predicted zinc-binding motif which is common to many DNA binding proteins (Berg, 1986). It has been suggested that this motif may participate in intra or intermolecular interactions which contribute to cooperative DNA-binding and/or to the stabilisation of the larger "core" DNA- binding region which is located near the C-terminus (Gao et al., 1988; Leinbach and Heath, 1988; Gao and Knipe, 1989; Wang and Hall, 1990). The presence of zinc however does not appear to be necessary for the mDBP's binding activity (Leinbach and Heath, 1988).

Attempts to map the nuclear localisation signals have

suggested that several regions of the protein may contribute (Gao and Knipe, 1989; Knipe, 1989). These signals appear to be distinct from those required for DNA binding as several ts mutants were isolated which either entered the nucleus but failed to bind ss-DNA, or retained their binding activity but failed to localise to the nucleus (Gao et al., 1988; Gao and Knipe, 1989; Knipe, 1989).

The identification of mutants which expressed mDBP which could bind DNA in vitro and localise to the nucleus in vivo yet failed to replicate DNA suggested that mDBP exhibits additional functions required for DNA synthesis (Gao et al., 1988; Gao and Knipe, 1989). These may include essential interactions with other viral or cellular factors involved in DNA replication or be connected with DNA duplex melting.

Other mDBP ts mutants tested had altered sensitivities to DNA polymerase inhibitors (Chiou et al., 1985) suggesting that mDBP may interact with the viral DNA polymerase and modulate its catalytic activity (Godowski and Knipe, 1985; 1986). Using activated DNA templates, Ruyechan and Weir, (1984), demonstrated that mDBP exhibited a small stimulatory effect on the activity of the viral UL30/UL42 DNA polymerse complex. A conflicting report from O'Donnell et al, (1987), however suggested that mDBP had the opposite effect and inhibited polymerase activity. More recent studies, in which the mDBP was shown to have a modest stimulatory effect on the action of the HSV-1 UL30 catalytic subunit (Gottlieb et al., 1990) and additionally on the polymerase holoenzyme (Hernandez and Lehman, 1990) appear to substantiate the former claim.

The use of monoclonal antibodies against mDBP has shown that the intranuclear distribution of mDBP throughout the viral replicative cycle is regulated in part, by the infected cell physiology and the state of viral DNA replication (Showalter et al., 1981; Knipe and Spang, 1982; Quinlan and Knipe, 1983; Quinlan et al., 1984; de Bruyn-Kops and Knipe, 1988).

In cells, in which viral DNA replication is blocked by the use of chemicals or ts mutants with lesions in the UL30 gene, mDBP localises to nuclear framework-associated structures called pre-replicative sites (Quinlan and Knipe, 1983; Quinlan et al,. 1984; deBruyn-Kops and Knipe, 1988). During viral DNA replication, however, mDBP is associated with replicating viral DNA and accumulates in structures referred to as replication compartments (Lee and Knipe, 1983; deBruyn-Kops and Knipe, 1988). Mutant viruses expressing altered mDBP failed to assemble the pre-replicative sites, suggesting that mDBP is involved in the organisation of these sites. Several cellular proteins are also re-localised to pre-replicative sites and replicative compartments in HSV-1 infected cells (deBruyn-Kops and Knipe, 1988; Bush et al., 1991; Wilcock and Lane, 1991).

Analysis of a mDBP mutant which significantly inhibited the replication of wt HSV-1 suggested that the protein may also play a role in the stimulation of late gene expression (Gao and Knipe, 1991).

(c) Helicase-Primase Complex (encoded by genes UL5, UL8 and UL52)

Crute et al., (1988), first described the induction of a novel DNA-dependent ATPase and helicase activity in HSV-1 infected cells. DNA-dependent ATPase activity is a known property of DNA helicases which function by coupling the unwinding of duplex DNA with the hydrolysis of ATP. The DNA helicase activity, which was purified to near homogeneity, contained three major polypeptides with molecular weights of 120 kDa, 97 kDa and 70 kDa. In addition to an ATPase and helicase activity, DNA-dependent GTPase and DNA primase activities were also found to co-purify with the three proteins (Crute et al., 1989).

The components of the HSV-1 helicase-primase complex were identified using specific antisera in Western blot assays as the products of the HSV-1 UL5, UL8 and UL52 genes (Crute et al., 1989). These proteins had already

been shown by Wu et al., (1988) to be essential for origin dependent DNA replication.

Attempts to reconstitute the enzyme activity in vitro from the individual components have not yet proved successful. However by triply infecting insect cells with three viruses expressing the UL5, UL8 and UL52 proteins, a functional HSV-1 helicase-primase complex was produced in vivo (Dodson et al., 1989; Calder and Stow., 1990). This complex exhibited identical enzymatic activities to the complex found in HSV-1 infected cells (Dodson et al., 1989).

Very little information is available regarding the functions of the individual subunits of the helicase-primase complex. Biochemical experiments have failed to detect any enzymatic activities in the individual UL5, UL8 or UL52 proteins (Calder and Stow., 1990; Dodson and Lehman., 1991).

The existence of a consensus ATP binding site within the UL5 gene suggests that this gene product may be involved in the ATPase and DNA helicase activities (McGeoch et al., 1988; Zhu and Weller, 1988). The ATP binding site represents two of the six sequence motifs present in the UL5 protein which are common to the members of a large superfamily of proteins which includes many DNA and RNA helicases (Gorbalenya et al., 1989). Results from mutational analysis of the most highly conserved regions of these helicase motifs demonstrated that all six are essential for UL5 function in a transient DNA replication assay (Zhu and Weller, 1992a). The ability of several UL5 mutants to form complexes with the other components of the helicase-primase complex suggested that the domains responsible for these protein/protein interactions are distinct from those required for helicase activity (Zhu and Weller, 1992a).

Although UL5 alone does not exhibit any enzyme activities, co-expression of the UL5 and UL52 gene products resulted in the formation of a complex which exhibited enzymatic properties virtually indistinguisable from the three subunit complex (Calder and Stow, 1990;

Dodson and Lehman, 1991). Since the UL8 protein appeared to be dispensable for the four known enzyme activities, its function was somewhat unclear. However recent intracellular localisation studies suggested that the UL8 protein may play a role in facilitating efficient nuclear uptake of the helicase-primase complex (Calder et al., 1992). In addition Sherman et al, (1992) recently demonstrated that although the UL5/UL52 complex can synthesise primers of the same size and at the same rate as the three subunit complex, the UL8 subunit is required for their efficient utilisation by DNA polymerase. Using a coupled polymerase/primase assay, M13 ss-DNA was converted to nicked or gapped ds-circles by either HSV-1 DNA polymerase holoenzyme or by E.coli polymerse I when the reaction was primed by the UL5/UL8/UL52 complex. Little or no elongation of primers was observed with either polymerase in the absence of the UL8 protein. DNA synthesis was restored by the addition of partially purified UL8 protein. On the basis of these observations, the UL8 protein has been proposed to function by increasing the efficiency of primer utilisation by stabilising the association between the primers and the template DNA (Sherman et al., 1992). This role for the UL8 protein may previously have been overlooked because poly dT was used as a template, and any primers dissociating from the template would have been able to re-anneal rapidly.

(d) Origin Binding Protein (encoded by gene UL9)

Using filter binding assays, Elias et al (1986), first detected a protein, in extracts from HSV-1 infected cells, which bound specifically to the HSV-1 origins of replication. This protein, found only in infected extracts, accumulated during viral infection at the same time as the viral-induced DNA polymerase and UL42 gene product suggesting that it may play an important part in the initiation of origin dependent DNA synthesis (Elias et al., 1986).

Identification of the gene encoding the origin

binding activity was achieved by expressing the genes required for viral DNA synthesis in insect cells using a baculovirus expression vector (Olivo et al., 1988) and also in BHK cells using a tsK vector (Weir et al., 1989) and testing the products for origin binding activity. Both approaches identified the UL9 gene as encoding the origin binding protein (OBP).

Experiments using rabbit antisera raised against synthetic decapeptides corresponding to the C-terminal seqences of four replication proteins (UL5, UL8, UL9 and UL52), confirmed this identification. Protein/DNA complexes formed between a labelled probe fragment containing the HSV-1 ori_S sequence and extracts from HSV-1 infected cells were immunoprecipitated only with the anti-UL9 antiserum (Olivo et al., 1988). Similarly the mobility of these complexes in a gel retardation assay was specifically reduced in the presence of UL9-specific antibody (Weir et al., 1989).

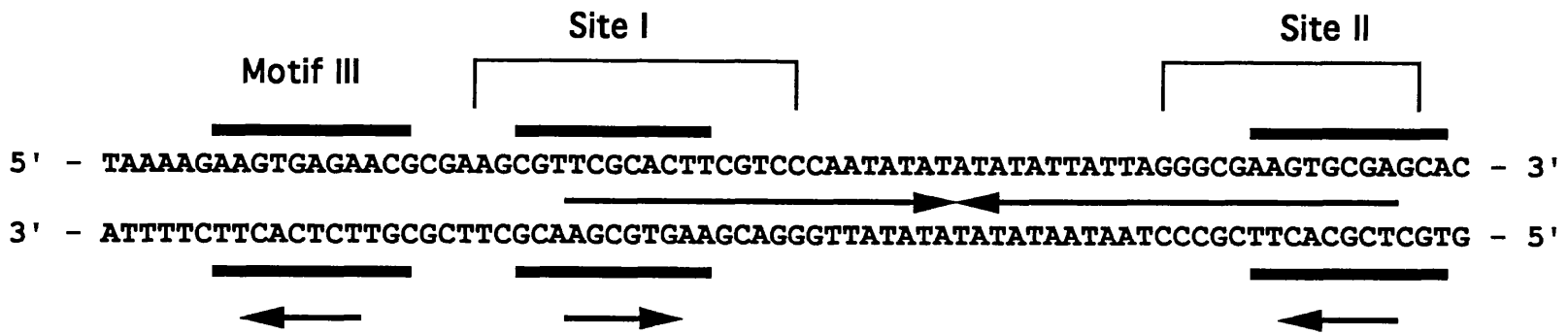
The size of the UL9 protein, predicted from DNA sequence analysis, is 94,246 (McGeoch et al., 1988b) and its apparent Mr based on SDS-PAGE gel mobility is 82 - 89 kDa (Elias and Lehman, 1988; Olivo et al., 1988; Calder, Ph.D thesis 1991). No modifications of the UL9 protein have been reported and experiments using C-terminal specific antisera suggest that the protein has an intact C-terminus (Olivo et al., 1989).

DNase 1 footprinting analysis, using purified UL9 protein, demonstrated that the protein specifically protects two sites within the HSV-1 ori_S region. Site I contains a 18 bp sequence which overlaps with the left end of the ori_S palindrome (**Figure 6**). Site II is identical in 15 out of the corresponding 18 bases to site I and is located on the right arm of the palindrome.

Within the 18 bp region of site I, lies an 11 bp sequence (5'-CGTTCGCACTT-3') which is conserved within ori_S of HSV-2 and VZV, and ori_L of HSV-1 and HSV-2 (Weller et al., 1985; Stow and Davison., 1986; Lockshon and Galloway., 1986; 1988). Two further, almost identical, copies of this sequence are present in HSV-1

Figure 6. Origin Binding Sites Within Ori_S

The 75 bp sequence of the HSV-1 minimal origin, as defined by Lockshon and Galloway, (1988), is shown opposite and the 45 bp near perfect palindrome indicated by the narrow arrows. The bracketed regions, labelled site I and site II, refer to sequences protected from DNase I digestion as reported by Elias and Lehman, (1988). The 11 bp conserved sequence present within site I, site II and motif III has been underlined and the orientation of each indicated by the arrows below.


Site I
Site II
Motif III
5' - TAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCAC - 3'
3' - ATTTTCTTCACTCTTGCGCTTCGCAAGCGTGAAGCAGGGTTATATATATATAATAATCCCGCTTCACGCTCGTG - 5'

ori_S, in inverted orientation to site I. One copy lies within binding site II and the other in a region outside the palindrome adjacent to site I (motif III). Methylation interference analysis, site directed mutagenesis and comparisons with other binding sites have indicated that an 8 bp sequence (5'-GTTCGCAC-3') within the 11 bp conserved region may represent the actual UL9 recognition sequence (Koff and Tegtmeyer., 1988; Deb and Deb., 1989). It has been proposed that the 8bp sequence corresponds to two, pentameric inverted repeats sharing a two base overlap (5'-GT(T/G)CG-3'), and that each pentamer may function as a UL9 protein recognition site. No direct evidence supporting this proposal exists, however it appears to be consistent with the observation that UL9 forms a stable dimer in solution (reviewed by Challberg and Kelly., 1989; Fierer and Challberg., 1992).

Binding studies using synthetic ds-oligonucleotides have demonstrated that UL9's affinity for an isolated site I is approximately 10x greater than for site II (Elias and Lehman., 1988). No binding to an isolated motif III (which differs from site I only at a single residue contacted by the UL9 protein) was observed.

The affinity of UL9 for site I within the origin was observed to decrease when binding site II was deleted suggesting that there may be cooperative interaction between proteins bound to the two sites. Further evidence for this came from studies which showed that the introduction of two point mutations within an isolated site I resulted in a 100 fold decrease in UL9 affinity whereas the same mutations introduced into a fragment containing the complete ori_S sequence produced only a two fold reduction in affinity for site I (Elias et al., 1990).

Mutational analyses of ori_S have also demonstrated a direct relationship between the ability of the UL9 protein to bind to site I and ori_S function (Deb and Deb., 1989; Weir and Stow., 1990). Binding of UL9 was abolished when the sequence within site I was changed

from GTTCGCAC to GTTCTCAC (Weir and Stow,. 1990). Koff and Tegtmeyer (1988) had previously reported that positions 4 and 5 within this recognition sequence form important contacts with OBP.

Deletion of site II resulted in a decrease in origin activity to about 5 - 10% of the wt level suggesting that two functional binding sites are required for efficient initiation of viral DNA replication (Lockshon and Galloway., 1988; Weir and Stow., 1990). A conflicting report by Deb and Doelberg (1988) which suggested that site II is not required for efficient origin activity is now considered to be erroneous. Deletion of motif III resulted in a 2 fold decrease in ori_S activity (Weir and Stow, 1990).

A series of mutant origins with varying numbers of AT dinucleotides inserted into the central AT-rich region of the ori_S, resulted in a cyclic change in origin efficiency (Lockshon and Galloway, 1988). Maximum ori_S activity was observed when integral numbers of helical turns of DNA were inserted, suggesting that UL9 molecules bound to sites I and II may have to be present on the same side of the DNA helix for efficient origin function. Thus, in addition to sequence specificity, the relative orientation of the binding sites may be important for efficient origin function.

Binding of UL9 to supercoiled origin-containing plasmids alters the structure of the origin without the requirement for ATP. Nuclease and chemical footprinting analyses have demonstrated that UL9 loops and distorts the AT-rich region between binding sites I and II when free energy is provided by positive supercoiling (Koff et al., 1991).

Nucleoprotein complexes consisting of purified UL9 and origin-containing DNA have been visualised by electron microscopy providing evidence for inter- and intramolecular interactions. The results suggest that following initial sequence-specific recognition multimers of UL9 protein may assemble at the replication origin (Rabkin and Hanlon, 1991).

Expression of various regions of the UL9 protein as fusion proteins in E.coli mapped the UL9 sequence-specific DNA binding domain to the C-terminal 317 amino acids of the protein (Weir et al., 1989). Subsequent studies have further narrowed the DNA binding domain to a 269 amino acid stretch amino acids 564 to 832). This region has been proposed to contain a pseudo-leucine zipper and a helix-turn-helix motif both of which are suggested to be involved in DNA binding (Deb and Deb, 1991).

Other functions are associated with the N-terminal two thirds of the UL9 protein. A consensus leucine zipper motif common to many DNA binding proteins and which is believed to be the site of protein/protein interactions involved in dimerisation is present at amino acids 150 - 173 (Landschulz et al., 1988). Studies of mutations which alter the leucines within the motif suggest that it too is essential for UL9 function (Martinez et al., 1992). Whether the zipper has a role in protein dimerisation or in the interaction with other components of the replicative machinery is not yet known.

The N-terminal portion of the UL9 protein also contains six sequence motifs found in all members of a superfamily of DNA and RNA helicases (Gorbalenya et al., 1988; 1989) distinct from the superfamily to which UL5 belongs (Zhu and Weller, 1992b). The importance of the helicase motifs in UL9 function was investigated using an in vivo complementation assay in which mutant UL9 proteins were tested for their ability to complement a replication deficient null mutant in the UL9 gene and allow amplification of a HSV-1 ori_S-containing plasmid. The introduction of site-specific mutations into the highly conserved regions of five of these motifs resulted in the loss of UL9 replicative function (Martinez et al., 1992) suggesting that UL9 helicase activity is likely to be essential for viral DNA replication.

Using partially ds substrates, an intrinsic 3′-5′ helicase activity was identified in the UL9 protein (Bruckner et al., 1991; Fierer and Challberg, 1992). This

suggests that UL9 may function analogously to SV40 T-antigen by specifically binding to the origin of replication and then using its intrinsic helicase activity to achieve local unwinding of the DNA duplex. This activity, however remains to be demonstrated (Fierer and Challberg, 1992).

The role of the UL9 protein in the initiation of viral DNA replication is thus still unclear. Nevertheless by analogy with other eukaryotic and prokaryotic systems, it seems likely that UL9 will be involved in the separation of the parental strands at the origin and the assembly of a multi-protein preinitiation complex leading to subsequent initiation of DNA synthesis (Elias et al., 1990).

Other Enzyme Activities Likely to be Required for HSV DNA Replication

In addition to the activation specified by the known virus-coded replication proteins it is likely that HSV DNA replication will require a topoisomerase activity to relieve torsional strain ahead of the replication fork and a DNA ligase activity to join Okazaki fragments following the removal of RNA primers. Whether these activities are provided by viral or host enzymes remains unknown.

1.3.3 Trans-acting Functions Indirectly Involved in HSV-1 DNA Synthesis

(a) Viral Enzymes Involved in Nucleotide Metabolism

Thymidine Kinase (encoded by gene UL23)

The HSV-1 encoded thymidine kinase (TK) was first described by Kit and Dubbs, (1963) and functions by phosphorylating thymidine, other pyrimidine deoxyribonucleosides and a variety of nucleoside analogues (Dubbs and Kit, 1964; Jamieson and Subak-Sharpe, 1974; Chen and Prusoff, 1978).

Studies using tk^- mutants indicated that TK is non-

essential for viral growth in dividing tissue culture cells as TTP is provided by host pathways (Dubbs and Kit, 1964). An impairment in growth was however observed in serum-starved cells indicating that the viral enzyme activity is essential in non-dividing cells (Jamieson et al., 1974; Field and Wildy, 1978).

Using a mouse model, several tk- mutants were shown to be unable to reactivate from a latent infection (Coen et al., 1989; Efstathiou et al., 1989). The identifiction of latency-associated transcripts in the ganglia from the infected mice, suggested that the virus encoded TK is not essential for the establishment of a latent infection in mice, but appears to be involved in the reactivation process (Coen et al., 1989; Tenser et al., 1989).

Ribonucleotide Reductase (encoded by genes UL39 and UL40)

Cohen, (1972) provided the first evidence demonstrating that HSV induced a novel RR activity. RR plays an important role in the formation of DNA precursors as it catalyses the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates (Cohen, 1972; Thelander and Reicard, 1979).

Biochemical analysis indicated that the viral-encoded RR consists of two non-identical subunits; a large 149 kDa subunit (RR_1), and a smaller 38 kDa subunit (RR_2) which are encoded by co-terminal transcripts from the UL39 and UL40 genes (McLauchlan and Clements, 1983; Bacchetti et al., 1984; Preston et al., 1984; Frame et al., 1985).

A lesion within the UL39 gene in mutant ts1207 was shown to impair viral growth at the NPT, which suggested that RR was essential for virus replication (Preston et al., 1984). Studies using two other UL39 mutants hrR3 and ICP6 however, indicated that the HSV-1 RR activity is dispensable for virus growth and DNA replication in dividing cells in culture at 34°C. RR does however appear to be essential for viral growth in non-dividing, serum-starved cells and also in cells at higher temperatures

(Goldstein and Weller, 1988a;b). These results suggest that host pathways involved in the synthesis of dNTPs may be inactivated in non-dividing cells and at elevated temperatures generating a requirement for the viral RR under such conditions. Indeed, it has been shown that cellular RR is not present in non-dividing and terminally differentiated cells (Thelander and Reichard, 1979).

Deoxyuridine 5′ triphosphatase (encoded by gene UL50)

Deoxyuridine 5′ triphosphatase (dUTPase) catalyses the hydrolysis of dUTP to dUMP and pyrophosphate (PPi), (Wohlrab and Francke, 1980; Caradonna and Cheng, 1981).

Studies on an HSV-1 mutant in which the dUTPase gene had been inactivated demonstrated that the viral enzyme is dispensable for viral growth in exponentially growing and serum-starved cells (Fisher and Preston, 1986).

dUTPase may play a role in providing a supply of dUMP for conversion into dTMP. However, because the enzyme is able to reduce the intracellular concentration of dUTP it has been suggested that its principal role is to prevent incorporation of uridine into nascent DNA.

(b) Other Enzyme Activities Induced in HSV-1 Infected Cells

Uracil-DNA Glycosylase (encoded by gene UL2)

Uracil-DNA glycosylase functions in a DNA repair mechanism by removing uracil residues from DNA which arise either from mis-incorporation of dUTP or from the deamination of cytosine residues in the DNA.

An HSV-1 encoded uracil-DNA glycosylase activity was first reported by Caradonna and Cheng, (1981). Analysis of an HSV-1 UL2 insertion mutant demonstrated that the viral enzyme is dispensable for viral growth in tissue culture (Mullaney et al., 1989).

Protein kinase

Blue and Stobbs, (1981) first reported a novel protein kinase activity in HSV-1 infected cells. Based on

It has been reported that autophosphorylation occurs in the N-terminal region of the HSV RR1 subunit.

amino acid sequence similarity with known eukaryotic kinases, the US3 protein was proposed to be a protein kinase (McGeoch and Davison, 1986). Biochemical and genetic studies have since confirmed this and demonstrated that the protein is dispensable for viral growth in tissue culture (Frame et al., 1987; Purves et al., 1987).

Subsequent studies have shown that the US3 encoded protein kinase phosphorylates a membrane protein encoded by the UL34 gene (Purves et al., 1991; 1992). Whether other substrates for the protein kinase exist remains to be determined, but it is possible that proteins indirectly or directly involved in DNA replication may be regulated by phosphorylation.

A second HSV induced protein kinase activity has been reported in infected cell nuclei. Recent work has shown that this activity, identified as the product of the UL13 gene is capable of autophosphorylation (Cunningham et al., 1992).

Alkaline Exonuclease (encoded by gene UL12)

Keir and Gold, (1963) first described an HSV-1 alkaline exonuclease activity in infected cells. This enzyme was later identified as the product of the UL12 gene (McGeoch et al., 1986; Draper et al., 1986) and consists of a single polypeptide which exhibits both 5'-3' exonuclease and endonuclease activities (Morrison and Keir, 1968; Hoffman and Cheng, 1979; Strobel-Fidler and Francke, 1980).

Early studies using UL12 ts mutants reported conflicting data concerning whether this enzyme is essential for viral growth (Moss et al., 1979; Francke and Garret, 1982; Moss, 1986). However, more recently, Weller et al, (1990), using an exonuclease null mutant, h ave provided definitive evidence that the exonuclease is essential for viral growth but not for viral DNA replication.

Interestingly, the enzyme appears to localise in replication compartments within the nucleus (Randall and

Dinwoodie, 1986) and has been shown to co-precipitate with the viral DNA polymerase (Vaughan et al., 1985).

Possible roles for the HSV-1 exonuclease have been proposed which include the cleavage of DNA concatemers, the provision of dNTPs for DNA synthesis and an involvement in recombination events (Weller et al., 1990).

1.4 INITIATION OF DNA REPLICATION

Detailed studies of DNA replication in relatively simple prokaryotic and eukaryotic organisms have proved invaluable in the understanding of the underlying mechanisms in other organisms. The development of origin-dependent in vitro DNA replication systems for E.coli (Fuller et al., 1981), bacteriophage lambda (Wold et al., 1982) and SV40 (Li and Kelly., 1984) provided evidence for a common series of events involved in the initiation of DNA replication: (i) the interaction of an origin binding protein with a specific sequence within the origin of replication results in the formation of an initial complex; (ii) the melting of a small stretch of DNA within the origin leads to an open complex (replication bubble); (iii) daughter strand synthesis is initiated and a helicase activity extends the replication bubble.

As described in Section 1.3.2(d), the events following the binding of UL9 to the HSV-1 origins that lead to the initiation of viral DNA replication are not fully understood. However, the discovery that UL9 exhibits functions similar to better-characterised DNA initiator proteins such as the E.coli DnaA protein, the lambda O protein and the SV40 T antigen, suggests that it may have a similar role to play in the initiation of viral DNA synthesis.

1.4.1 Initiation of E.coli DNA replication

E.coli DNA replication is initiated at a unique site within the chromosome called oriC. The minimal

region essential for replication, comprising all the necessary cis-acting sequences, encompasses 245 bp (Oka et al., 1980) and contains several sequence blocks which are highly conserved among the oriC regions of Enterobacteriaceae (Zyskind et al., 1983). Mutational analyses of this region revealed the presence of two types of sequence element which are essential for the initiation of DNA synthesis (**Figure 7**). Four 9-mer tandem repeats (dnaA boxes) are located towards one end of oriC (Oka et al., 1984) and function as specific recognition sites for the E.coli dnaA initiator protein (Fuller et al., 1984; Kaguni and Kornberg., 1984). Three tandem repeats of an AT-rich 13-mer (Left, Middle, Right) are found at the opposite edge of oriC (Oka et al., 1980) and have been proposed to contain the duplex opening sites for initiation of DNA synthesis.

The dnaA protein is a monomer of 52 kDa which binds ATP tightly and hydrolyses it very slowly (Sekimizu et al., 1987). Both the ATP- and ADP-bound forms of dnaA bind specifically to the consensus sequence 5′-TTAT(C/A)CA(C/A)A-3′ found within the four dnaA boxes of oriC and also in the control regions of several E.coli genes, including the dnaA gene itself (Zyskind and Smith., 1986; Fuller et al., 1984). DNase I footprinting studies identified a region protected by dnaA encompassing about 200 bp of the origin, including all but the three AT-rich 13mers (Fuller et al., 1984).

The formation of an initial dnaA-oriC complex (Fuller and Kornberg, 1983; Fuller et al., 1984), consisting of a core of about 20-40 dnaA monomers wrapped in supercoiled DNA (Fuller and Kornberg., 1983; Funnell et al., 1987; Baker and Kornberg., 1988), causes partial unwinding of the duplex to form an open complex (See **Figure 8**). This process requires ATP hydrolysis, carried out by the dnaA protein itself (Bramhill and Kornberg., 1988a,b), a negatively supercoiled template (Baker and Kornberg., 1988) and the presence of a histone-like DNA binding protein, HU (Dixon and Kornberg, 1983; Ogawa et al., 1985) which may stabilise a desirable bend in the DNA

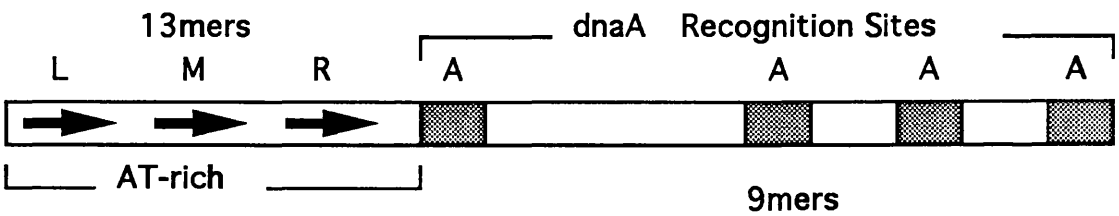
Figure 7. Replication Origins of E.coli, Bacteriophage Lambda and Simian virus 40

A schematic representation depicting the minimal E.coli origin, OriC, is shown in the top figure. It contains three 13mer tandem repeats (L [Left], M [Middle], R [Right]) indicated as solid arrows, within an AT-rich segment. The shaded boxes adjacent to this region represent four 9mer tandem repeat dnaA binding sites (dnaA boxes).

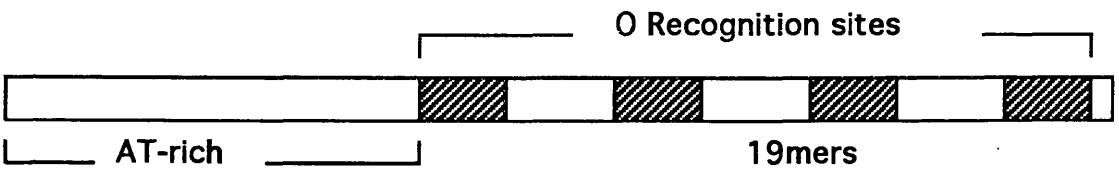
The Bacteriophage Lambda origin (centre figure) is schematically drawn and contains four nearly perfect 19mer repeats (shaded boxes) which bind the bacteriophage lambda O protein. Adjacent to this region lies a 49 bp AT-rich domain.

The SV40 origin (bottom figure) is schematically drawn opposite. The core origin contains three functionally distinct domains, the early palindrome, central domain and AT-rich domain, as indicated. The T antigen binding sites (GAGGC motifs) are represented by the small solid arrows and are located within the central domain and also within a region outside the core origin adjacent to the early palindrome.

E.coli oriC



Bacteriophage lambda origin



SV40 origin

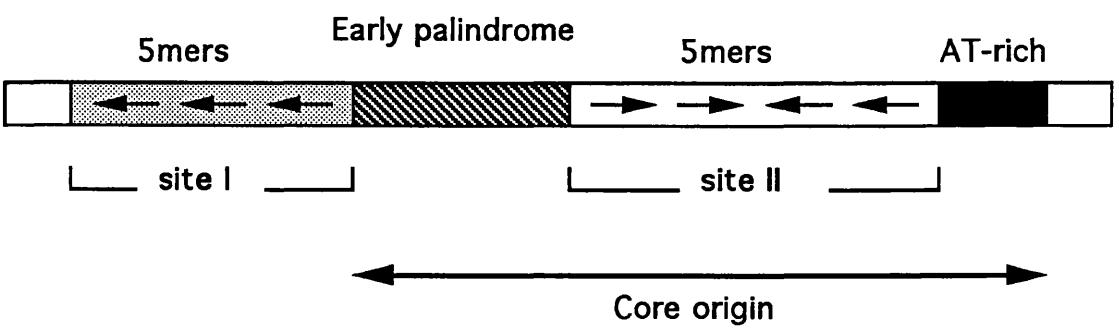
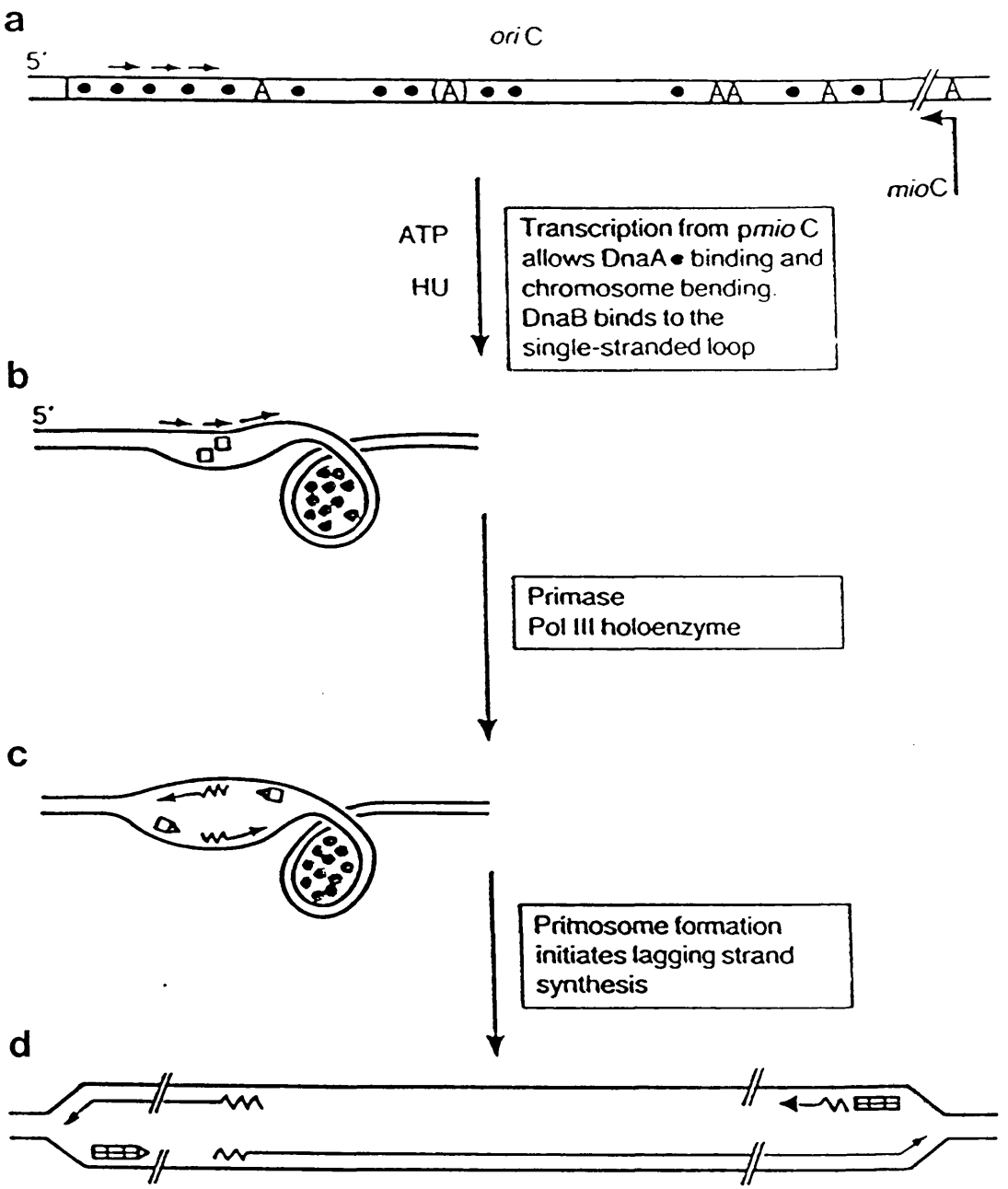


Figure 8. Initiation of E.coli DNA Replication

A schematic illustration of the E.coli origin of replication, OriC is shown in **Figure a.** DnaA binding sites (A) and the three AT-rich 13mer repeats (arrows) are indicated. Gene transcription from a promoter located close to the origin facilitates binding of dnaA which initiates duplex melting at the AT-rich repeats. In the presence of ATP and HU, dnaB molecules can bind to the ss DNA loop (**Figure b.**). Primase generates primers for the holopolymerase III thereby initiating bidirectional replication (**Figures c. and d.**), (reproduced from Adams, 1991, DNA replication, Ed. D.Rickwood.).


a
oriC
5'
mioC
ATP
HU
Transcription from pmioC allows DnaA• binding and chromosome bending. DnaB binds to the single-stranded loop
b
5'
Primase
Pol III holoenzyme
c
Primosome formation initiates lagging strand synthesis
d

(Skarstad et al., 1990). Regions of single-stranded DNA which are sensitive to single-strand specific endonuclease digestion have been detected at the three AT-rich 13-mers (Kowalski and Eddy, 1989), but the mechanism by which the dnaA protein/oriC complex forms the open complex is still unclear. One proposal involves a specific interaction between the dnaA protein and one or more of the 13-mers. This interaction has not, however, been detected by DNase I footprinting. The reduced stability of dnaA-oriC complexes on templates from which the 13-mers had been deleted (Yung and Kornberg, 1989), the ability of a synthetic 13-mer oligonucleotide to compete with dnaA binding to oriC, and the presence of 13-mer sequences near dnaA boxes in other origins (Bramhill and Kornberg, 1988) all suggest some specificity in the reaction between the 13-mers and dnaA. It has been suggested that only the right hand AT-rich region is recognised by the dnaA protein. Melting is initiated and unwinding of the DNA in the region of the non-contacted 13-mers ensues as a result of their high AT content conferring a reduced helical stability. This hypothesis is consistent with mutational studies which revealed that the right 13-mer is the only AT-rich element required for dnaA/HU opening of oriC (Bramhill and Kornberg., 1988), although all three were required for the formation of the pre-priming complex. Further evidence supporting this proposal is that the sequence of the right 13-mer and the spacing between it and the leftmost dnaA box is completely conserved in the origins of the enteric bacteria whereas the left and middle 13-mers tolerate base substitutions (Asada et al., 1982; Kowalski and Eddy., 1989).

The binding of dnaA to oriC allows the assembly of a multi-protein pre-priming complex in preparation for initiation of DNA synthesis (Baker et al., 1986; 1987; Funnell et al., 1987). Once an open complex is formed, the dnaB helicase, with the help of dnaC binds to both DNA strands in the single-stranded region. Although absolutely essential for complex assembly, the dnaC

protein inhibits the helicase activity of dnaB and must therefore be removed before replication can proceed (Kornberg and Baker, 1992). Electron microscopy has shown that the pre-priming intermediate is larger and less symmetrical than the initial complex and also encompasses the region containing the three 13-mer repeats (Funnell et al., 1987).

In addition to its role in opening the duplex evidence exists which suggests that dnaA also plays a direct role in the loading of dnaB onto the DNA. DnaB binds very poorly to ss-DNA and is normally unable to do so if single stranded DNA binding protein (SSB) is bound (Wahle et al., (1989). DnaB with the aid of dnaC can only utilise this DNA as a template when dnaA associates with the DNA prior to SSB addition. DnaC is always required for this process suggesting that protein-protein interactions between dnaA and either dnaB or dnaC (or a complex of both) may be essential for dnaB loading.

The helicase action of dnaB melts the DNA bi-directionally forming a replication bubble and providing the templates for initiation of DNA synthesis (Baker et al., 1987; Bramhill and Kornberg, 1988a;b). An interaction between dnaG primase and dnaB is thought to produce a mobile primosome which generates primers for both leading and lagging strand synthesis. The primosome moves with 5′-3′ polarity in the direction of the replication fork allowing multiple primings to take place during discontinuous synthesis (Kornberg and Baker, 1992). Primers are extended by DNA polymerase III on both the leading and lagging strands and replication proceeds bi-directionally from the origin (Baker et al., 1986; 1987).

DNA gyrase and SSB are also required during fork movement. Following initial strand separation and extention of the replication bubble, the displaced DNA strands are maintained in an extended conformation by SSB providing a suitable template for DNA primase and DNA polymerase activity (Kornberg, 1980; 1982; 1988).

Topoisomerase II (DNA gyrase) cuts and reseals the

duplex to release the torsional strain of positive supercoiling ahead of the advancing fork (Funnel et al., 1987; Baker, 1986; 1987).

The identification of dnaA boxes within the promoter of the dnaA gene as well as in other genes led to the suggestion that in addition to its role in initiation of DNA synthesis, dnaA may also play a part in regulating the transcription of its own and other genes. Indeed Schaefer and Messer, (1989) demonstrated that insertion of a dnaA box site into the middle of the dnaA ORF resulted in a transcriptional block of dnaA and downstream gene expression (Schaefer and Messer, 1989).

1.4.2 Initiation of Bacteriophage Lambda DNA replication

Following entry into E.coli, linear phage lambda DNA circularises and bidirectional replication is initiated from a single origin within the phage genome, oriλ (Schnos and Inman, 1970). Initiation of phage DNA replication resembles initiation at E.coli. oriC, except that the host proteins dnaA and dnaC are not required. Instead two virally-coded proteins, O and P, interact with oriλ and with other host replicative proteins to initiate DNA synthesis (Eisen et al., 1966; Ogawa and Tomizawa, 1968; Friedman et al., 1984).

Mutational analysis defined a region within the viral O gene which contained the essential sequences required for origin function. Two major characteristic domains were identified consisting of four nearly perfect repeats of 19 bp, and an adjacent 49 bp AT-rich segment (Schnos and Inman, 1970; Scherer, 1978). (See **Figure 7**).

The O protein may be regarded as the functional analogue of E.coli dnaA as it too recognises and binds to the origin. Nuclease and methylation protection analyses demonstrated that the O protein binds to the four 19 bp repeat elements (Tsurimoto and Matsubara, 1981; Zahn and Blattner, 1985). The internal symmetry of each element also suggests that the O protein may bind as a dimer (Zylicz et al., 1984).

Electron microscopy experiments indicated that the

formation of specialised nucleoprotein complexes at ori is responsible for localising the initiation of DNA synthesis (Dodson et al., 1985). Binding of O protein to the origin results in the formation of an initial nucleoprotein structure called the "O-some" whose condensed appearance under the electron microscope suggested that the DNA was bent or wrapped around the protein (Dodson et al., 1985; 1986; Zahn and Blattner, 1985).

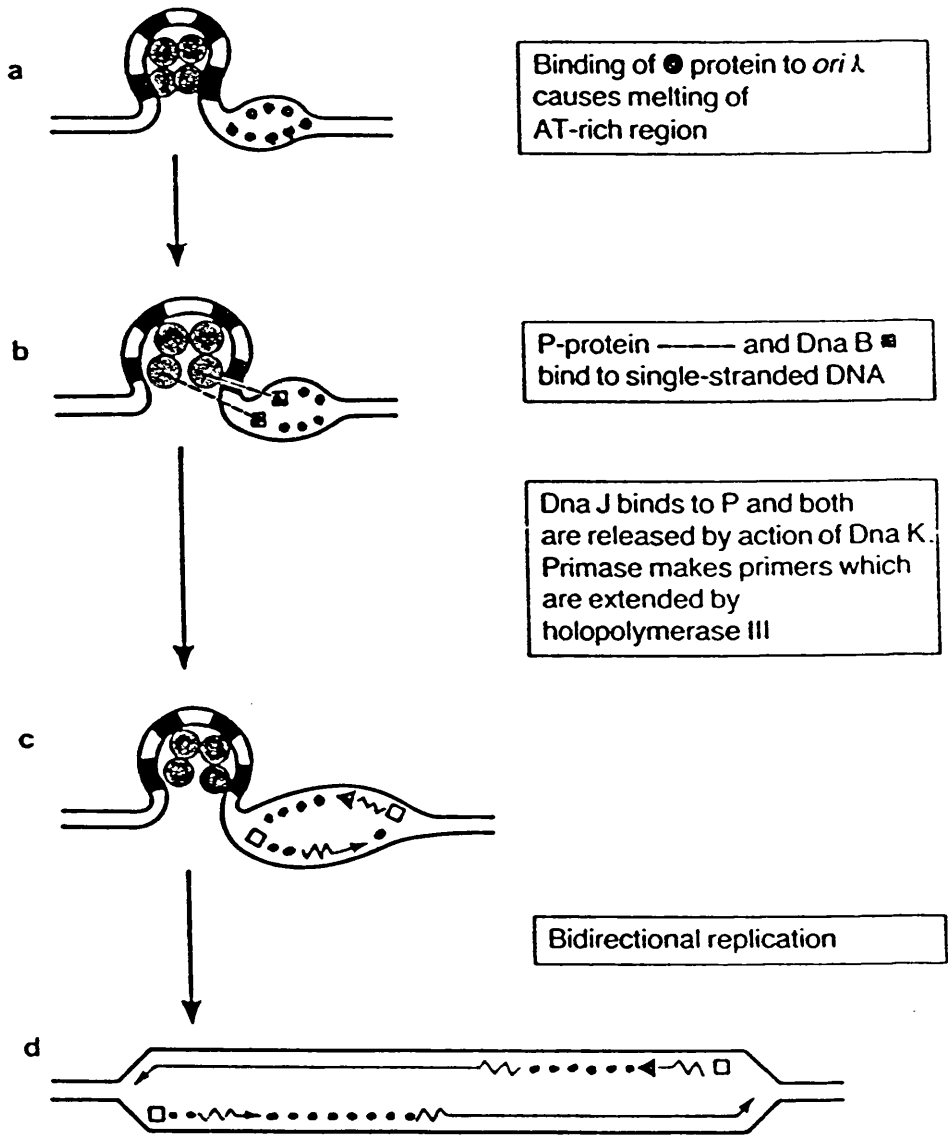
Once bound, the O protein initiates melting of the flanking AT-rich region, (demonstrated by an increased sensitivity to ss-specific nuclease digestion), forming an open complex into which the rest of the replicative machinery can enter (Schnos et al., 1988), (**Figure 9**). Unlike the E.coli dnaA/oriC complex, the lambda ori /O protein complex does not require the binding of ATP for helix de-stabilisation at the AT-rich region (Schnos et al., 1988). The mechanism by which the localised strand separation occurs has been suggested to be dependent on the tension arising from duplex supercoiling. How this energy or that arising from bending the origin is transferred into strand separation is still unclear (Schnos et al., 1988; Dodson et al., 1989).

Studies using hybrid phage indicated that the O protein origin binding domain lay within the N-terminus of the protein while the C-terminus was responsible for its interaction with the viral P protein (Furth et al., 1978). The P protein, an analogue of the E.coli dnaC protein, binds host dnaB and interacts with the "O-some", thus recruiting a helicase actvity to the origin for duplex unwinding (Dodson et al., 1986). The binding of the O protein to the origin acts as the catalyst for the unwinding reaction as the dnaB helicase activity can only function on pre-formed open complexes (LeBowitz and McMacken, 1986).

Initiation of DNA synthesis depends on these reactions and on the host dnaJ and dnaK proteins causing transition of the open complex into a form competent for localised activity of the dnaG primase and DNA polymerase

Figure 9. Initiation of Bacteriophage Lambda DNA Replication

A schematic drawing of the bacteriphage lambda origin of replication is illustrated in **Figure a.** The binding of O-protein (large circles) to the four 19mer repeat motifs (black boxes) initiates unwinding of the duplex at the AT-rich region within the origin. Duplex opening allows binding of P-protein (dashed line)/dnaB (squares) complexes which link the ss DNA region to the O-protein (**Figure b.**). Disassembly of the complex by dnaJ and dnaK allows the dnaB helicase function to extend the ss region (**Figure c.**). DnaG generates primers (zig-zag lines) for the holopolymerase III thereby initiating leading strand synthesis for bidirectional replication (**Figure d.**), (reproduced from Adams, 1991).


a
Binding of ● protein to ori λ causes melting of AT-rich region
b
P-protein ——— and Dna B ■ bind to single-stranded DNA
Dna J binds to P and both are released by action of Dna K. Primase makes primers which are extended by holopolymerase III
c
Bidirectional replication
d

III holoenzyme (Wold et al., 1982; Tsurimoto and Matsubara, 1982; LeBowitz and McMacken, 1984; LeBowitz et al., 1985). Subsequent replication requires the same proteins as E.coli oriC dependent DNA synthesis suggesting that identical replication forks are assembled (Mensa-Wilmot et al., 1989).

1.4.3 Initiation of SV40 DNA replication

The SV40 genome consists of a circular DNA duplex of approximately 5000 bp which complexes with histones forming nucleosomes which are indistinguishable from those of host cellular chromatin. DNA replication is initiated bi-directionally from a single origin, and, since SV40 encodes only a single replication protein, (T antigen), the virus relies almost entirely on the host cell replication machinery (Tegtmeyer, 1972; Stillman, 1989).

Deletion and substitution analysis of SV40 origin sequences revealed a 64 bp core origin containing all the elements required for initiation of DNA replication in vitro (Stillman et al., 1985; Deb et al., 1986a; Smale and Tjian, 1986; Dean et al., 1987a). Further mutational studies indicated that the core origin contained at least three functionally distinct domains (Deb et al., 1986a;b;c).

The central domain contains four pentanucleotide motifs (5'-G(A/G)GGC-3') arranged on the two arms of a 27 bp palindrome. These sequence elements are recognised by the viral initiation protein T antigen, and each one is bound by a single monomer (Tjian, 1978; DeLucia et al., 1983; Mastrangelo et al., 1985). Flanking the central domain lies an imperfect palindrome (early palindrome) of unknown function and a 17 bp AT-rich tract. Additional T antigen binding site sequences are also present to the left of the early palindrome.

The SV40 origin therefore exhibits some striking similarities to the origins of E.coli and bacteriophage lambda. All three genomes contain multiple binding sites, recognised by the relevant initiator proteins, with

adjacent AT-rich tracts.

Binding of the 95 kDa T antigen to the four pentameric repeats in the origin, in the presence of ATP, leads to the assembly of an organised nucleoprotein structure which is competent for replication initiation (Mastrangelo et al., 1985). This multimeric structure consists of a double T-antigen hexamer complex covering the entire core origin plus 12 bp on either side (Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988; Mastrangelo et al., 1989).

The presence of ATP induces specific conformational changes in the DNA at the early palindrome and AT-rich segments (Borowiec and Hurwitz, 1988) and the result is localised duplex melting within the former region (**Figure 10.**). This reaction does not require the hydrolysis of ATP (Dean et al., 1987; Borowiec and Hurwitz, 1988; Roberts, 1989).

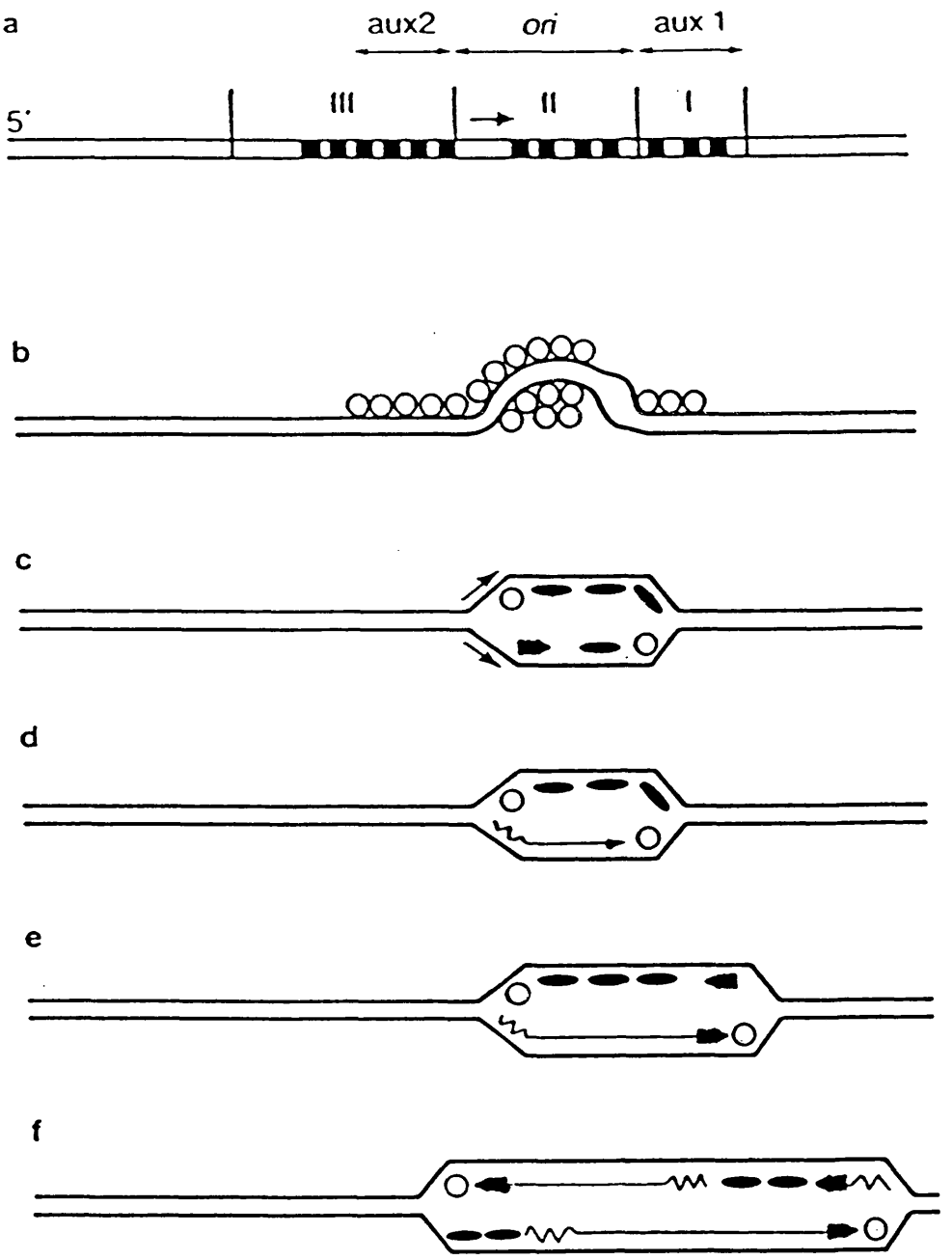
In addition to catalysing localised melting of duplex DNA, T-antigen, which exhibits an intrinsic helicase activity, has been shown to continue bi-directional DNA unwinding during the progression of the SV40 replication fork (Hurwitz, 1990; Wessel et al., 1992). This process requires the hydrolysis of ATP, and the presence of a cellular SSB (RF-A) and topoisomerase I (Dodson et al., 1987; Parsons et al., 1990). It has been suggested that the dodecamer complex acts at the centre of the replication fork and that the duplex DNA is threaded through the intact complex which then extrudes single-stranded DNA loops (Wessel et al., 1992).

The SV40 T-antigen does not bind tightly to ss DNA and the whole initiating complex would fall apart if it were not stabilised by the binding of other T-antigen molecules to auxiliary sites out with the core origin (Gutierrez et al., 1990). Changes in the phosphorylation status of T-antigen have been suggested to play a role in the binding and release of the protein (McVey et al., 1989).

Following the opening of the origin region, priming of both leading and lagging strand DNA synthesis is

Figure 10. Initiation of SV40 DNA Replication

The SV40 origin of replication and flanking regions are schematically outlined in **Figure a.** where the black boxes indicate the positions of T antigen (Tag) binding sites within the core origin (site II) and also within auxilary regions (sites I and III). The AT-rich segment within site II is also indicated by the arrow above. Tag (circles) binds firmly to site II (less firmly to other sites) and opens up the duplex at a region between sites I and II (**Figure b.**). Functioning as a helicase Tag, along with an SSB complex (ovals) opens up the whole origin and allows polymerase alpha/primase to bind (**Figure c.**) and initiate replication of the early template strand (**Figure d.**). Polymerase delta/PCNA complex takes over leading strand synthesis while polymerase alpha/primase initiates lagging strand synthesis (**Figure e.**) and bidirectional replication ensues (**Figure f.**), (reproduced from Adams, 1991).


a
aux2
ori
aux 1
5'
III
II
I
b
c
d
e
f

believed to be carried out by the DNA polymerase alpha/primase complex. Leading and lagging strand DNA synthesis is then catalysed by two mammalian DNA polymerases Pol delta and Pol alpha respectively (Prelich et al., 1987; Decker et al., Wold et al., 1988; Lee et al., 1989). PCNA is required as an auxiliary factor for Pol delta which allows highly processive synthesis of the leading strand. It is not clear whether T-antigen has a role in directing the Pol enzymes and other replication proteins to the origin region, although an association between T antigen and DNA pol alpha has been reported (Smale and Tjian, 1986; Donreiter et al., 1990).

1.5 Aims of the Work Presented in this Thesis

The previous sections describe several features that UL9 shares with the more intensely studied initiator proteins required for initiation of E.coli., phage lambda and SV40 DNA synthesis.

Understanding the precise role of UL9 in the initiation of HSV DNA synthesis will contribute to our overall knowledge of the mechanisms of viral DNA replication and hopefully also assist the development of an in vitro system for the study of HSV DNA replication.

The work presented in this thesis is concerned with the mechanism by which UL9 recognises and binds to the HSV origins. Weir et al. (1989) demonstrated that a protein A-UL9 fusion containing the C-terminal one-third of the UL9 protein contained all the information necessary for sequence-specific DNA binding activity. The aim of this study was to construct deletion and insertion mutants within this region and to study their interactions with the origin in an attempt to define the important regions of UL9 involved in the DNA/protein interaction. In addition, selected mutations were introduced into full length copies of the UL9 gene which were expressed using recombinant baculoviruses. These viruses were analysed in a transient viral origin-dependent replication assay in insect cells in which

amplification of an ori$_S$-containing plasmid is driven by superinfection with a mixture of seven recombinant baculoviruses which express the full complement of essential HSV-1 DNA replication proteins. By this means it was possible to assess the ability of the mutant UL9 proteins to participate in viral DNA replication.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and Reagents

The majority of chemicals were obtained from either **BDH CHEMICALS Ltd.**, or **SIGMA CHEMICALS Ltd** and were of analytical grade or better. Exceptions to this were : Tissue culture reagents were obtained from **GIBCO LIFE TECHNOLOGIES Ltd.**; Ammonium persulphate, TEMED (N,N,N'N tetramethylethylenediamine), gelatin, Bio-Rad protein assay dye reagent from **BIO-RAD LABS Ltd**; Ampicillin (penbritin) from **BEECHAM RESEARCH LABS Ltd.**; HPLC grade dimethyl-sulphoxide and formamide from **FLUKA CHEMICALS Ltd.**; Absolute alcohol and methanol from **HAYMEN Ltd.**; Glacial acetic acid, hydrochloric acid, chloroform and glycerol from **MAY and BAKER Ltd.** DEAE-sephacel and sequencing solutions from **PHARMACIA Ltd.**; Prestained Protein molecular weight markers from **STRATAGENE**; Horse radish peroxidase-protein A and alkaline phosphatase-protein A antibody detection kits from **PROMEGA Ltd.**; Nitrocellulose membrane from **SCHLEICHER and SCHUELL.**; Kodak X-OMAT XS-1 and duplicating films from **KODAK Ltd.**; wacker silane GF 38 from **WACKER CHEMICALS** and dialysis membrane from **MEDICELL INTERNATIONAL Ltd.**

2.1.2 Solutions

Blocking buffer:

1x TBST plus 3% gelatin heated to 37°C.

Blotting (Towbin) buffer (Towbin et al., 1979):

25 mM Tris.HCl (pH 8.3), 192 mM glycine, 20% (v/v) methanol.

Cell lysis buffer (CLB):

0.5% SDS, 20 mM Tris.HCl (pH 7.5), 2 mM EDTA.

Denhardt's reagent (50x):

1% polyvinylpyrrolidone, 1% BSA, 1% Ficoll 400.

Electrophoresis buffer (SDS-PAGE):

52 mM Tris, 53 mM glycine, 0.1% SDS.

Gel soak I:

200 mM NaOH, 600 mM NaCl.

Gel soak II:

1 M Tris.HCl (pH 8.0), 600 mM NaCl.

GTE buffer:

50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl (pH 8.0).

HBS (lipofection):

150 mM NaCl, 20 mM hepes.NaOH (pH 7.4).

HeBS buffer:

137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM D-Glucose, 21 mM hepes.NaOH (pH 7.05).

Hybridisation buffer:

6x SSC, 5x 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, 1 mM EDTA.

Optimem pH 6.0:

265 ul conc HCl was added to 100 ml optimem and vigo rously stirred for 1 hour.

Phenol/Chloroform solution:

50% TE saturated phenol (v/v), 50% chloroform (v/v).

Pre-hybridisation buffer:

6x SSC, 5x Denhardt's solution, 0.1% SDS, 20 ug/ml denatured calf thymus DNA.

4x Resolving gel buffer (RGB):

1.5 M Tris.HCl (pH 8.9), 0.4% SDS.

SSC (20x):

3 M NaCl, 0.3 M trisodium citrate.

4x Stacking gel buffer (SGB):

0.488 M Tris.HCl (pH 6.7), 0.4% SDS.

STET buffer:

8% (w/v) sucrose, 0.5% (v/v) TritonX-100, 50 mM Tris.HCl (pH 8.0), 50 mM EDTA.

TBE buffer:

90 mM Tris base, 89 mM boric acid, 1 mM EDTA.

TBS (Tris buffered saline):

20 mM Tris.HCl (pH 7.5), 500 mM NaCl.

TBST:

1x TBS plus 0.05% TWEEN.

TE buffer:

10 mM Tris.HCl (pH 8.0), 1 mM EDTA.

TM buffer:

10 mM Tris.HCl (pH 7.5), 10 mM $MgCl_2$.

TSB (Transformation and storage buffer):

L-broth (pH 6.1), (Section 2.1.8), 10% (w/v) PEG (mw 3350), 5% (v/v) DMSO, 10 mM $MgCl_2$, 10 mM $MgSO_4$.

2.1.3 Plasmids

The following plasmids were provided by Dr. N. D. Stow.

Plasmid **pAcYM1** is a AcNPV transfer vector (Matsuura et al., 1987) containing all the upstream sequences of the polyhedrin gene promoter, the untranslated leader and the A of the initiating ATG codon but lacking the rest of the polyhedrin coding sequences. This plasmid contains a unique BamHI site for cloning genes which are to be translated from their own ATG translational start site.

Plasmid **pBL91** consists of a HSV-1 DNA fragment containing the UL9 gene (nucleotides 20670 to 23542; McGeoch et al., 1988a), ligated to XhoI linker oligonucleotides and cloned into the filled-in unique BamHI site of pAcYM1.

Plasmid **pTZ19U** (Mead et al., 1986) is a standard cloning vector conferring ampicillin resistance and containing a multiple cloning site.

Plasmid **pEC8** (also referred to as pST19; Weir and Stow, 1990) consists of a 100 bp fragment specifying a functional HSV-1 ori_S replication origin inserted into the vector pTZ19U.

2.1.4 Enzymes

Restriction enzymes were manufactured by **BOEHRINGER MANNHEIM** and **NEW ENGLAND BIOLABS**, T7 DNA polymerase by **PHARMACIA** and nuclease Bal-31 by **BETHESDA RESEARCH LABS**.

2.1.5 Synthetic Oligonucleotides

Oligonucleotides were synthesised by Dr. J. McLauchlan using a model 8600 Biosearch DNA synthesiser.

Oligonucleotides used during the course of this study to sequence UL9 mutants are listed below :

Oligonucleotide 1 : 5′- AACAACGTAACGGCTTC -3′
Oligonucleotide 2 : 5′- AAATTGTCGCGCTCATG -3′
Oligonucleotide 3 : 5′- TCAACGTCACCGGAGAG -3′
Oligonucleotide 4 : 5′- TGACGTGCTGGACCTAC -3′
Oligonucleotide 5 : 5′- CCGTTAGCGAGACGGAC -3′
Oligonucleotide 6 : 5′- TGTTGGACGCCCACCAC -3′
Oligonucleotide 7 : 5′- TGTTTGCGCACGCACTC -3′

Oligonucleotides used in gel retardation assays are:

Oligonucleotide I :

5′- GATCCGCGAAGCGTTCGCACTTCGTCCCA
GCGCTTCGCAAGCGTGAAGCAGGGTCTAG -5′

Oligonucleotide II :

5′- GATCTGGGGCGAAGTGCGAGCACTTCGCG
ACCCCGCTTCACGCTCGTGAAGCGCCTAG -5′

2.1.6 Radiochemicals

All radio-labelled compounds were supplied by **AMERSHAM INTERNATIONAL PLC**.

		Specific Activity
(i)	5′ [α-^{32}P]- deoxyribonucleoside triphosphates	>3000 Ci/mmol
(ii)	L-[^{35}-S]-Methionine	>1000 Ci/mmol
(iii)	[^{35}-S]- dATP	>1000 Ci/mmol

2.1.7 Viruses, Cells and Tissue Culture Media

Spodoptera frugiperda (Sf) insect cells (strain IPLB-SF-21 : Kitts et al., 1990) were routinely used for the growth of baculoviruses, preparation of DNA and protein extracts and plasmid amplification assays. Sf cells were maintained in TC100 medium, containing 5% (v/v) foetal calf serum, penicillin (100 units/ml) and streptomycin (100 ug/ml).

Recombinant baculoviruses AcUL5, AcUL8, AcUL9, AcUL29, AcUL30, AcUL42 and AcUL52 containing the HSV-1 UL5, UL8, UL9, UL29, UL30, UL42 and UL52 genes

respectively have been previously described by Stow, (1992). The parental virus used in constructing recombinants expressing mutant UL9 genes was AcRP23lacZ (Possee and Howard, 1987).

Recombinant baculoviruses containing mutant HSV-1 UL9 genes were constructed during this study as described later in the text.

2.1.8 Bacterial Strains and Culture Media

The E.coli strains used were DH5 and K12 Δ H1 Δ trp. The following culture 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.

Ampicillin, when appropriate, was added to the above solutions at a concentration of 50 ug/ml.

2.1.9 Antisera

Rabbit antiserum raised against a decapeptide corresponding to the C-terminus of the UL9 gene product was provided by Dr. M.D Challberg (Olivo et al., 1988).

2.2 METHODS

2.2.1 Isolation and Propagation of Recombinant Baculoviruses

(a) Growth of Cells

Spodoptera frugiperda (Sf) insect cells were grown in supplemented TC100 medium at a temperature of 28°C. Cells were detached and passaged routinely every three to four days.

(b) Preparation of Virus Stocks

Stocks of recombinant baculoviruses were propagated in Sf cells. Cell monolayers in 500 ml flasks were infected with virus at a moi of 1 - 5 pfu per cell in 50 ml of supplemented TC100 medium. Infected cultures were

harvested after 3 - 4 days when an extensive cytopathic effect was observed. Cells were pelleted at 2000 rpm (Beckman JA-14 rotor) and the resulting supernatant centrifuged at 14000 rpm for 5 hours at 4°C. The pellet of cell released virus was resuspended in approximately one tenth the original volume of supplemented TC100 medium, sonicated in a Cole Palmer ultrasonic bath and aliquots stored at -70°C. Sterility checks were carried out using blood agar plates.

(c) Titration of Virus

The titration of baculovirus stocks was carried out as described by Brown and Faulkner, (1977).

All virus stocks were titrated on Sf cell monolayers in 35 mm petri dishes. Serial 10-fold virus dilutions were prepared using supplemented TC100 medium and 0.1 ml of each dilution inoculated after the removal of the growth medium. After one hour at RT, the inoculum was carefully removed and replaced with 1.5 ml of overlay medium (made by mixing equal volumes of TC100 medium and melted 3% (w/v) low gelling temperature agarose prewarmed to 37°C) which was allowed to solidify. 1.5 ml of supplemented TC100 medium was then added as a liquid overlay and the plates incubated at 28°C for 4 days. 0.5 ml of a 1 in 25 dilution of stock neutral red stain (0.5% w/v) was then added to the plates which were returned to 28°C and plaques counted the following day.

(d) Transfection of Cells with DNA

Recombinant viruses were generated by co-transfecting Sf cell monolayers with viral and plasmid DNAs using the calcium phosphate precipitation method, (Graham and van der Eb, 1973).

Sf cell monolayers were set up in 35 mm petri dishes one day prior to transfection. 1 ug plasmid DNA and 1 ug Bsu36I-cleaved AcRP23lacZ DNA were gently mixed in 1 ml of HeBS (pH 7.05) in a 15 ml Falcon tube. 70 ul of 2 M $CaCl_2$ was then added and the mixture left at RT for 3 min during which time a fine precipitate appeared. Monolayers

from which the growth medium had been removed were inoculated with the calcium phosphate precipitate, incubated for 1 hour at RT then overlaid with 2 ml of growth medium and moved to a 28°C environment for a further 3 hours. The $CaPO_4$ precipitate was then removed, the cells washed once with growth medium then returned to 28°C with 2 ml of fresh medium as an overlay. The virus progeny were harvested 3 days later by scraping cells into the medium and stored at -70°C until required.

(e) Plaque Purification of Recombinant Baculoviruses

Recombinant viruses were purified by titration of the progeny from transfected monolayers as described above. X-gal (0.5 mg/ml) was added to the neutral red stain to identify recombinant viruses in which the B-galactosidase gene of the parental virus had been disrupted. Well isolated white plaques (putative recombinants) were picked using sterile Pasteur pipettes and placed into 0.5 ml of supplemented TC100 medium and sonicated. Stocks of virus were derived from the progeny of individual plaques and the presence of recombinants was confirmed by Western blot assays and radiolabelling of infected cell proteins.

2.2.2 Manipulation of DNA

(a) Restriction Enzyme Digestion of DNA

DNA samples (1 - 5 ug) were usually digested in a 20 ul volume containing the appropriate enzyme buffer and a suitable amount of restriction enzyme (usually 3 units/ug DNA). Most digestions were carried out at a temperature of 37°C for 1.5 - 2 hours.

For the analysis of small scale plasmid DNA preparations (see section 2.2.4b), 2 ul samples were digested in a total volume of 20 ul as above but with the addition of 10 ug/ml RNase A.

(b) Ligation of Linker Oligonuleotides to Plasmid DNA

Linear plasmid DNA molecules were treated with 1 unit of calf intestinal alkaline phosphatase at 37°C for 1

hour to prevent recircularisation during ligation. After phenol/chloroform extraction and ethanol precipitation, 2.5 ug DNA was ligated overnight at RT to 0.25 ug phosphorylated synthetic linker oligonucleotides in a total volume of 20 ul containing ligase buffer and 1 unit of T4 DNA ligase.

The T4 DNA ligase was inactivated by heating the reaction to 70°C for 10 min. A restriction enzyme digestion was then carried out at 37°C for 3 hours to cleave ligated linker oligonucleotides and produce "sticky" ends. The reaction was extracted with phenol/chloroform, and the ligated DNA precipitated with 0.5 vol of isopropanol for 4 hours at RT and then recircularised in a 30 ul vol using 1 unit of T4 DNA ligase at 37°C for 4 hours. 1 ul samples of ligated DNA were used to transform competent E.coli bacteria.

(c) Digestion of Plasmid DNA with Nuclease Bal-31

35 ug of plasmid pB1 DNA was linearised with the restriction enzyme SalI in a total volume of 100 ul for 3 hours at 37°C then serially extracted with phenol and chloroform and precipitated with 2.5 vols of ethanol. Pelleted DNA was lyophilised and resuspended in 180 ul H_2O. 45 ul 5x Bal-31 buffer (100 mM Tris.HCl [pH8.0],60 mM $MgCl_2$, 60 mM $CaCl_2$, 5 mM EDTA [pH 8.0],1 M NaCl) and 1 unit of the enzyme Bal-31 were added. A 35 ul sample (zero time point) was immediately removed into 350 ul 0.02 M EGTA (pH 8.0), 0.15 M NaCl, extracted with phenol/ chloroform and ethanol precipitated. The remainder of the reaction was incubated at 31°C and 35 ul samples were withdrawn at various times (from 1 to 10 min) and treated as above. Pelleted DNA was lyophilised and resuspended in 22 ul H_2O. 5 ul of each sample was digested with BamHI and electrophoresed in a mini agarose gel to determine the extent of deletion.

Plasmid DNA digested to an appropriate extent with Bal-31 was treated with calf intestinal alkaline phosphatase and ligated to 14 bp XbaI linker oligonucleotides containing termination codons in all

three frames. Following ligation, the reaction products were digested with XbaI and recircularised as described in section b, prior to transformation of E.coli.

(d) Linker Insertion Mutagenesis

Plasmid pB1 DNA was partially digested with HaeIII or MvnI (which leave blunt ends) using either short incubation periods or digestion in the presence of ethidium bromide.

In the first case, 10 ug of pB1 were cleaved with 10 units of enzyme in a total vol of 50 ul at 37°C for various times. Partial cleavage in the presence of ethidium bromide was carried out using 10 ug of pB1 and 50 or 10 units of enzyme in the presence of 20 or 50 ug/ml of ethium bromide in a volume of 100 ul. These reactions were incubated at 37°C for 22 min.

4 ul samples from each reaction were then run on 1% agarose gels alongside plasmid pB1 cleaved with a single-cut enzyme to check the extent of linearisation. DNA samples with the highest concentration of linear molecules were dephosphorylated with calf intestinal alkaline phosphatase and the linears purified by agarose gel electrophoresis and DEAE-sephacel column chromatography (see section 2.2.5a). Purified linear molecules were then ligated to 12 bp EcoRI linker oligonucleotides overnight at RT (see section 2.2.2 b), cleaved with EcoRI and the resulting molecules recircularised in preparation for transformation into competent E.coli cells.

2.2.3 Preparation and Transformation of Competent E.coli DH5 Cells

The method was essentially as described by Chung and Miller (1988).

50 ml of L-broth was inoculated with 300 ul of an overnight culture of E.coli DH5 cells and grown to an O.D of 0.3 at 630 nm. Bacteria were pelleted at 2,300 rpm for 10 min at 4°C, resuspended in 2 ml TSB and incubated on ice for 10 min. 200 ul aliquots were placed in

polypropylene tubes and stored on ice.

DNA was added in a small volume (usually < 7ul) to the competent cells which were incubated on ice for about 25 min. 800 ul of TSB containing 20 mM glucose was then added and the bacteria incubated at 37°C in an orbital shaker for 1.5 hours. 200 ul of undiluted and diluted transformed cells were spread onto agar plates containing 50 ug/ml of ampicillin and incubated overnight at 37°C.

2.2.4 Preparation of Plasmid DNA

(a) Large Scale Preparation of Plasmid DNA

4 ml of L-broth containing 50 ug/ml ampicillin was inoculated with either a picked bacterial colony from an ampicillin agar plate or 10 ul of bacterial stock (stored in 7% DMSO at -70°C) and incubated overnight at 37°C. These cultures were added to 300 ml of L-broth containing 50 ug/ml ampicillin and shaken at 37°C in a 2 L conical flask until an optical density measured at 630 nm of 0.6 was reached. 1 ml of chloramphenicol solution (34 mg/ml dissolved in absolute alcohol) was then added to the culture and shaking was continued overnight.

Bacterial cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C in a Sorvall GS3 rotor. Cell pellets were resuspended in 9 ml GTE buffer (50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA), 1 ml of lysozyme solution (20 mg/ml prepared in 0.2 M Tris-HCl pH 8.0) added and the cells incubated with gentle mixing for 5 min at RT. 20 ml of freshly prepared 1% SDS/0.2 M NaOH was then added and incubation continued for a further 5 min at 4°C. Finally, 10 ml of ice cold 5 M potassium acetate (pH 4.8) was added and the cellular DNA and bacterial debris pelleted by spinning at 18,000 rpm (Sorvall SS34 rotor) for 15 min at 4°C. 16.5 ml isopropanol was then added to the supernatant for 5 min at RT. The DNA was recovered by centrifugation at 10,000 rpm (Sorvall SS34 rotor) for 30min at 4°C. The resulting DNA pellet was washed in 70% ethanol, air dried and redissolved in 2 ml TE.

The plasmid DNA was further purified by centrifugation to equilibrium on a caesium chloride-ethidium bromide density gradient.

2.3 g of CsCl was dissolved in the DNA solution (2 ml) and 50 ul of ethidium bromide solution (10 mg/ml) added. The mixture was allowed to stand for a few min at RT before spinning for 3 min at high speed in a microfuge. The resulting supernatents were removed and placed in "quick seal" Beckman TL100 tubes. After heat sealing, the tubes were spun at 80,000 rpm overnight at 20°C in a Beckman TL100 vertical rotor. Two DNA bands were observed and the lower one, consisting of closed circular plasmid DNA, was removed with a syringe. Ethidium bromide was extracted by mixing four times with an equal volume of isoamyl alcohol. The aqueous phase was dialysed against TE for 2 hours and the DNA precipitated in ethanol overnight. The DNA pellet was redissolved and subsequently treated with RNase A (50 ug/ml) for 30 min at 37°C. After phenol/chloroform extraction and ethanol precipitation, the final air dried pellet of plasmid DNA was dissolved in 200 ul of TE.

Plasmid DNA was quantified by UV absorption at 260 nm taking an absorbance of 1.0 to correspond to a concentration of 50 ug/ml DNA.

(b) Small Scale Preparation of Plasmid DNA (Maniatis, 1982)

1.5 ml of a 2.0 ml overnight shaken E.coli culture was spun for 12 sec in a microcentrifuge. After removal of the supernatant the cell pellet was resuspended in 100 ul of STET buffer by vortexing and 16 ul of a 20 mg/ml solution of lysozyme was added for 30 sec. The tube was placed in a boiling water bath for 50 sec and spun at high speed in a microcentrifuge for 10 min. The glutinous cell pellet was removed and 100 ul isopropanol added to the supernatant. After 15 mins at -20°C, precipitated nucleic acids were recovered by spinning again at high speed for 10 min, washed in 70% ethanol, lyophilised then redissolved in 35 ul TE buffer.

2.2.5 Gel Electrophoresis of DNA

(a) Non-denaturing Agarose Gel Electrophoresis

For analysis of DNA restriction fragments horizontal 1% agarose gels were prepared in TBE buffer or Loening's buffer containing 0.5 ug/ml of ethidium bromide. Before loading, DNA samples were mixed with one quarter volume 5x tracker dyes (5x appropriate electrophoresis buffer, 50% sucrose and 0.2% bromophenol blue (BPB)). Loaded gels were then electrophoresed at 100 V for 3 - 4 hours (TBE gels) or 35 V overnight (Loening's buffer gels). Rapid analysis of small amounts of DNA was carried out on 1% agarose mini-gels (20 ml) run in TBE buffer at 50 V for approximately 45 min. DNA bands in both cases were visualised under short wave UV light and photographed using Polaroid film (type 667).

DNA fragments were purified by excising the required gel slice from an agarose gel (observed under long wave UV light) and placing it in boiled dialysis tubing along with 1 ml of 0.5x TBE buffer. The closed bag was immersed in an electrophoresis tank containing 0.5x TBE perpendicular to the direction of the current flow. DNA was electroeluted from the gel slice for 2 hours at 200 V. The buffer containing DNA was removed and passed through a 0.5 ml bed volume DEAE-sephacel column equilibriated with 0.1 M NaCl in TE. The column was washed with 5 ml of 0.1 M NaCl/TE solution. The DNA was then eluted from the column with 2x 0.4 ml aliquots of 1 M NaCl/TE solution, precipitated with 2 ml of ethanol. The DNA was pelleted, lyophilised and dissolved in TE.

(b) Non-denaturing Polyacrylamide Gel Electrophoresis

Purification of radio-labelled DNA fragments or analysis of small DNA restriction fragments was carried out on vertical 6% polyacrylamide gel slabs in TBE buffer. Stock solutions of 30% acrylamide were prepared in water with an acrylamide to bisacrylamide ratio of 28.5:1.5 and deionised by stirring with amberlite resin. Final gel solutions (50 ml) were polymerised by addition

of 500 ul of 10% (w/v) ammonium persulphate solution and 50 ul TEMED. Gels were electrophoresed at 150 V for 2.5 - 3 hours.

Resolved radio-labelled DNA fragments were located by autoradiography of the wet polyacrylamide gel and purified as described in section 2.2.4a. The radio-labelled DNA was stored at -20°C.

Separated unlabelled DNA restriction fragments were visualised on acrylamide gels under UV light after soaking the gel in 1x TBE containing ethidium bromide (0.5 ug/ml).

(c) Denaturing Polyacrylamide Gel Electrophoresis

DNA sequencing reactions were resolved on 6% polyacrylamide, 7.7 M urea, 0.5 - 2.5x TBE gradient gels (Hong, 1987).

Prior to pouring the gel, both sequencing plates were washed with 100% ethanol. The front notched plate was then covered with approximately 5 ml replecote™ while 5 ml "Wacker" solution (25 ul Wacker silane GF38, 150 ul 10% acetic acid, 5 ml ethanol) was applied to the back plate followed by 5 washes with 100% ethanol.

DNA samples were boiled for 2 - 3 min in 95% deionised formamide, 0.05% BPB and 0.05% xylene cyanolFF, 20 mM EDTA (pH 7.5) before loading 3 ul into the previously rinsed wells. Gels were electrophoresed at 60 W for approximately 2 hours in 1x TBE tank buffer until the BPB dye front reached the bottom of the gel. After removal of the siliconised front gel plate, the gel was soaked in 10% acetic acid for 30 min then dried onto the "wackered" back plate in a 140°C oven for 1 - 1.5 hours. The dried gel was then exposed to autoradiography film overnight at RT.

2.2.6 Plasmid DNA sequencing

Plasmid DNA was sequenced by the dideoxy chain termination sequencing protocol (Sanger et al., 1977) using denatured plasmid DNA templates (Hattori and Sakaki, 1986).

(a) Preparation of Denatured Plasmid DNA

This method was derived from that described by Hattori and Sakaki (1986).

Bacterial cells from a 15 ml overnight culture were pelleted by spinning for 15 min at 9000 rpm (SS34 rotor) and resuspended in 1 ml of lysozyme solution (2 mg/ml lysozyme, 50 mM glucose, 25 mM Tris.HCl (pH 8.0), 10 mM EDTA) for 5 min at RT. 2 ml of 1% SDS/0.2 M NaOH was then added for 5 min at 4°C followed by 1.5 ml of ice-cold 5 M potassium acetate (pH 4.8), for a further 5 min. After a centrifugation for 15 min at 4°C and 9000 rpm, the supernatant was removed and phenol/chloroform extracted. The DNA was precipitated with 2 vols of ethanol for 30 min at RT and pelleted for 15 min. The washed and lyophilised pellet was resuspended in 100 ul TE containing 10 ug RNase A and incubated for 30 min at 37°C. 60 ul of 20% PEG/2.5 M NaCl was added for 1 hour on ice. The DNA was pelleted, washed, lyophilised and resuspended in 18 ul TE. The plasmid DNA was denatured in 0.2 M NaOH for 5 min at RT, ammonium acetate added to 0.3M and the DNA precipitated with ethanol. The final pellet of denatured plasmid DNA was washed, lyophilised and resuspended in 40 ul H_2O.

(b) Dideoxy sequencing

4 ul of denatured plasmid DNA was annealed with 10 ng of primer oligonucleotide in a final vol of 10 ul containing 0.1 M Tris.HCl (pH 8.0) and 0.1 M $MgCl_2$ for 30 min at 37°C. Meanwhile, 2.5 ul of each of 4 termination mixes (i.e., G,A,T and C) were dispensed into separate microtitre wells. These mixes comprised 150 uM dGTP, dATP, dTTP, dCTP and 15 uM of the appropriate dideoxy (dd)NTP (ie G,A,T or C) in 20 mM $MgCl_2$, 40 mM Tris.HCl (pH 7.5) and 50 mM NaCl. The annealed DNAs were radio-labelled by addition of 1 ul 0.1 M DTT, 2 ul labelling mix (2 uM dGTP, dTTP and dCTP), 20 uCi [^{35}S]-dATP and 2 units T7 DNA polymerase for 5 min at RT (final vol 17 ul). 3.8 ul aliquots of the labelled annealed DNAs were transferred to each of the four termination mixes and

incubated at 37°C for 15 min. 4 ul of formamide dye mix were added to the reactions and the products analysed on denaturing polyacrylamide gels (section 2.2.5c).

2.2.7 Radioactive Labelling of DNA

(a) Synthesis and Purification of Oligonucleotides

Oligonucleotides were synthesised on a Biosearch 8600 DNA synthesiser and the DNA was eluted in 1 ml of ammonia.

Following a 5 hour incubation at 55°C, the ammonia was removed by lyophilising overnight. The DNA pellet was resuspended in 50 ul of H_2O and 25 ul removed and stored at -70°C. The remainder was mixed with an equal vol of 90% formamide, 1x TBE. The sample was boiled for 2 - 3 min and loaded immediately onto a 15% non-denaturing polyacrylamide gel (acrylamide:bis-acrylamide, 19:1). 5 ul of formamide dye mix was also loaded into an adjacent well to act as a migration marker. The gel was electrophoresed in 1x TBE at 300 V for about 4 hours or until the bromophenol blue had reached about 3/4 of the way down the gel. The gel was transferred onto cellophane and the DNA observed over a silica gel thin layer chromatography plate illuminated with an angled UV short wavelength lamp. The DNA was observed as a shadow on the fluorescent plate as a result of absorption of UV light. The oligonucleotide DNA band was excised and eluted with 0.5 ml H_2O overnight at 37°C. The DNA solution was subsequently phenol/chloroform extracted, ethanol precipitated, lyophilised and the oligonucleotide resuspended in 50 ul H_2O.

The concentration of the oligonucleotide was estimated by taking an absorbance value of 1 at 260 nm to correspond to 20 ug/ml of oligonucleotide.

(b) End-labelling of Synthetic Oligonucleotides and DNA Fragments

Complementary synthetic oligonucleotides were annealed by mixing 5 ug of each in 50 ul H_2O, boiling for

2 min and incubating for 30 min at 37°C. 0.2 ug annealed DNA was radio-labelled in a 40 ul vol of NT buffer (50 mM Tris.HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT) containing 20 uCi of [$-^{32}P$]dATP, 0.1 mM dGTP, dTTP, dCTP and 2 units of the Klenow fragment of DNA polymerase I for 20 min at RT. Cold dATP was then added to 0.05 mM and the reactions left for a further 10 min and extracted with phenol. DNA was separated from unincorporated nucleotides by passage through a G-50 sephadex column.

Larger DNA fragments were purified from 5% non-denaturing polyacrylamide gels and radio-labelled as above.

(c) Internal Labelling of Plasmid DNA by Nick Translation

Plasmid DNA (200 - 300 ng) was radio-labelled by nick translation (Rigby et al., 1977) using 10 uCi each of [$-^{32}P$] dCTP and [$-^{32}P$] dGTP, 2 units of E.coli DNA polymerase I and $1x10^{-7}$ mg DNase I in a final volume of 30 ul NT buffer containing 40 nM each of cold dATP and dTTP. After a 1 hour incubation at 14°C, the reaction was brought to 150 ul with H_2O, extracted with phenol and labelled DNA purified on a G-50 sephadex column.

2.2.8 Analysis of Proteins

(a) Expression and Preparation of Protein A/UL9 Fusion Proteins

A 3 ml E.coli culture was shaken overnight at 37°C. 100 ml of L-broth plus ampicillin was inoculated with 500 ul of the overnight culture and incubated at 37°C in an orbital shaker until an O.D of 0.5 at 630 nm was reached. 50 ml of the culture was spun at 2300 rpm for 10 min at 4°C in a Sorvall bench top centrifuge. The cell pellet was washed twice with 2 ml TE and resuspended in 1 ml TE. The cells were pelleted in a microfuge at high speed for 30 secs and resuspended in 400 ul 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 0.6 M KCl. The cell

suspension was subjected to 3x 1 second bursts with a soniprobe. After centrifugation at 15000 rpm for 15 min (using SS34 eppendorf adaptors in a Sorvall rotor), the supernatant protein extract was removed, flash frozen and stored at -70°C.

(b) Estimation of Protein Concentration

Estimates of total protein concentration in bacterial extracts were obtained using Bio-Rad protein assay dye reagent according to the method of Bradford, (1976). 1 ul of the bacterial cell extract was diluted in 1 ml of H_2O, 800 ul of which was mixed with 200 ul of the dye reagent and left at RT for 10 min. The absorbance of the solution was measured at 620 nm and the concentration calculated from a standard curve produced using BSA solutions of known concentration.

(c) SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Vertical polyacrylamide gels were set up in a Biorad mini gel protein kit. Stocks of 30% acrylamide with a ratio of acrylamide to bisacrylamide of 28.5:1.5 were made up in H_2O. A 9% acrylamide resolving gel mix was prepared in 375 mM Tris.HCl (pH 8.9) and 0.1% SDS. 10% ammonium persulphate (APS) and TEMED were added to final concentrations of 0.1% and the resolving gel poured. Butan-2-ol was layered on top of the gel solution to ensure uniform polymerisation. After polymerisation the butan-2-ol was rinsed off using unpolymerised stacking gel solution. A stacking gel consisting of 5% acrylamide (28.5:1.5) in 122 mM Tris.HCl (pH 6.7), 0.1% SDS was layered on top of the resolving gel immediately after addition of APS and TEMED. Wells were formed using a teflon comb. Before electrophoresis, protein samples were boiled for 5 min in sample buffer (final concentration 50 mM Tris.HCl (pH 6.7), 2% SDS, 700 mM 2-mercaptoethanol 10% glycerol and sufficient BPB for visualisation of the dye front). Electrophoresis was carried out at RT in electrophoresis tank buffer (52 mM Tris, 53 mM glycine and 0.1% SDS) at 100 V until the dye front reached the

bottom of the gel (approximately 60 min).

(d) Staining of Separated Proteins

(i) Coomassie brilliant blue staining

Following electrophoresis, protein gels were fixed and stained in a solution of methanol : acetic acid : water (50:7:50) containing 0.2% Coomassie brilliant blue R250 for 30 min. Gels were then destained in 10% methanol, 7.5% acetic acid for at least 1 hour.

(ii) Silver staining

Silver staining was performed essentially as described by McLean et al., (1990).

Following electrophoresis gels were fixed in 30% ethanol, 10% acetic acid for 30 min. Gels were then incubated for a further 30 min 30% ethanol, 0.5 M sodium acetate, 0.5% glutaraldehyde and 0.2% sodium thiosulphate followed by 3x 10 min washes in water. The gels were soaked next in 0.1% silver nitrate/0.02% formaldehyde for 15-30 min and developed in a solution of 2.5% sodium bicarbonate and 0.01% formaldehyde for around 5 min until bands were visible. Band development was stopped by addition of EDTA to 0.05 M for 5 min followed by rinsing in deionised water. All solutions for silver staining were prepared using high purity filtered HPLC grade water.

(e) Elecroblot Transfer of Proteins to Nitrocellulose

SDS polyacrylamide mini gels were used to resolve polypeptides prior to electroblot transfer to nitrocellulose (Towbin et al., 1979).

The gel was washed briefly in blotting buffer then placed on 3 mm Whatman paper soaked in buffer. A piece of wetted nitrocellulose, cut to the same size as the gel, was carefully placed on top ensuring no air bubbles were trapped between the layers. Another piece of pre-soaked Whatman paper was added and the sandwich placed in the Biorad mini transblot cell. The

polypeptides were electoblotted either at 0.2 A for 3 hours or 40 mA overnight according to the manufacturers instructions.

(f) Detection of Protein A Fusions Using Antibodies

After electroblotting, nitrocellulose membranes were rinsed in TBST buffer then blocked in a solution of 3% gelatin in TBST buffer overnight at 37°C. The blocking solution was removed and replaced with a 1 in 10000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate in TBST buffer for 30 min at RT (this antibody can bind to the protein A domain of fusion proteins via its Fc region). The membrane was then washed 3x in TBST buffer for at least 5 min to remove unbound antibody. After the removal of excess liquid, the membrane was added to 10 ml of freshly prepared colour development solution (Promega). Once the desired intensity of bands was obtained, the membrane was washed thoroughly in deionised H_2O for a few min, air dried and stored in aluminium foil to prevent colour deterioration.

(g) Radiolabelling of Proteins

For the preparation of radiolabelled polypeptides, 70 - 80% confluent Sf cell monolayers in 35 mm petri dishes were used. After removal of medium, cell monolayers were infected with 20 pfu/cell of appropriate recombinant baculovirus in supplemented TC100 medium for 1 - 2 hours at RT with occasional gentle agitation. 2 ml fresh medium were added and incubation continued for 50 hours at 28°C. The medium was removed and replaced with 1 ml TC100 salt solution (43 mM KCl, 10 mM $CaCl_2$, 6 mM glucose, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$, 4.6 mM $NaHCO_3$, 8 mM NaH_2PO_4 pH 6.2) containing 25 uCi [^{35}S]-L-methionine. Cells were harvested into ice cold TC100 (without antibiotics and foetal calf serum), washed three times and resuspended in boiling mix. Radiolabelled polypeptides were separated by SDS-PAGE on 8.5% gels overnight at 10 mA. Gels were fixed for 10 min, treated with EnHance for 60 min, dried and exposed to

autoradiographic film overnight at -70°.

2.2.9 Gel Retardation Assay

3′ end-labelled probe fragments were purified either by passage through a G-50 column or by elution from a 6% non-denaturing polyacrylamide gel, extraction with phenol and chloroform and ethanol precipitation.

1 ng of labelled ori_S-containing oligonucleotide or 5 ng of a labelled ori_S-containing fragment, 2 ug of sonicated calf thymus DNA and 6 ul extraction buffer containing 10 ug of protein extract were incubated at 22°C in a final vol of 24 ul of 60 mM Hepes, pH 7.5; 1.5 mM DTT; 3 mM EDTA (150 mM KCl is contributed by the extraction buffer). After 20 min, 5 ul loading buffer (25% glycerol, 10 mM DTT and 0.01% BPB in TBE) were added and the samples loaded onto a 5% polyacrylamide gel (55:1 acrylamide: N,N′-methylene bisacrylamide). Gels were run in TBE at 150 V for 2.5 - 3 hours, fixed in 10% acetic acid, dried and exposed to autoradiography film overnight at - 70°C.

2.2.10 Plasmid Amplification Assay

(a) Transfection of Cells

50% confluent monolayers of Sf cells in 8 mm diameter linbro wells were transfected with plasmid pEC8 DNA containing a functional HSV-1 ori_S fragment by lipofection.

2 ug plasmid DNA were mixed with 30 ul of liposome preparation (1 mg/ml DOPE, 1 mg/ml DDAB prepared by N.Stow as described by Rose et al., 1991) in 160 ul of HBS buffer and incubated at RT for 10 min. 2 ml of optimem pH 6.0 were added to the liposome/DNA mixture and 300 ul of the resulting solution added per linbro well after first washing the cells with pH 6.0 optimem. The plates were incubated at 28°C for 4 hours after which the inoculum was removed and the cells washed once with supplemented TC100 medium. Each well was then inoculated with a virus mix containing 2.5x 10^6 pfu of each

indicated baculovirus recombinant and incubated at RT for 60 min with occasional gentle agitation. 1 ml of supplemented TC100 medium was then added and the cells incubated at 28°C for 50 hours.

(b) Preparation of Total Cellular DNA

Growth medium was removed from the linbro wells 50 hours post-transfection and replaced by 400 ul of cell lysis buffer (0.6% SDS, 10 mM EDTA, 10 mM Tris-HCl pH 7.5) containing 0.5 mg/ml protease (Sigma Type XIV). 32 ul of 4 M NaCl, 0.05 M EDTA was added and the lysate sequentially extracted with phenol and chloroform. The DNA was precipitated with 1 ml of ethanol overnight at -20°C, pelleted, lyophilised and resuspended in TE (usually 60 ul).

One third of the total cellular DNA was digested with 3 units each of the restriction enzymes DpnI and EcoRI overnight at 37°C.

(c) Southern Transfer of DNA to a Nylon Membrane

Digested DNA fragments were separated on a 1% agarose gel in Loening's buffer prior to Southern transfer (Southern, 1975). Following electrophoresis, the gel was photographed to confirm that digestion had occurred and then placed in Gel soak I for 45 min with agitation followed by 45 min in Gel soak II. The DNA was then blotted overnight onto a nylon membrane by capillary action using a wick of 3 mm Whatman paper in 6x SSC solution below the gel and a weighted stack of absorbent paper towelling on top of the membrane. The nylon filter was air dried and the DNA fixed to the filter by UV irradiation using a Stratalinker (Stratagene) prior to hybridisation.

(d) DNA/DNA hybridisation

The membrane was incubated in a plastic bag in 20 ml of pre-hybridisation buffer for at least 2 hours at 65°C with constant agitation. About 1-2x 10^7 cpm of nick translated probe DNA in 1 ml H_2O was denatured by

addition of 0.2 ml 1 M NaOH for 10 min. The mixture was then neutralised by addition of 0.2 ml 1 M HCl and added to 10 ml of hybridisation buffer. The prehybridisation solution was removed and the probe added to the bag containing the nylon filter. Hybridisation was carried out at 65°C overnight with constant agitation. The filter was then washed 4x in 1x SSC/0.25% SDS for 20 min each at 65°C. After air drying, the filter was exposed to autoradiography film overnight at -20°C with an image intensifying screen.

It should be noted that all figures in the following Results section are representative of repeat experiments.

CHAPTER 3: RESULTS

3.1 MUTAGENESIS OF THE UL9 DNA BINDING DOMAIN

3.1.1 Introduction

The experiments presented in this section are concerned with the mutagenesis of the DNA binding domain of the UL9 protein and utilise an expression system and binding assays based upon those previously described by Weir et al., (1989).

The parental plasmid employed in these studies is pB1 which Weir et al., (1989) previously shown encodes functional origin binding activity.

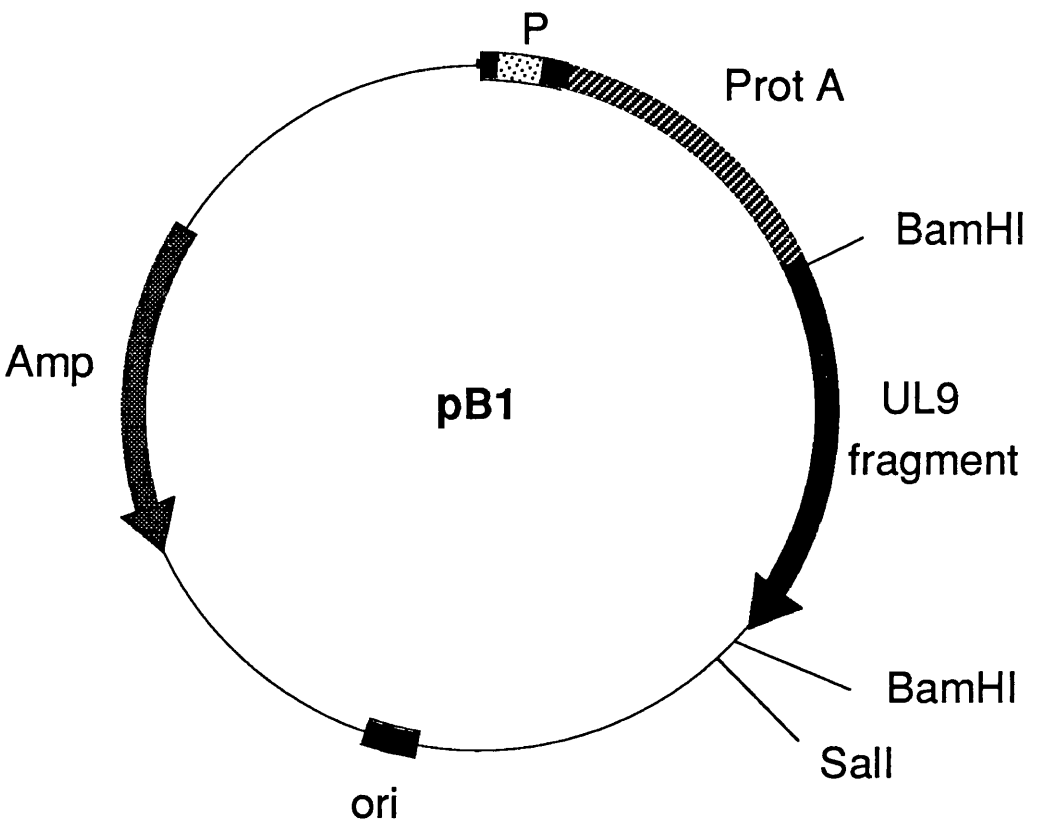
Plasmid pB1 (**Figure 11**) consists of an HSV-1 DNA fragment (nucleotides 21655 - 20666, McGeoch et al., 1988) encoding the C-terminal 317 amino acids of the wt UL9 protein inserted, in-frame, into the BamHI site of a derivative of the fusion protein expression vector pRIT2T (Weir et al., 1989). pRIT2T allows the expression of fusion proteins (driven by the bacteriophage lambda P_R promoter) which contain sequences from the Staphylococcus aureus A protein at their amino terminus. The origin binding product encoded by pB1 consists of the N-terminal 260 amino acids of the Staphylococcus aureus protein A followed by amino acids 535 - 851 of UL9.

As in the previous work (Weir et al., 1989), I have assessed the ability of the fusion proteins to bind specifically to radiolabelled oligonucleotides containing ori_S sequences using a gel retardation assay. This technique, developed by Fried and Crothers (1981), involves incubating protein extracts with DNA fragments or oligonucleotides containing putative protein binding sites. Following incubation, the products are analysed by electrophoresis through non-denaturing polyacrylamide gels during which protein/DNA complexes are separated from unbound DNA on the basis of their lower electrophoretic mobility. The detection of sequence-specific binding activity is possible, even in crude extracts, if an excess of unlabelled carrier DNA is added

Figure 11. Structure of plasmid pB1

The protein A-UL9 fusion is expressed under the control of the phage lambda promoter P_R and consists of the N-terminal 260 amino acids of the Staphylococcus aureus A protein linked to amino acids 535 - 851 of UL9. The BamHI and SalI sites are marked.

"Ori" indicates the presence of an origin of replication.


P
Prot A
BamHI
UL9
fragment
BamHI
SalI
ori
Amp
pB1

(e.g. calf thymus DNA), to the binding reactions, to compete for the binding of non-sequence specific DNA binding proteins (Garner and Revin., 1981).

Before the mutagenesis of pB1 was commenced I performed some preliminary experiments in which two host strains of E.coli were compared for expression of origin binding activity (Section 3.1.2) and also developed a Western blot assay for the presence of pB1 fusion protein in E.coli extracts (Section 3.1.3).

3.1.2 Expression of pB1 Fusion Protein In E.coli Strains DH5 and K12 Δ HI Δ trp

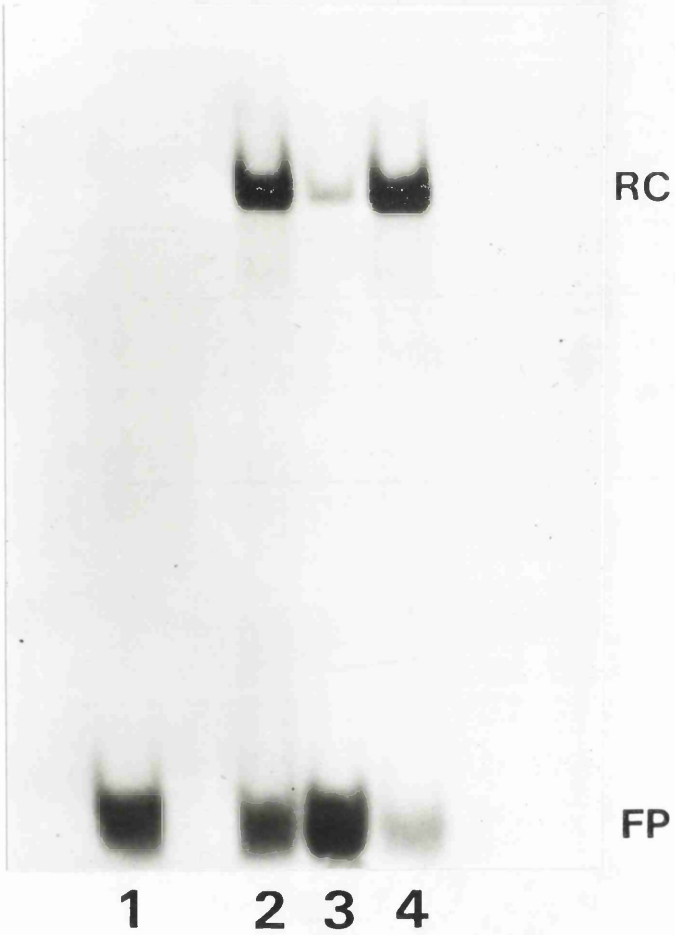
In the experiments reported by Weir et al., (1989) expression of the pB1 fusion protein was specifically induced by temperature upshift of an E.coli host (strain K12 Δ HI Δ trp) containing a ts bacteriophage lambda repressor. Initial experiments were performed to determine whether constitutive expression of the pB1 fusion protein occurred in the E.coli strain DH5 which is routinely used in our laboratory for cloning purposes and does not contain the lambda repressor. Gel retardation assays were used to compare origin binding activity in pB1-transformed DH5 cells grown at 37°C with pB1-transformed K12 Δ HI Δ trp cells grown at 37°C or grown at 28°C and induced at 42°C. Cells were grown to an OD 0.5, harvested and extracts prepared by sonication. Extracts were incubated with labelled oligonucleotide I containing ori_S binding site I for 20 min at 22°C and binding analysed on 5% polyacrylamide gels. **Figure 12** shows the presence of retarded complexes in all samples containing pB1-transformed cells (lanes 2 - 4). Since DH5 cells efficiently expressed origin binding activity and were more convenient to use because temperature upshift was not required they were employed as host cells in the experiments described throughout this study.

3.1.3 Development of a Western Blot Assay for pB1 Fusion Proteins

Figure 12. Gel Retardation Analysis of pB1 Extracts

Protein extracts were incubated with ^{32}P-labelled oligonucleotide I containing the HSV-1 ori_S site I for 20 min at 22°C and the products separated by electrophoresis through a 5% polyacrylamide gel. Extracts were from *E.coli* strain K12 ΔHI Δtrp cells (lanes 1 - 3) or strain DH5 cells (lane 4): lane 1, untransformed cells; lane 2, cells transformed with plasmid pB1 and grown at 28°C with a temperature upshift to 42°C; lane 3, cells transformed with pB1 and grown at 37°C; lane 4, cells transformed with plasmid pB1 and grown at 37°C.

"RC" represents the position of the major retarded complex and "FP", free unbound probe.


RC
FP
1
2
3
4

In order to assess the binding ability of mutant and wt pB1 fusion proteins, it was necessary to have a method for confirming the presence of fusion proteins with E.coli extracts. Several methods based upon the Western blot were attempted. Extracts were resolved on 9% polyacrylamide gels and electroblotted on to nitrocellulose membranes prior to detection using antibodies.

Initially the method for detecting fusion proteins involved incubating the blot first with an antibody raised in rabbits against a peptide corresponding to the C-terminal 10 amino acids of the UL9 protein, a kind gift from M. Challberg. Bound antibody was then visualised by incubating the blot with a goat anti-rabbit IgG/horse radish peroxidase antibody conjugate followed by washing and addition of colour development substrates. This procedure however resulted in non-specific staining of a large number of protein bands (even in untransformed DH5 cell extracts) which greatly complicated the detection of UL9 fusion proteins (data not shown). Also, because the mutational analysis of the UL9 DNA binding domain was to include constructing C-terminal deletion mutations which would remove the antibody's epitope a different detection method had to be employed which did not rely on the presence of specific UL9 sequences within the fusion protein.

A different detection method was therefore developed. This involved incubating the Western blot directly with the IgG/horse radish peroxidase conjugate followed by washing and addition of colour development substrates. Fusion proteins were specifically detected using this procedure due to the interaction of the protein A domain of the protein with the Fc region of the antibody conjugate (**Figure 13**). Very low backgrounds were obtained.

Two antibody detection kits supplied by Promega (Western blot HRP system and Western blot AP system) were compared (data not shown) and the sensitivity of the IgG/AP system was found to be greater. As a result, this

detection method was used to confirm the presence of protein A fusions in subsequent experiments.

An example of a Western blot assay carried out using this detection method is illustrated in **Figure 14.** Extracts were from bacteria transformed with either plasmid pB1 encoding the intact fusion protein (A-9CT), (**lane 1**), or plasmid pRIT2T encoding the protein A moiety alone (**lane 2**). Single protein bands corresponding to polypeptides of the expected sizes (approximately 63 kDa and 30 kD respectively) were detected. Although not shown in this figure, no corresponding bands were detected in extracts prepared from untransformed DH5 cells (e.g. see **Figure 22**).

3.1.4 Mutagenesis of Plasmid pB1

The aim of the following experiments was to carry out a mutational study of the UL9 DNA binding domain in an attempt to characterise further the regions involved in the interaction with its target sequence. This involved generating series of mutants with either C-terminal deletions or small in-frame insertions within the UL9 DNA binding domain.

(a) Construction and Characterisation of pB1 Deletion Mutants

Plasmid pB1 was linearised at the unique SalI site downstream of the UL9 translation stop signal and digested with the exonuclease Bal-31. Bal-31 digestion for different times resulted in the production of a family of fragments with deletions extending varying lengths into the UL9 coding sequence. The extent of deletion was analysed by agarose gel electrophoresis after digestion with BamHI (**Figure 15**).

BamHI digestion of Sal I cleaved pB1 yields two fragments of approximately 4250 bp and 989 bp in size and the degree of Bal-31 digestion was monitored by the reduction in size of the smaller fragment. By comparison with HinfI cleaved pAT153 DNA molecular weight marker fragments, Bal-31 digestion for 4 min was estimated to

Figure 14. Western Blot Assay of pB1 and pRIT2T Extracts

1 ug samples of protein extract were resolved on a 9% denaturing polyacrylamide gel with protein molecular size markers and blotted onto nitrocellulose. The blot was probed with alkaline phosphatase-conjugated goat anti-rabbit IgG which was visualised by the addition of colour development substrates supplied in the Promega Western blot AP detection kit. Extracts were from E.coli DH5 bacteria transformed with the following plasmids: Lane 1, pB1; lane 2, pRIT2T which contains sequences encoding the N-terminal 260 amino acids of the Staphylococcus aureus protein A only.

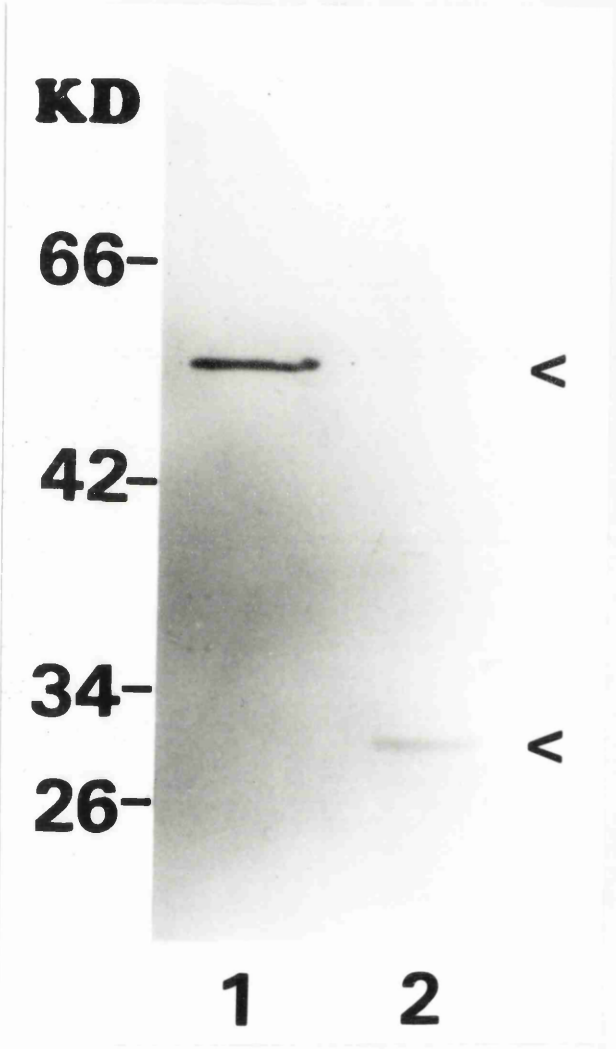
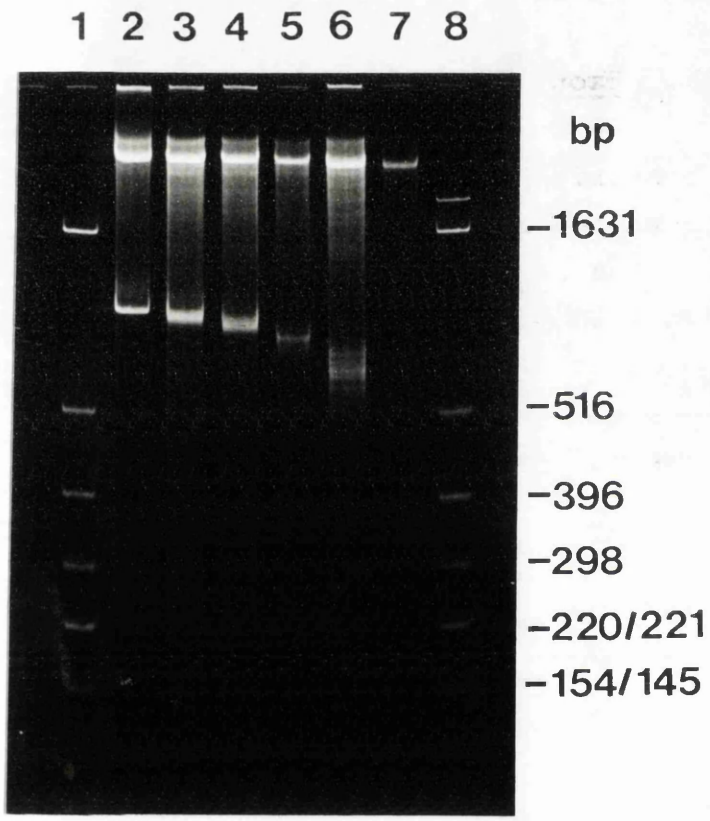

KD
66-
42-
34-
26-
<
<
1
2

Figure 15. Exonuclease Bal-31 Digestion of pB1

SalI linearised pB1 was treated with the exonuclease Bal-31 for various times at 31°C. After phenol extraction, the DNA samples were digested with BamHI and analysed on a 1% agarose gel alongside HinfI digested pAT153 molecular weight marker fragments (lanes 1 and 8), the sizes of which (bp) are indicated at the side. Bal-31 digestions were carried out for the following times: lane 2, 0 min; lane 3, 1 min; lane 4, 2 min; lane 5, 4 min; lane 6, 7 min; lane 7, 10 min.


1 2 3 4 5 6 7 8
bp
1631
516
396
298
220/221
154/145

decrease the length of the smaller fragment by about 200 bp.

Linear DNA molecules from the 4 and 7 min incubation periods were treated with calf intestinal phosphatase (to prevent direct recircularisation of plasmid) prior to ligation to 14 bp phosphorylated XbaI oligonucleotides (5'-CTAGTCTAGACTAG-3') containing stop codons in all three frames. Multiple linkers were excised by re-cleaving with Xba I. The products were then recicularised and transformed into competent E.coli DH5 bacteria. Colonies were selected for ampicillin resistance. Small scale plasmid preparations from resulting colonies were analysed. The extent of Bal-31 digestion in each plasmid was estimated by comparing the size of the smaller BamHI/XbaI fragment with the smaller BamHI fragment of plasmid pB1. Several plasmids with estimated deletions ranging from 50 bp - 200 bp were selected and the exact deletion end points determined by dideoxynucleotide DNA sequence analysis using two 17mer oligonucleotide primers (nucleotides 20956 - 20940 and 20828 - 20812). Six plasmids referred to as pD17, pD27, pD32, pD34, pD50 and pD62, (where the number represents the number of codons of UL9 deleted as determined from DNA sequence analysis) were subsequently chosen for further study. All but one of these mutants (pD62) were found to contain single linker insertions.

The products of BamHI plus XbaI double digests of all six plasmids resolved on a 1% agarose gel are shown in **Figure 16.** A gradual increase in the electrophoretic mobility of the smaller BamHI/XbaI fragment indicates an increasing extent of deletion.

An example of a DNA sequence determination is presented in **Figure 17,** in which a portion of the UL9 sequence of mutant pD62 is shown. The sequence of plasmid pD62 clearly diverges from the known wt sequence at nucleotide 20895 and the presence of the linker sequence which contains a novel XbaI restriction site and three termination codons marks the limit of Bal-31 digestion.

The mutations within the six deleted plasmids and the

Figure 16. Restriction Enzyme Analysis of Deletion Mutant DNA Preparations

Mini preparations of deletion mutant plasmid DNA were double restricted with BamHI and XbaI and analysed on a 0.8% agarose gel alongside HinfI restricted pAT153 molecular weight marker fragments (lane 1), the sizes of which (bp) are indicated at the side. BamHI/XbaI digestions were performed on the following plasmid preparations: lane 2, pB1; lane 3, pD12; lane 4, pD17; lane 5, pD27; lane 6, pD32; lane 7, pD34; lane 8, pD50; lane 9, pD62.

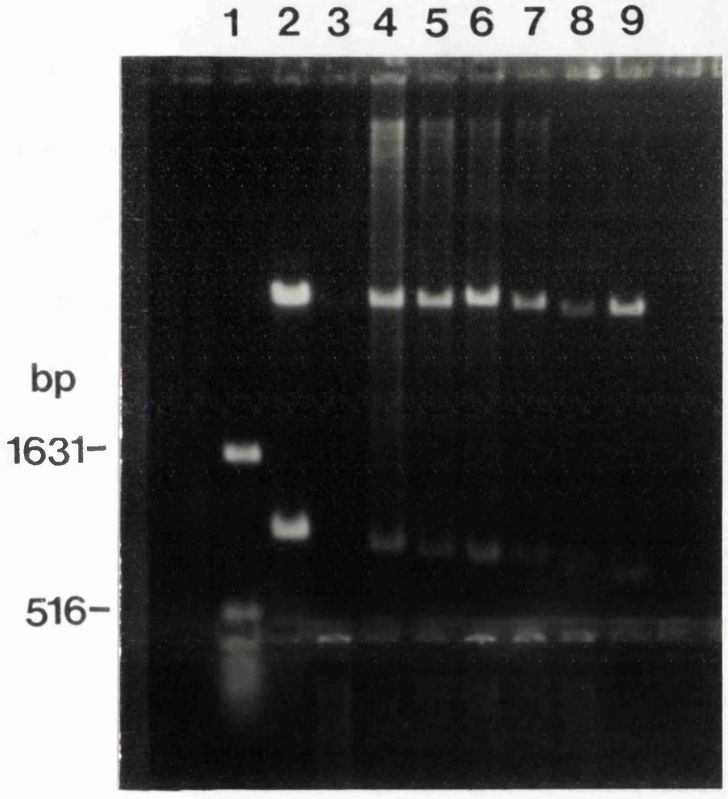
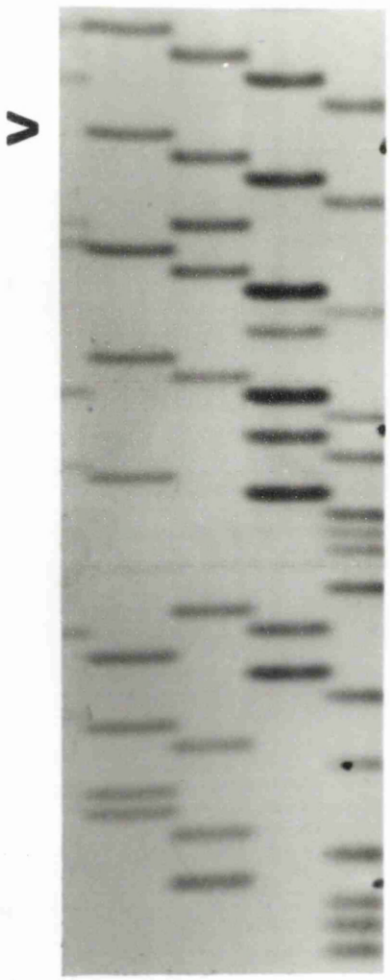

1 2 3 4 5 6 7 8 9
bp
1631–
516–

Figure 17. Mapping of Bal-31 Deletion End-points

Dideoxynucleotide DNA sequence analysis was carried out on deletion mutant plasmid preparations and the reaction samples analysed on a 6% denaturing urea/polyacrylamide gel. The DNA sequence, read upwards from nucleotide 20920 for plasmid pD62 is indicated below along with the sequence of the wt UL9 gene (McGeoch et al., 1988). The Bal-31 end-point at position 20896 where the sequence diverges from that of the wt is marked with a * and the location of the 14bp XbaI oligonucleotide linker indicated between the two arrows.

```
                                    XbaI Linker
(pD62)                          <             >

5'-CCCACAGGCAGCTGTACGCCCTGCTCTAGTCTAGACTAG-3'
                                *
5'-CCCACAGGCAGCTGTACGCCCTGCTTATGGCCCACAAGC-3'
(wt)
```



20920

G A T C

alterations to the encoded products are summarised in **Table 1.**

Initially 10 mutants with deletions corresponding to between 12 and 62 amino acids from the C-terminus were analysed by DNA sequencing. Protein extracts were prepared and examined in Western blot assays. Three of the mutants (with deletions of 22, 40 and 46 amino acids from the C-terminus) failed to yield a UL9 fusion protein while another (D25) yielded only low levels of protein. The reason for this is unclear, but may result from the introduced mutations creating an insoluble or unstable protein product or a product whose resulting secondary structure is unrecognisable to the antibody conjugate. In addition, a defect in the host cell may be responsible. Subsequent re-transformation of plasmid pD25 resulted in a level of fusion protein expression similar to the others suggesting a host cell defect in this case. One other deletion mutant (with a 12 amino acid deletion) expressed a protein which suffered degradation upon storage. This mutant and the three non-expressing mutants (D22, D40 and D46), were therefore omitted from further study.

(b) Construction and Characterisation of In-frame Insertion mutants

A series of pB1 mutants encoding products with 4 amino acid insertions were prepared by partially digesting plasmid pB1 with two multi-cut restriction enzymes, HaeIII (cleaves at GG/CC sequences) and MvnI (cleaves at CG/CG sequences) under conditions which produced a maximum of singly cut linear molecules with blunt ends. Partially digested pB1 samples were analysed on 1% agarose gels and those exhibiting the highest proportion of linear molecules chosen for use. Linear molecules were excised from a preparative gel and purified by electroelution and DEAE-sephacel column chromatography. Following treatment with calf intestinal phosphatase, linear molecules were ligated to phosphorylated, blunt-ended 12 bp EcoRI linker

Table 1. Plasmids Used in these Studies: Deletion Mutants

PLASMID	LINKER[1]	POSITION[2]	CHANGE[3]
pB1	-	-	-
pD16	T	20758/20757	834 LPTE-->LP*
pD27	T	20791/20790	823 ADII-->AD*
pD32	T	20807/20806	818 SANPNA-->SASLD*
pD34	T	20810/20809	817 SSANPN-->STSLD*
pD50	T	20859/20858	800 RFKLR-->RFLV*
pD62	T	20896/20895	788 LLMA-->LL*

[1] T denotes termination linker (CTAGTCTAGACTAG).

[2] Position of linker within HSV-1 DNA sequence (McGeoch et al., 1988).

[3] Alteration to UL9 amino acid sequence. The number refers to the position of the adjacent amino acid. * represents the C-terminus.

oligonucleotides. The linkers inserted at MvnI and HaeIII sites had the sequences 5'-CCGGAATTCCGG-3' and 5'-CCCGAATTCGGG-3' respectively. Linker multimers were reduced to single copies by EcoRI digestion followed by ligation to circularise the plasmid molecules. The products were then transformed into E.coli cells and colonies selected for ampicillin resistance. Plasmids containing linker insertions within the UL9 coding region were identified by screening small-scale plasmid preparations from selected bacterial colonies by digestion with EcoRI plus BamHI.

The sites of linker insertions were first mapped using an EcoRI/PstI double digestion then verified by dideoxynucleotide sequencing of the entire UL9 C-terminal fragment. Within the C-terminal one third of the UL9 gene there are 15 MvnI and 13 HaeIII restriction sites spanning the region (**Figure 18**). Unfortunately, enzyme analysis of mini DNA preparations of bacterial colonies transformed with mutagenised pB1 revealed that linker insertions were only present in 12 of the available 28 sites. Indeed, the two enzymes appeared to cleave preferentially at three of these sites in particular. Dideoxy sequencing of the entire UL9 specific region of mutants with insertions in these 12 sites revealed that each one contained a single linker insertion although one mutant (with a linker insertion at amino acid 594) also contained an internal deletion. The latter was omitted from further study.

Figure 19 shows part of the DNA sequence of one insertion mutant (pI838) and compares it with the known wt sequence. The linker insertion occurs at position 20750 and a novel EcoRI restriction site is present.

Ten plasmids, pI581, pI591, pI630, pI652, pI668, pI691, pI708, pI719, pI799 and pI838, containing insertions spanning the UL9 C-terminal sequence were selected for further analysis (the number represents the amino acid position within the UL9 polypeptide corresponding to the site of insertion). The products of EcoRI plus PstI double digests of the 10 mutant plasmids

Figure 18. Location of HaeIII and MvnI Restriction Sites Within the C-terminal One Third of the UL9 Gene

A diagramatical representation of the UL9 gene is shown. The C-terminal one third of the gene has been enlarged below and the approximate position of the HaeIII and MvnI restriction sites within this region are indicated. The numbering of the scale refers to positions within the HSV-1 UL9 gene (McGeoch et al., 1988). Those marked with a diamond represent sites where EcoRI linker oligonucleotides were inserted.

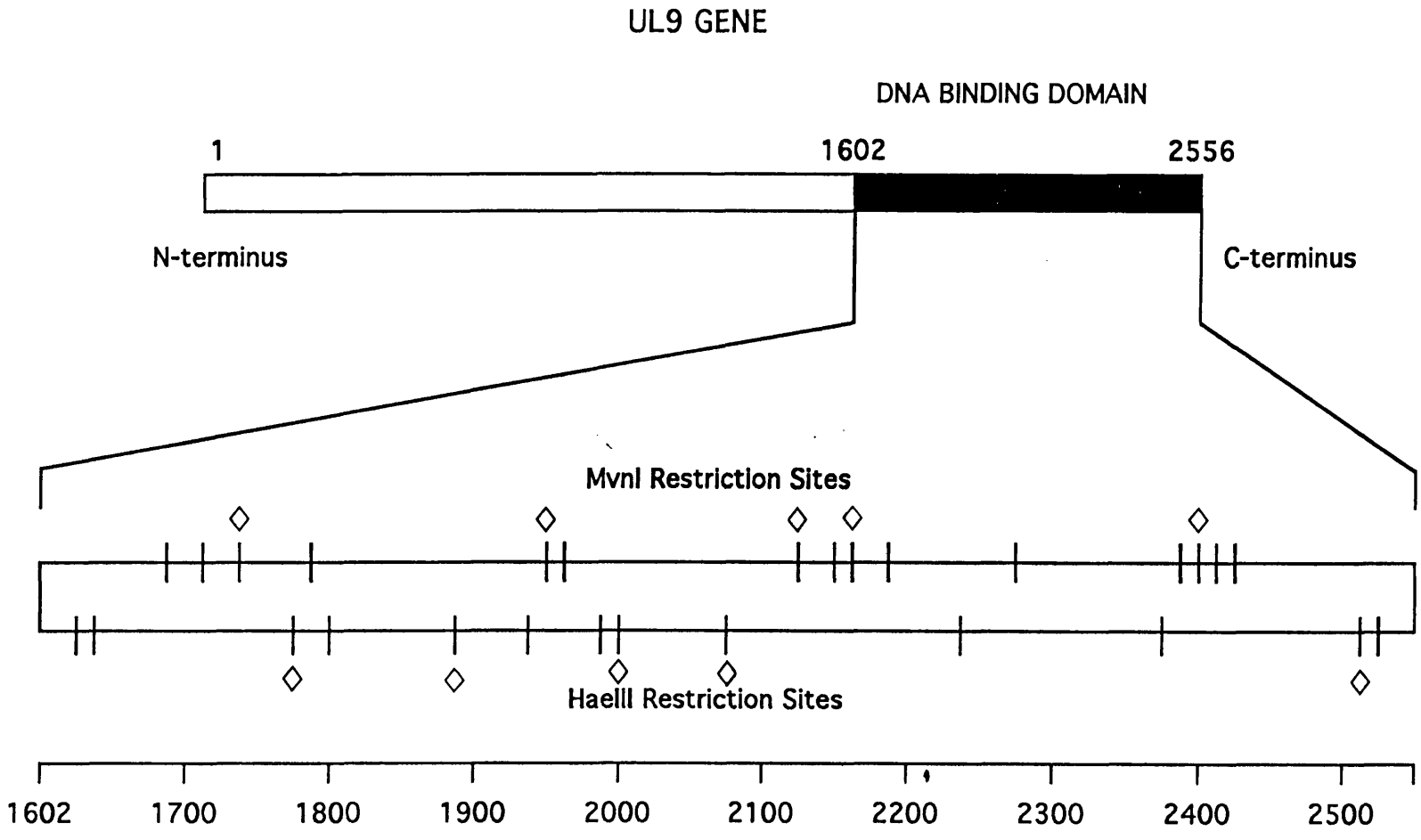
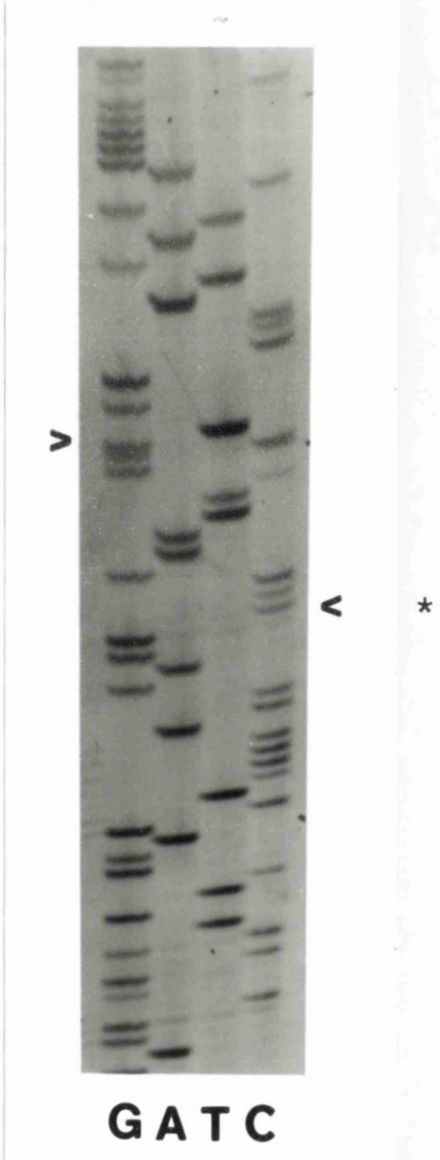

UL9 GENE
DNA BINDING DOMAIN
1
1602
2556
N-terminus
C-terminus
MvnI Restriction Sites
HaeIII Restriction Sites
1602
1700
1800
1900
2000
2100
2200
2300
2400
2500

Figure 19. Mapping of EcoRI Linker Oligonucleotide Insertion Sites

Dideoxynucleotide DNA sequence reactions were carried out on insertion mutant plasmid preparations and the reaction samples resolved on a 6% denaturing, urea/polyacrylamide gel. The DNA sequence read upwards from nucleotide 20766 for mutant pI838 is indicated below. The site of the linker insertion at position 20750 is marked with a * and the 12bp EcoRI linker itself is indicated between the arrows.

```
                        EcoRI linker
(pI838)                 <          >
5'-GGAGCTCCCCACCGAGGCCCGAATTCGGGCCTGGCCCATGATGCA-3'
                        *
5'-GGAGCTCCCCACCGAGG              CCTGGCCCATGATGCA-3'
(wt)
```


20766 –
>
<
*
GATC

resolved on a 1% agarose gel are shown in **Figure 20.**

The resulting alterations to the protein coding sequence are described in **Table 2.**

Initially eleven insertion mutants were tested for expression of UL9 fusion proteins in Western blot assays. Two of these (with linker insertions at amino acids 799 and 807) failed to express detectable UL9 fusion protein. Re-transformation of mutant I799 but not I807 into DH5 cells resulted in fusion protein expression. It is possible that the I807 mutation results in production of an unstable protein. Interestingly this insertion lies close to or within the region where mutants with deletions terminating there also failed to express a UL9 fusion protein. Indeed, Martinez et al., (1992) reported that a mutant containing a 2 amino acid insertion within this region (at amino acid 798) failed to express a stable UL9 protein.

3.1.5 Analysis of pB1 Deletion Mutants

The ability of the pB1 deletion mutant proteins (A-9CT mutant proteins) to bind to the HSV-1 origin of replication was examined in gel retardation assays.

Extracts were prepared from untransformed E.coli DH5 cells and transformants carrying the wt plasmid pB1 or plasmids pD16, pD27, pD32, pD34, pD50 and pD62 which express C-terminally truncated forms of the protein A-UL9 fusion. The presence of fusion proteins was confirmed by Western blots and gel retardation assays were performed to examine sequence-specific DNA binding activity. ^{32}P-labelled oligonucleotides containing either the sequences of ori$_S$ binding site I or II (**Figure 21**), or a ^{32}P-labelled 100 bp fragment from plasmid pEC8 (Weir et al., 1989) containing the complete ori$_S$ (i.e both binding sites) were used as probe fragments. The labelled DNA fragments were incubated with the extracts at 22^{o}C and resulting complexes resolved on 5% polyacrylamide gels.

(a) Western Blot Analysis

To determine whether a pB1-related product was

Figure 20. **Restriction Enzyme Analysis of Insertion Mutant DNA Preparations**

Mini plasmid preparations of insertion mutant DNA were double resticted with EcoRI and PstI and analysed on a 0.8% agarose gel alongside HinfI restricted pAT153 molecular weight marker fragments (lanes 1 and 13), the sizes of which (bp) are indicated at the side. Enzyme restrictions were performed on the following plasmid preparations: Lane 2, pB1; lane 3, pI581; lane 4, pI591; lane 5, pI630; lane 6, pI652; lane 7, pI668; lane 8, pI691; lane 9, pI708; lane 10, pI719; lane 11, pI799; lane 12, pI838.

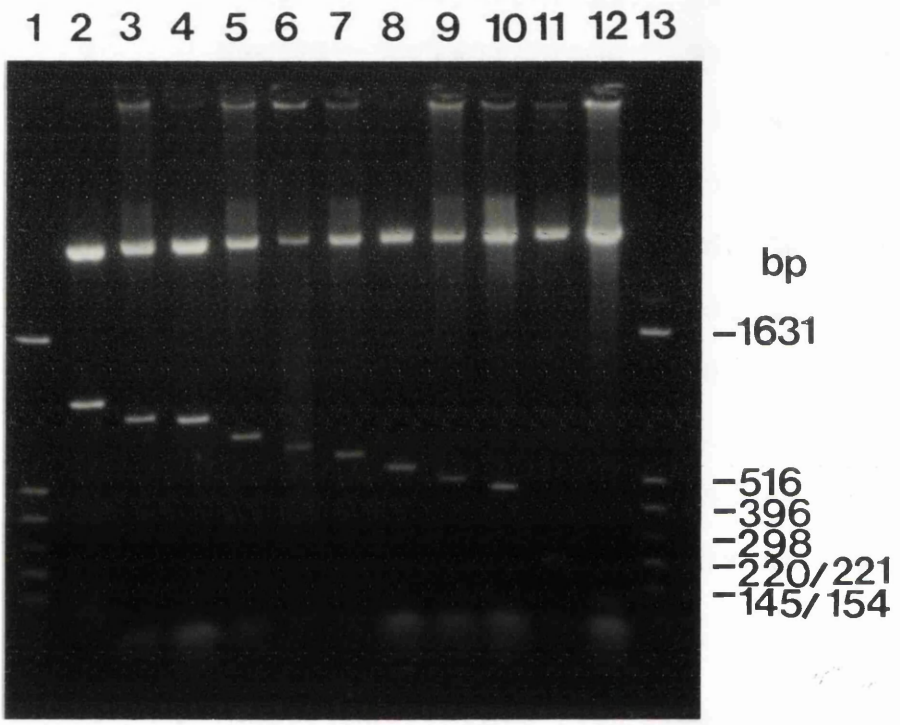

1 2 3 4 5 6 7 8 9 10 11 12 13
bp
1631
516
396
298
220/221
145/154

Table 2. Plasmids Used in these Studies: Insertion Mutants

PLASMID	LINKER[1]	SITE[2]	POSITION[3]	CHANGE[4]
pB1	-	-	-	-
pI581	I_1	MvnI	21519/21518	581 TR-->TPEFRR
pI591	I_2	HaeIII	21491/21490	591 AC-->ARIRAC
pI630	I_2	HaeIII	21375/21374	630 AL-->ARIRAL
pI652	I_1	MvnI	21308/21307	652 AR-->AGIPAR
pI668	I_2	HaeIII	21259/21258	668 GP-->GPNSGP
pI691	I_2	HaeIII	21190/21189	691 GH-->GPNSGH
pI708	I_1	MvnI	21139/21138	708 RV-->RRNSGV
pI719	I_1	MvnI	21106/21105	719 RG-->RRNSGG
pI799	I_1	MvnI	20865/20864	799 AR-->APEFRR
pI838	I_2	HaeIII	20750/20749	838 AW-->ARIRAW

[1] I_1 and I_2 denote insertion linkers CCGGAATTCCGG or CCCGAATTCGGG respectively.

[2] Enzyme cleavage sites at which I_1 and I_2 linkers were inserted.

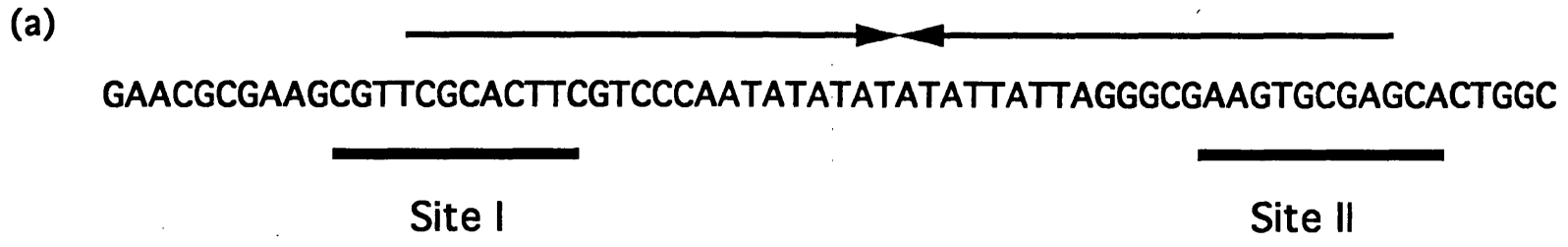
[3] Position of linker within the HSV-1 DNA sequence (McGeoch et al., 1988).

[4] Alteration to UL9 amino acid sequence. The number refers to the position of the adjacent amino acid.

Figure 21. **Recognition Sequences for the HSV-1 UL9 Protein within HSV-1 Ori_S**

(a) DNA sequence of the ori_S region.

(b) DNA sequence of oligonucleotides containing binding sites I and II.


(a)
GAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGC
Site I
Site II
(b)
Oligonucleotide I
5' GATCCGCGAAGCGTTCGCACTTCGTCCCA
GCGCTTCGCAAGCGTGAAGCAGGGTCTAG
Oligonucleotide II
5' GATCCGCGAAGTGCGAGCACTTCGCCCCA
GCGCTTCAGCCTCGTGAAGCGGGGTCTAG

Minor degradation products of the pD50 product can be seen in lane 7.

present in each of the extracts a Western blot was performed and probed directly with an IgG-alkaline phosphatase conjugate.

UL9 fusion protein was present in all six extracts from transformed DH5 cells, and a gradual increase in electrophoretic mobility was apparent correlating with the increasing extent of deletion (**Figure 22**).

(b) Gel Retardation Analysis: Interaction with Binding Site I

The results of a gel retardation assay examining the ability of the A-9CT deletion mutants to bind to a ^{32}P-labelled probe containing only binding site I of ori$_S$ are presented in **Figure 23.** As shown previously (Weir et al., 1989), extract from cells containing pB1 forms a major retarded complex (RC), (**lane 2**) which is absent from untransformed cells (**lane 1**) or cells transformed with the vector pRIT2T (data not shown). Retarded complexes were also obtained with extracts from cells harbouring plasmids pD16, pD27, pD32, and pD34 but not pD50 or pD62 (**lanes 3 - 8**).

(c) Gel Retardation Analysis: Interaction with Binding Site II and the Complete Ori$_S$ Fragment

The ability of the mutant proteins to interact with ori$_S$ binding site II and with the pEC8 ori$_S$ fragment (containing both binding sites) at 22^{o}C was also tested (**Figures 24 and 25,** respectively). Comparison of **Figures 23,** showing the interaction with site I, and **Figure 24** reveals that the mutant proteins showed similar behaviour towards the two probe fragments, i.e. only those mutant proteins showing a positive interaction with site I bound efficiently to site II. Gel retardation experiments using a probe containing both binding sites (**Figure 25**), also showed a similar behaviour of the various extracts. However, with the proteins exhibiting positive binding the bands appear more smeared and no free probe remains. The reason for the lack of free probe is not obvious. It could reflect higher affinity binding possibly as a

Figure 22. Western Blot Analysis of pB1 Deletion Mutant Extracts

1ug samples of protein extract were resolved on a 9% denaturing polyacrylamide gel alongside protein molecular size markers and blotted onto nitrocellulose. The blot was probed with alkaline phosphatase-conjugated anti-rabbit IgG which was visualised by the addition of colour development substrates supplied in the Promega western blot AP detection kit. Extracts were from bacteria transformed with the following plasmids : Lane 1, untransformed DH5 cells; lane 2, pB1; lane 3, pD16; lane 4, pD27; lane 5, pD32; lane 6, pD34; lane 7, pD50; lane 8, pD62. The positions of the protein molecular size markers are indicated on the left.

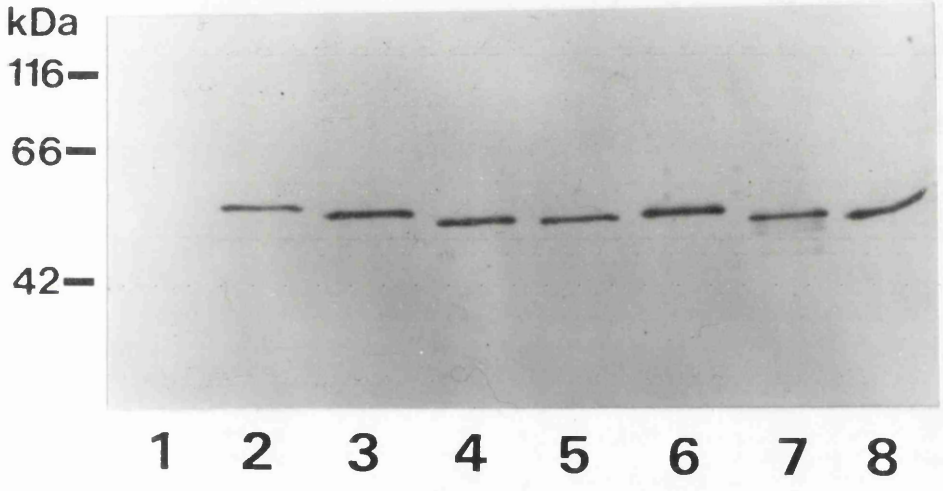

kDa
116
66
42
1
2
3
4
5
6
7
8

Figure 23. **Gel Retardation Analysis of pB1 Deletion Mutants: Interaction with Binding Site I**

Bacterial protein extracts were incubated with ^{32}P end-labelled oligonucleotide I (see **Figure 21.**) containing the HSV-1 ori_S binding site I for 20 min at 22°C and the products separated by electrophoresis through a 5% polyacrylamide gel as described in materials and methods. Extracts were from E.coli DH5 bacteria transformed with the following plasmids: Lane 1, untransformed DH5 cells; lane 2, pB1; lane 3, pD16; lane 4, pD27; lane 5, pD32; lane 6, pD34; lane 7, pD50; lane 8, pD62.

"RC" indicates the major retarded complex and "FP", free unbound probe.

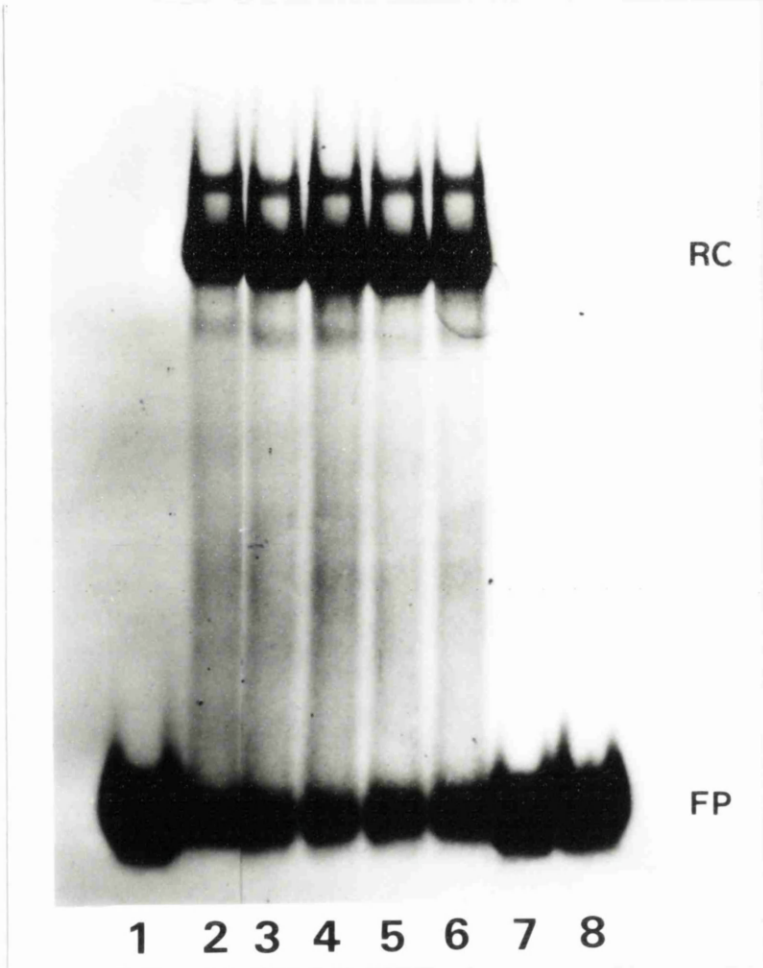

RC
FP
1 2 3 4 5 6 7 8

Figure 24. Gel Retardation Analysis of Deletion Mutants: Interaction with Binding Site II

Binding was analysed as described in **Figure Legend 23,** except oligonucleotide I was replaced by oligonucleotide II containing ori_S binding site II (**Figure 21**). Extracts were from E.coli DH5 bacteria transformed with the following plasmids: Lane 1, untransformed DH5 cells; lane 2, pB1; lane 3, pD17, lane 4, pD27; lane 5, pD32; lane 6, pD33; lane 7, pD50; lane 8, pD62.

"RC" indicates the major retarded complex and "FP", free unbound probe.

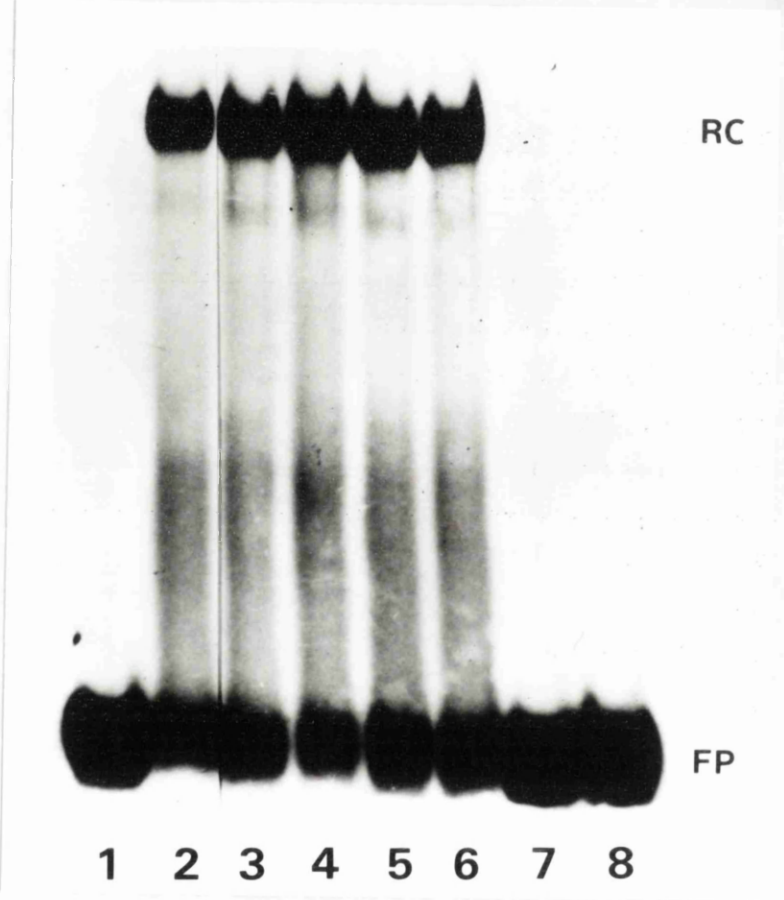
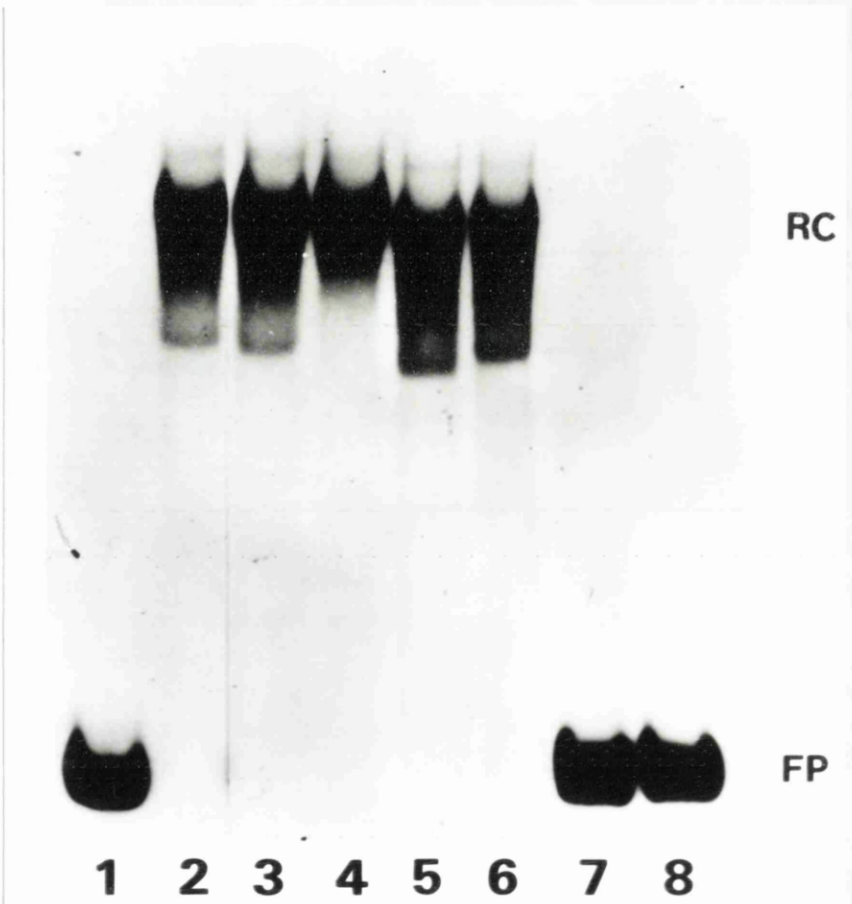

RC
FP
1 2 3 4 5 6 7 8

Figure 25. **Gel Retardation Analysis of Deletion Mutants: Interaction with Complete Ori_S**

Binding was analysed as described in **Figure Legend 23.** except that oligonucleotide I was replaced with a 100 bp fragment from plasmid pEC8 containing the whole ori_S sequence. Extracts were from bacteria transformed with the following plasmids: lane 1, untransformed DH5 cells; lane 2, pB1; lane 3, pD17; lane 4, pD27; lane 5, pD32; lane 6, pD33; lane 7, pD50; lane 8, pD62.

"RC" indicates the major retarded complex and "FP", free unbound probe.

In repeat experiments the slightly faster migrating retarded complex was seen with the pD27 extract.


RC
FP
1 2 3 4 5 6 7 8

result of cooperativity between proteins bound to both sites. The smeared appearance of bands is probably due to a mixed population of complexes comprising DNA fragments with protein bound to both sites (lower electrophoretic mobility complex) or to only one site (higher mobility complex).

The results from the gel retardation analysis demonstrate that removal of 34 amino acids from the C-terminus of the UL9 protein does not impair its ability to interact with sequences from the HSV-1 ori_S region. Since the product specified by plasmid pD50 is non-functional, residues essential for this interaction must lie between amino acids 801 and 818 of UL9.

3.1.6 Analysis of pB1 Insertion Mutants.

The abilities of the A-9CT insertion mutants to interact with HSV-1 origin sequences were also examined using gel retardation assays.

Extracts were prepared from untransformed E.coli DH5 cells and transformants carrying the wt plasmid pB1 or plasmids pI581, pI591, pI630, pI652, pI668, pI691, pI708, pI719, pI799 and pI838 which express fusion proteins containing insertions of four amino acids within the UL9-specific portion. Extracts were tested for the expression of pB1-related UL9 fusion proteins using Western blot assays.

(a) Western Blot Analysis

A Western blot was carried out to determine whether the extracts from cells containing the 10 insertion mutant plasmids contained stable pB1-related fusion proteins (**Figure 26a and b**). Fusion proteins with electrophoretic mobilities indistinguishable from the pB1 product (**lane 2 in both Figures**) were observed in extracts from the bacterial cells transformed with each of the insertion mutants.

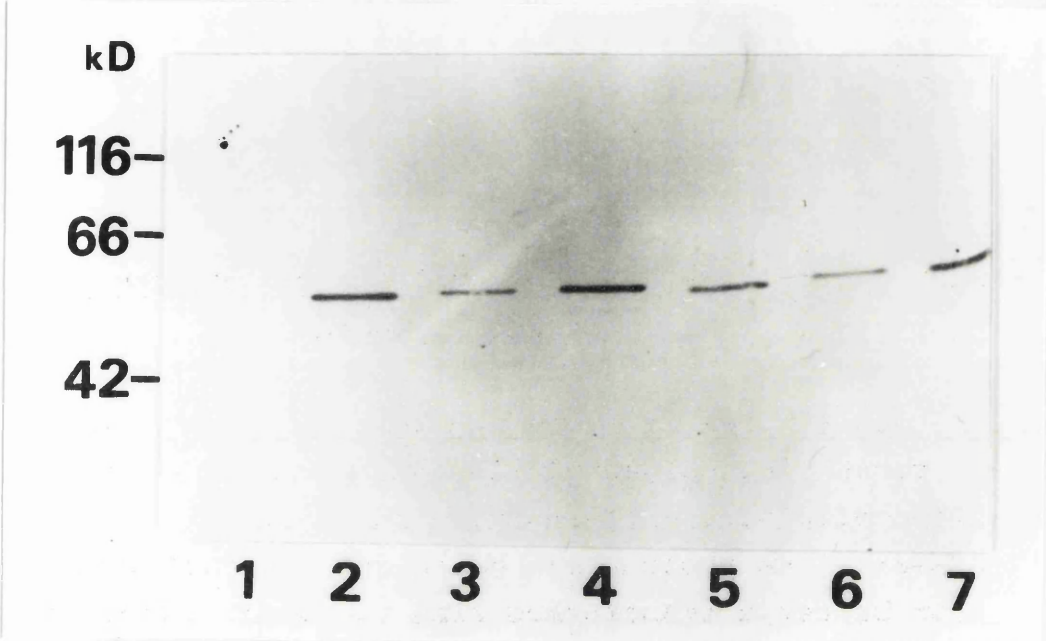
(b) Gel Retardation Analysis: Interaction with Site I at 22°C and 37°C

Figure 26. Western Blot Analysis of pB1 Insertion Mutant Extracts

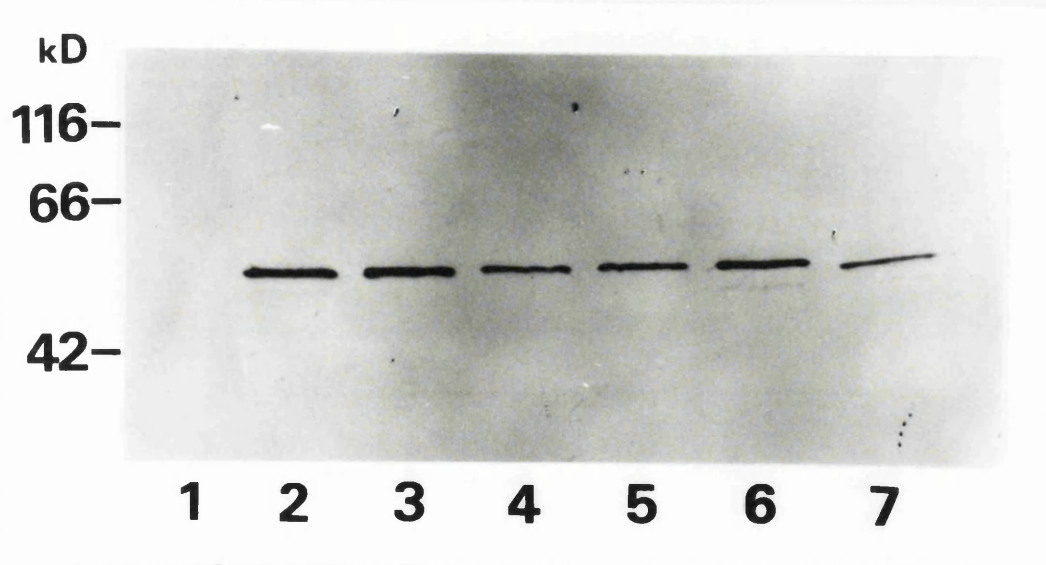
1ug samples of protein extracts were resolved on a 9% denaturing polyacrylamide gel and blotted onto nitrocellulose. Proteins were detected using the Promega IgG/alkaline phosphatase detection kit. Extracts were from bacteria transformed with the following plasmids :
Figure a. Lane 1, untransformed DH5 cells, lane 2, pB1, lane 3, pI581; lane 4, pI591; lane 5, pI630; lane 6, pI651; lane 7, pI668.
Figure b. lane 1, untransformed DH5 cells; lane 2, pB1; lane 3, pI691; lane 4, pI708; lane 5, pI719; lane 6, pI799; lane 7, pI838.

Protein molecular size markers are indicated on the left.

a)



b)



The ability of the mutated proteins to bind to a ^{32}P-labelled probe containing the HSV-1 ori$_S$ binding site I sequence was examined and the results of the binding assays performed at 22°C are presented in **Figure 27a.** Although each of the extracts from cells transformed with a mutant plasmid contained a fusion protein which exhibited an electrophoretic mobility indistinguishable from the pB1 product, only the pI691, pI708, pI719 and pI838 products were unaffected in their binding ability (**Figure a; lanes 8, 9, 10, 12**). The products of pI799 and pI630 showed slightly, and more severely impaired binding (**lanes 11 and 5**, respectively) whereas no binding was detected with the remaining pI581, pI591, pI652 and pI668 encoded proteins (**lanes 3, 4, 6, 7**).

When similar assays were performed at 37°C using the ^{32}P-labelled oligonucleotide containing site I, the ability of the pI630 product to form a protein/DNA complex at the higher temperature was abolished while the relative binding activities of the other proteins remained unchanged (**Figure 27b**).

(c) <u>Gel Retardation Analysis: Interaction with Site II and the Complete Ori$_S$ at 22°C and 37°C</u>

The ability of the mutant proteins to interact with ori$_S$ binding site II at 22°C and 37°C was also examined (**Figure 28a and b**). Comparison of **Figures 27a** and **28a** in which binding was at 22°C reveals that the mutant proteins showed similar behaviour towards the two probe fragments with the exception that the pI630 and pI799 products formed much lower amounts of retarded complex with binding site II than site I (**lanes 5 and 11**). Repetition of the experiment at 37°C demonstrated that the pI630 protein was unable to form a complex with binding site II at the higher temperature while the relative binding activities of the other proteins were not affected (**Figure 28b**). Thus, the pI630 expressed protein exhibits temperature sensitivity in its binding to both sites I and II.

The ability of the mutant proteins to bind to

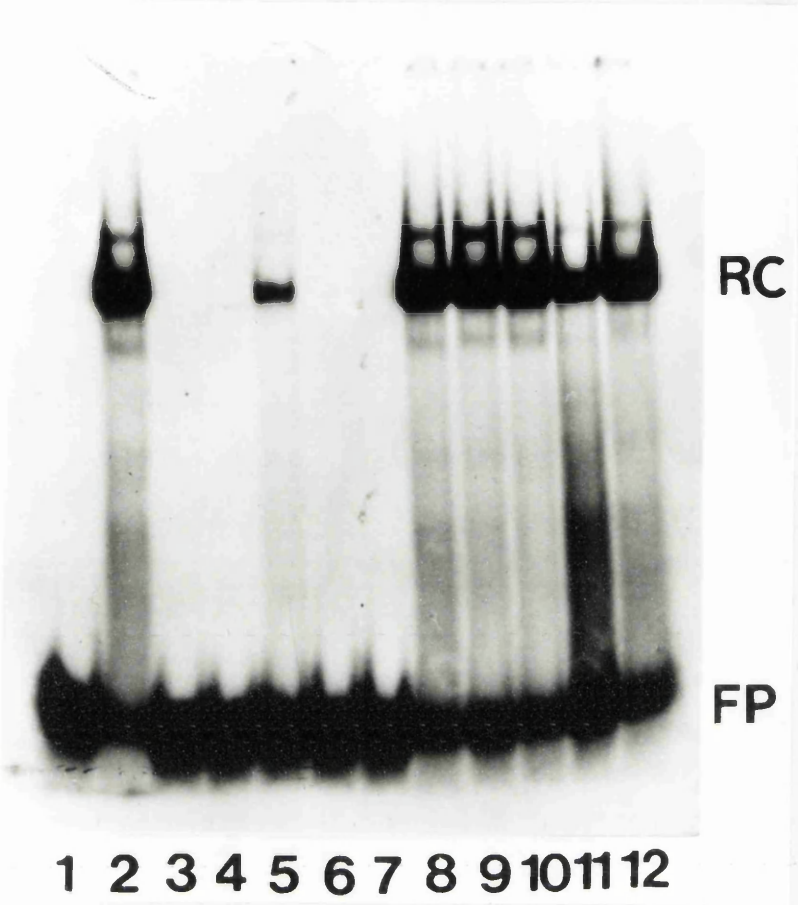
Figure 27. **Gel Retardation Analysis of Insertion Mutants: Interaction with binding site I at 22°C and 37°C**

Bacterial protein extracts were incubated with ^{32}P end-labelled oligonucleotide I (**Figure 21**) containing the HSV-1 ori_S binding site I for 20 min at either 22°C (**Figure a.**) or 37°C (**Figure b.**). The products were separated by electrophoresis through a 5% polyacrylamide gel as described in materials and methods. Extracts were from E.coli DH5 bacteria transformed with the following plasmids: Lane 1, untransformed DH5 cells; lane 2, pB1; lane 3, pI581; lane 4, pI591; lane 5, pI630; lane 6, pI652; lane 7, pI668; lane 8, pI691; lane 9, pI708; lane 10, pI719; lane 11, pI799; lane 12, pI838.

"RC" indicates the major retarded complex and "FP", free unbound probe.

a)

22°C



b)

37°C

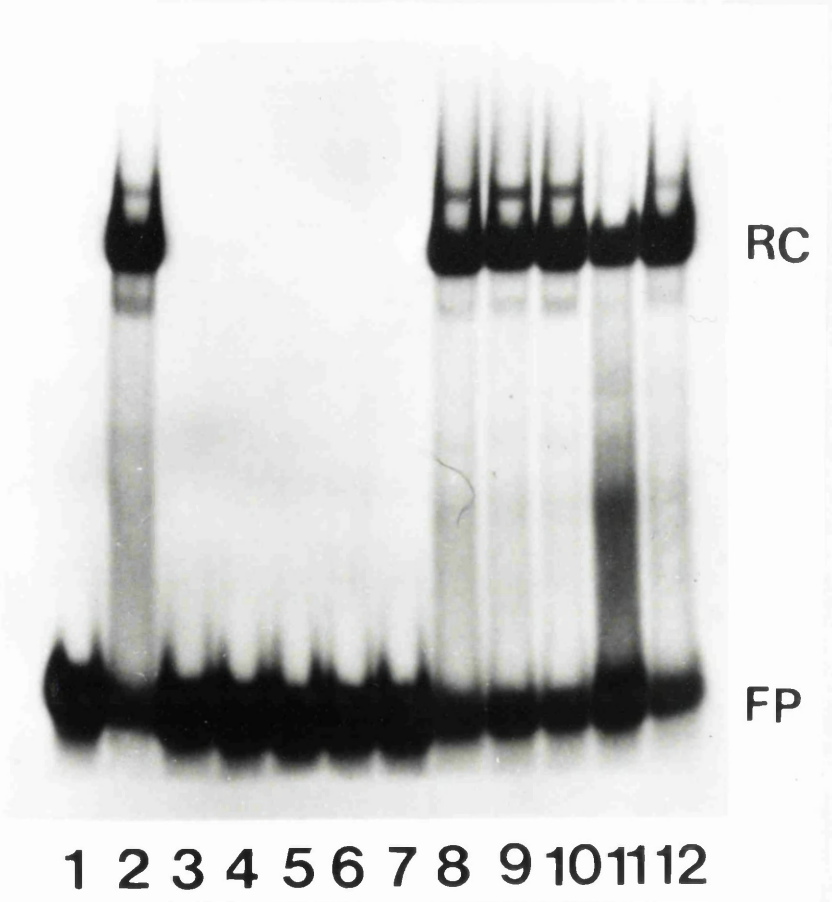


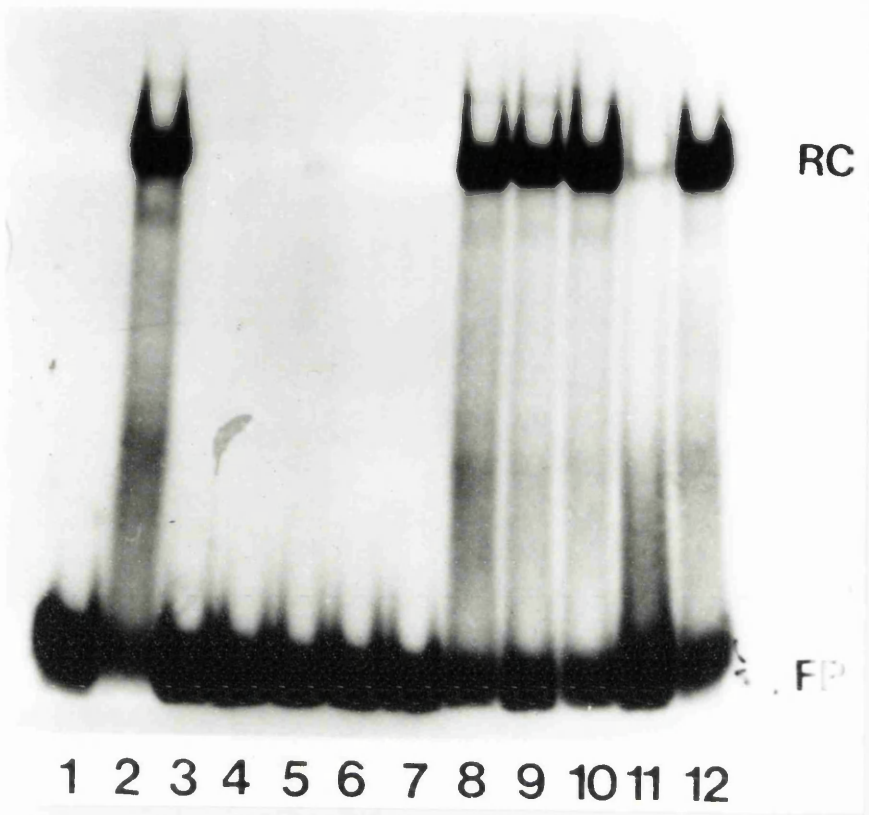
Figure 28. **Gel Retardation Analysis of Insertion Mutants: Interaction with site II at 22°C and 37°C**

Binding was analysed as described in **Figure legend 27,** except that oligonucleotide I was replaced with oligonucleotide II. Extracts for binding analysis performed at 22°C (**Figure a.**) and 37°C (**Figure b.**) were from E.coli DH5 cells transformed with the following plasmids: lane 1, untransformed DH5 cells; lane 2, pB1; lane 3, pI581; lane 4, pI591; lane 5, pI630; lane 6, pI652; lane 7, pI668; lane 8, pI691; lane 9, pI708; lane 10, pI719; lane 11, pI799; lane 12, pI838.

"RC" indicates the major retarded complex and "FP", free unbound probe.

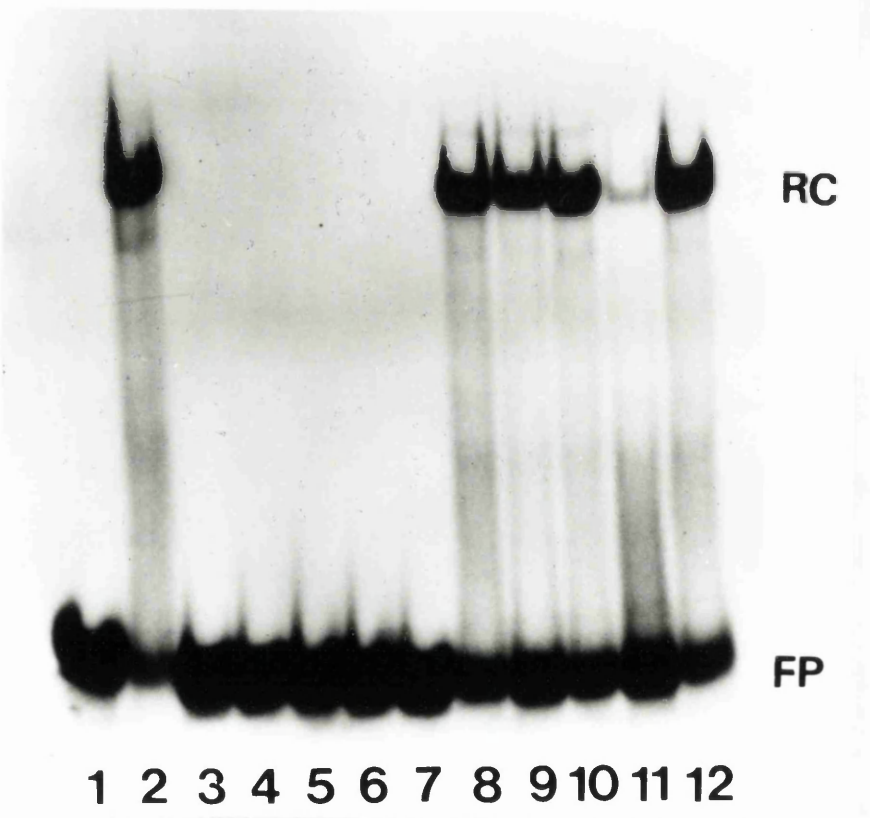
a)

22°C



b)

37°C



It would also appear that pI691 (lane 8) and pI719 (lane 10) mutant extracts exhibit a temperature sensitive effect with binding to the complete ori_S fragment as an increase in temperature resulted in a change in the amount of the two electrophoretically distinct complexes being formed.

fragment III which contains a functional ori_S with both binding sites was tested at 22°C and 37°C (**Figure 29a and b**).

At 22°C (**Figure a**), the behaviour of the extracts was similar to that observed with binding to site II and in general all the proteins exhibiting positive binding were slightly impaired compared with wt. Binding of mutant I630 to the complete ori_S fragment produced a single retarded complex of higher electrophoretic mobility than that observed by the wt A-9CT protein. Binding of the remaining mutants also produced this higher mobility complex as a minor band, in addition to a major complex of equal mobility to that produced by wt A-9CT protein. The position of the major complex of mutant I799 appears to be intermediate between the major and minor complexes of the other mutants. It is most likely that the higher mobility complex represents binding to a single site and the lower mobility complex binding to both sites.

Binding to the complete ori_S fragment at 37°C (**Figure b**), confirmed that the weak binding of mutant I630 was ts as it was unable to form a complex at the higher temperature.

These results indicate that 4 amino acids can be inserted at 4 separate sites within the DNA binding domain without affecting interactions with the UL9 recognition sequence (mutants pI691, pI708, pI719 and pI838). However, insertions at 4 different sites abolished detectable interactions (mutants pI581, pI591, pI652, pI668) and at two further positions (mutants pI630 and pI799), generated products which exhibited reduced binding, particularly to the lower affinity site II sequence.

3.1.7 The Effect of Competitor DNA on Fusion Protein Binding Activity

Competition experiments were carried out to compare the relative binding affinity of the A-9CT and I719 proteins to ori_S site I.

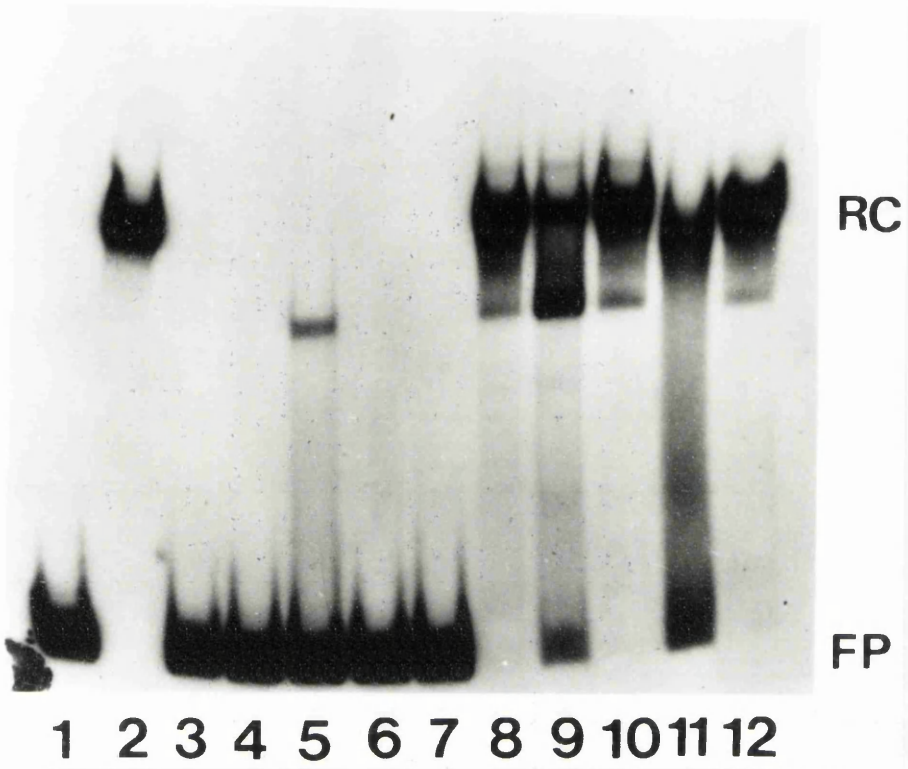
Figure 29. **Gel Retardation Analysis of Insertion Mutants: Interaction with complete oris at 22°C and 37°C**

Binding was analysed as described in **Figure legend 27,** except that oligonucleotide I was replaced with a 100 bp fragment containing both oris binding sites I and II. Extracts for binding analysis performed at 22° (**Figure a.**) and 37°C (**Figure b.**) were from E.coli DH5 cells transformed with the following plasmids: lane 1, untransformed cells; lane 2, pB1; lane 3, pI581; lane 4, pI591; lane 5, pI630; lane 6, pI652; lane 7, pI668; lane 8, pI691; lane 9, pI708; lane 10, pI719; lane 11, pI799; lane 12, pI838.

"RC" indicates the major retarded complex and "FP", free unbound probe.

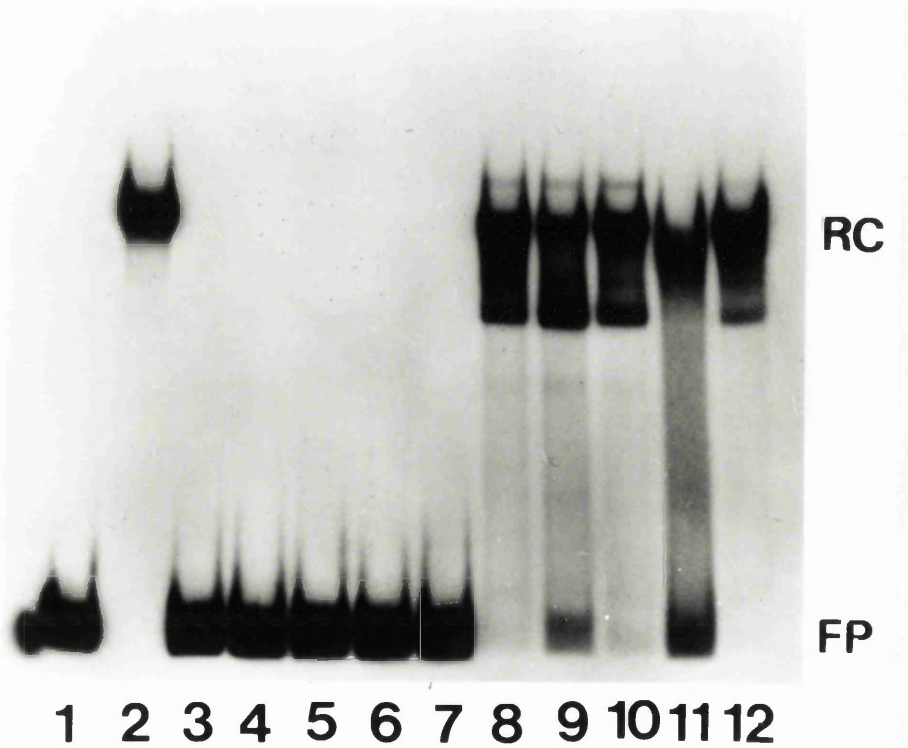
a)

22°C



b)

37°C



Protein extracts from E.coli DH5 cells transformed with either plasmid pB1 or pI719 were incubated with ^{32}P-labelled oligonucleotide I either in the presence or absence of unlabelled oligonucleotide I DNA (500 fold excess), for 30 min to allow binding to reach equilibrium. A 500-fold excess of unlabelled oligonucleotide I was then added to the reaction lacking competitor DNA. Samples were taken at various time intervals (from 0 - 10 min) after addition of unlabelled probe and immediately loaded and subjected to electrophoresis through a 5% polyacrylamide gel.

The samples containing A-9CT extract are shown in **Figure 30.** When competitor DNA was added together with the labelled probe (**lane 1**), the signal intensity of the retarded complex was very weak. The sample taken just before addition of competitor DNA (**lane 2**) demonstrated a retarded complex of high intensity, similar to those observed in other gel retardation assays. The samples taken at each of the times (from 1 min) after addition of competitor DNA, however demonstrated that the signal intensity of the retarded complex fell rapidly to the level observed when the unlabelled probe was included at the start of the reaction. Similar results were observed when the effect of competitor DNA on the binding activity of I719 extracts was analysed (data not shown).

These results indicate that the A-9CT protein must have a rather low affinity for ori_S site I (since the half-life of the complex is less than one minute). For this reason it was not possible to determine whether the I719 had a reduced affinity compared with A-9CT.

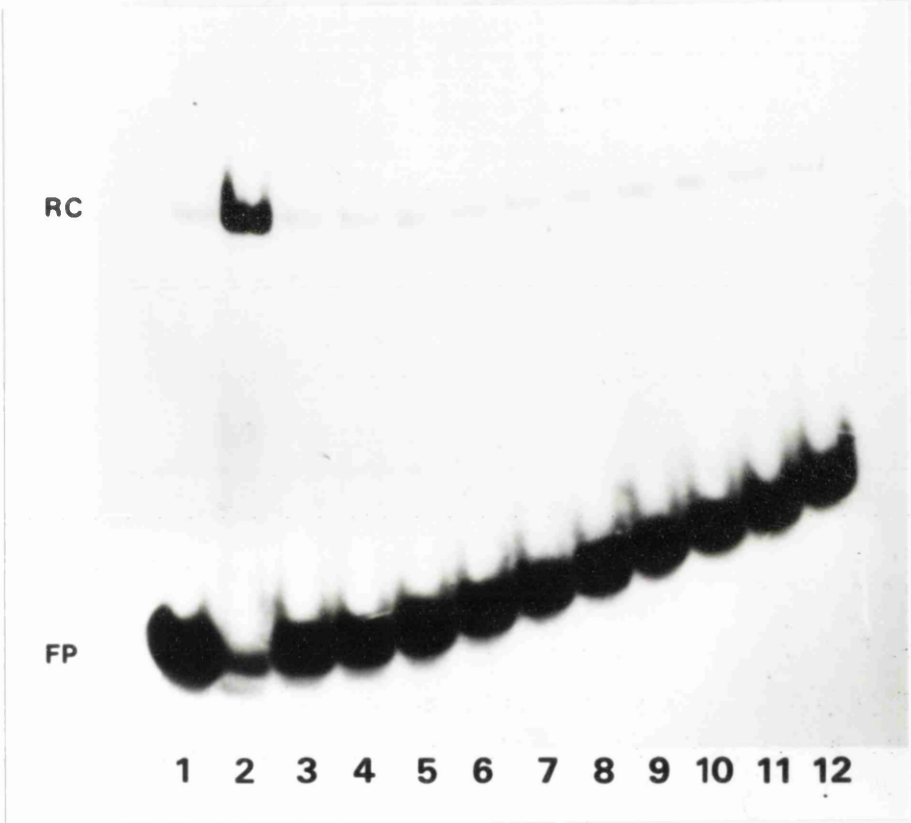
3.2 CONSTRUCTION AND ANALYSIS OF BACULOVIRUS RECOMBINANTS EXPRESSING MUTANT UL9 PROTEINS

3.2.1 Introduction

Although the experiments in which mutated fusion proteins were expressed in E.coli enabled the effects of the mutations on origin binding to be studied it was not possible to determine the effects of these mutations on

Figure 30. The Effect of Competitor DNA on Fusion Protein Binding Activity

Protein extract prepared from E,coli DH5 cells transformed with plasmid pB1 was incubated with ^{32}P-labelled oligonucleotide I containing the HSV-1 ori_S binding site I in the presence (lane 1) or absence of 500-fold excess of unlabelled oligonucleotide I. After 30 min at 22°C, 500-fold unlabelled oligonucleotide I DNA was added to the binding reaction lacking competitor DNA. Samples were taken at one minute intervals after the addition of unlabelled probe and analysed immediately on a 5% polyacrylamide gel. An autoradiograph of the dried gel is shown.


RC
FP
1 2 3 4 5 6 7 8 9 10 11 12

HSV-1 origin dependent DNA synthesis. A selection of the mutations described in the previous sections were therefore introduced into the full length UL9 ORF to allow the investigation of whether the alterations had any effect on the replicative function of the intact protein. The resulting full length UL9 proteins were then expressed under the control of the strong late polyhedrin promoter of the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV). This enabled the replicative ability of the mutated proteins to be assessed in a transient replication assay in Sf insect cells in which recombinant baculoviruses are used to supply all the HSV-1 DNA replication proteins.

3.2.2 Cloning of DNA Fragments into a Baculovirus Transfer Vector

Due to the large size of baculovirus genomes, recombinant viruses are produced by first cloning the gene to be expressed into a bacterial plasmid transfer vector. Foreign genes are cloned into the transfer vector downstream of a strong viral promoter and flanked by baculovirus sequences. When the modified transfer vector and infectious baculovirus DNA are introduced into insect cells homologous recombination, involving the flanking sequences in the transfer vector, allows insertion of the foreign gene into an infectious recombinant virus.

(a) Construction of Transfer Vector pY9N2/3

In order to introduce deletion and insertion mutations into an intact UL9 gene an intermediate plasmid (pY9N2/3) was first constructed which included the the N-terminal two thirds of the UL9 gene inserted into the baculovirus transfer vector pAcYM1 (Matsuura et al., 1987). This was achieved by using a previously described plasmid pBL91, in which the intact UL9 gene is located downstream of the AcNPV polyhedrin promoter. The small BstEII to BamHI fragment of plasmid pBL91 encoding the amino-terminal two thirds of the HSV-1 gene (nucleotides 23542 - 21655), was ligated to the large BstEII to BamHI

fragment of the baculovirus transfer vector pAcYM1 which provides the remainder of the vector sequences (Matsuura et al., 1987), (**Figure 31**). DH5 cells were transformed to ampicillin resistance, colonies selected and mini DNA preparations analysed. Plasmid pY9N2/3, with the desired structure, was selected for further use. The plasmid pY9N2/3 contains a unique BamHI restriction site into which the previously described mutated fragments encoding the C-terminal portion of UL9 could be inserted, in the correct orientation, to generate a complete UL9 gene.

(b) Insertion of Fragments into pY9N2/3

BamHI fragments corresponding to the 3' portion of the UL9 gene from seven of the insertion mutants, pI581, pI591, pI630, pI651, pI668, pI719 and pI799, were cloned into the unique BamHI site of plasmid pY9N2/3 (**Figure 31**).

The seven pB1 derivatives were digested with BamHI and the smaller of the two resulting DNA fragments purified by agarose gel electrophoresis followed by DEAE sephacel column chromatography. The purified fragments were then ligated to pY9N2/3 DNA which had been linearised with BamHI and treated with calf intestinal phosphatase.

E.coli DH5 cells were transformed with the ligation products and ampicillin resistant colonies selected. Mini plasmid preparations were analysed for the presence and correct orientation of the inserted fragment. An example of this analysis is shown in **Figure 32** in which the BamHI fragment from plasmid pI719 was inserted into pY9N2/3. Digestion of plasmid DNA with SmaI should produce fragments of 9860 bp, 1412 bp, 584 bp, 75 bp and 45 bp in size if the inserted fragment is present in the correct orientation (**Figure 32, lane 9; Figure 33a**). The presence of fragments of 10185 bp, 1000 bp, 584 bp, 75 bp and 45 bp is however indicative of the reverse orientation (**Figure 32, lanes 5, 7, 11; Figure 33b**). Digestion of a plasmid with no insert present produces fragments of 10987 bp, 584 bp, 75 bp and 45 bp only (**Figure 32,**

Figure 31. Cloning of DNA Fragments into a Baculovirus Transfer Vector

The cloning of a DNA fragment from plasmid pI581 into a baculovirus transfer vector is outlined opposite. An intermediate vector pY9N2/3 was constructed first by ligating the large BstEII to BamHI fragment of the baculovirus transfer vector pAcYM1 (Matsuura et al., 1987) to the small BstEII to BamHI fragment of plasmid pBL91 encoding the amino terminal two thirds of the UL9 gene (nucleotides 23542 - 21655). Plasmid pY9N2/3 contains a unique BamHI restriction site into which the mutated C-terminal fragments of the UL9 gene (e.g. from plasmid pI581) could be inserted, in the correct orientation, thus completing the sequence of the full length gene.

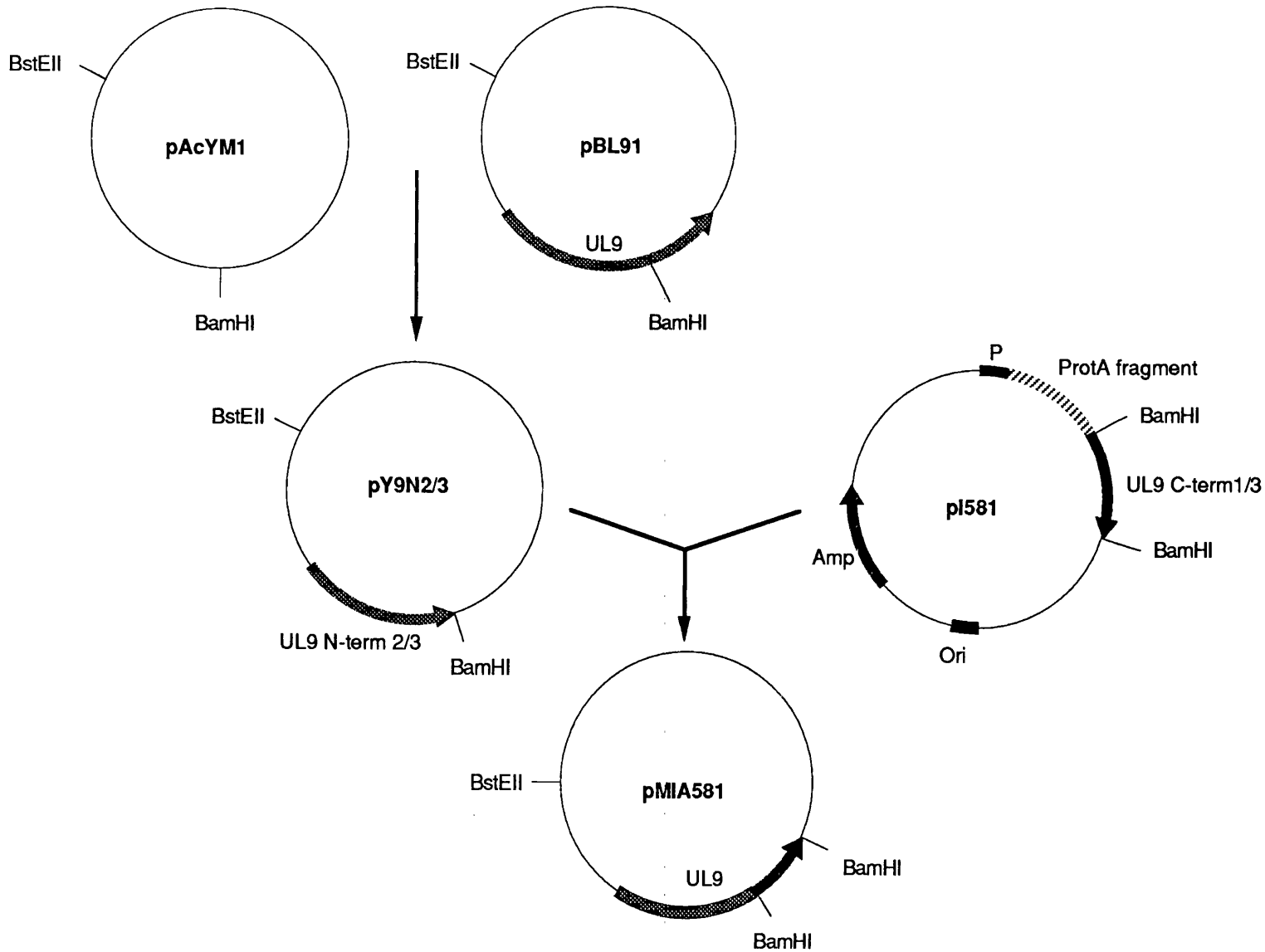

BstEII
pAcYM1
BamHI
BstEII
pBL91
UL9
BamHI
BstEII
pY9N2/3
UL9 N-term 2/3
BamHI
P
ProtA fragment
BamHI
UL9 C-term1/3
pI581
BamHI
Amp
Ori
BstEII
pMIA581
UL9
BamHI
BamHI

Figure 32. SmaI Digestion of pY9N2/3 Cloned UL9 Mutants

This figure shows a SmaI digestion of mini plasmid preparations of a UL9 mutant in which the BamHI fragment from plasmid pI719 was inserted into plasmid pY9N2/3 which contains the N-terminal two thirds of UL9 gene (nucleotides 23542 - 21655). Uncut and SmaI digested DNA samples of mini preps 1, 2, 3 and 4 (lanes 4 - 11) were resolved on a 6% polyacrylamide gel alongside uncut and SmaI digested pY9N2/3 (lanes 2 and 3 respectively) and HinfI digested pAT153 molecular weight markers (lanes 1 and 12), the sizes of which are indicated at the side.

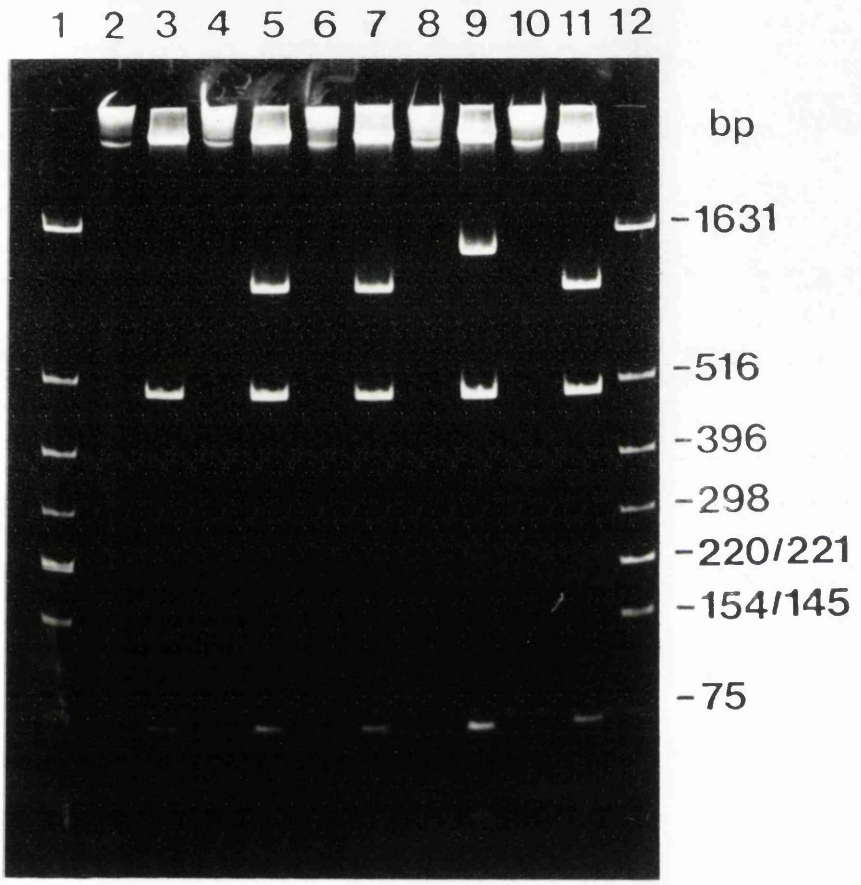
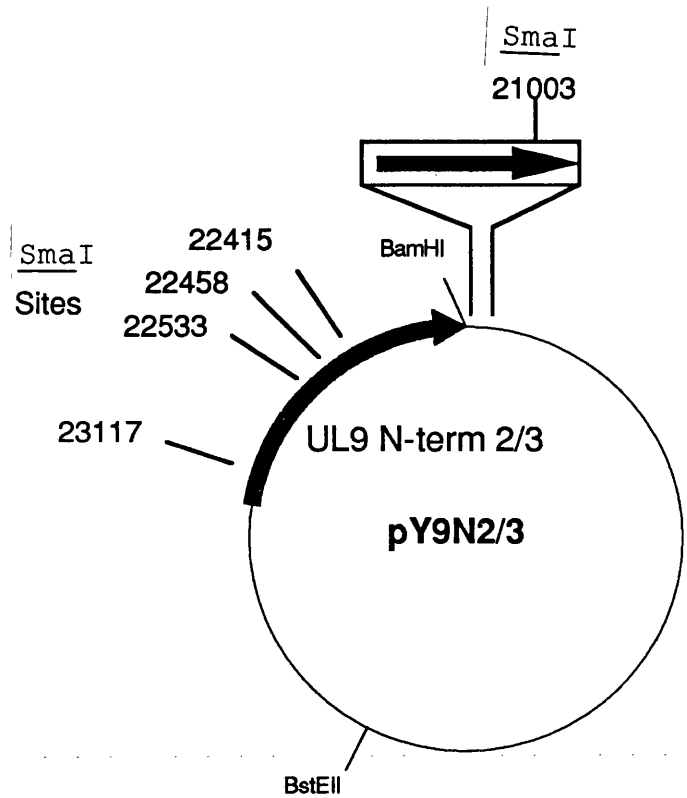

1 2 3 4 5 6 7 8 9 10 11 12
bp
1631
516
396
298
220/221
154/145
75

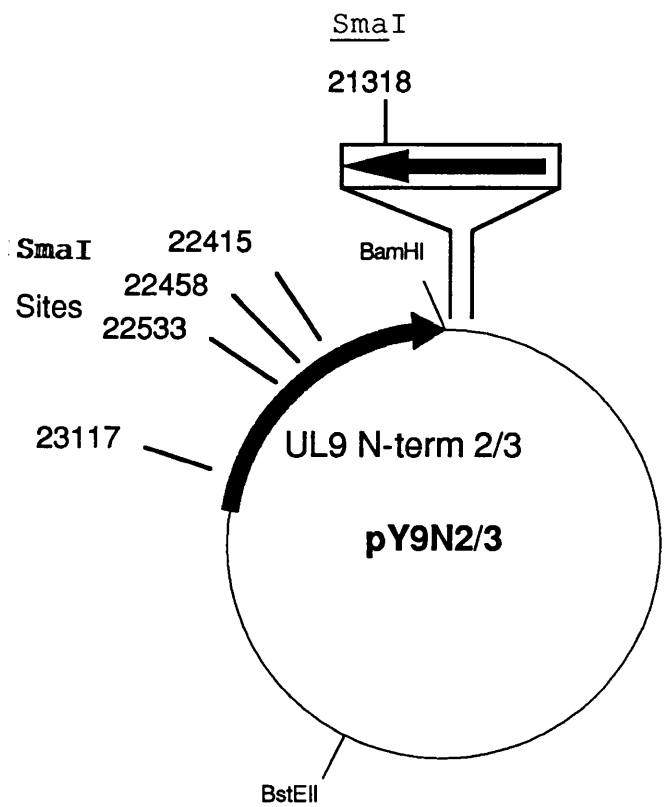
Figure 33. Correct and Incorrect Orientations of the UL9 C-terminal Fragment

This figure shows the correct and incorrect orientations of the UL9 C-terminal fragment; SmaI restriction sites as indicated were used to determine the direction of the inserted fragment. A correctly inserted C-terminal fragment would result in a SmaI digestion producing fragments of 9860 bp, 1412 bp, 584 bp, 75 bp and 45 bp (**Figure a.**). An incorrectly inserted fragment would result in a SmaI digestion producing fragments of 10185 bp, 1097 bp, 584 bp, 75 bp and 45 bp **(Figure b)**.

(a) **Correct Orientation of Inserted Fragment**



(b) **Incorrect Orientation of Inserted Fragment**



lane 3).

The resulting plasmids pMAI581, pMAI591, pMAI630, pMAI668, pMAI719 and pMAI799 contain full length UL9 genes which contain the insertion mutations derived from plasmids pI581, pI591, pI630, pI668, pI718, and pI799 repectively.

BamHI/XbaI restriction fragments of DNA containing UL9 C-terminal deletions from two mutant plasmids pD50 and pD62 were similarly cloned into the unique BamHI restriction site of plasmid pY9N2/3 after the addition of synthetic BamHI oligonucleotide linkers to the XbaI terminus. Plasmids pD50 and pD62 were cleaved with XbaI and BamHI. Blunt ends were produced by a klenow "fill in" reaction and BamHI linkers ligated. The products were then recleaved with BamHI and the fragment purified by sephacel chromatography. These mutant transfer vectors were named pMAD50 and pMAD62 respectively.

3.2.3 Isolation of Recombinant Baculoviruses

Co-transfection of insect cells with modified transfer vector and baculovirus DNA linearised at the insertion locus results in a great reduction in the level of non-recombinant virus obtained (Kitts et al., 1990). This is because linear AcNPV DNA is of very low infectivity, and as a consequence there is a selection for the production of infectious circular genomes by recombination with the transfer vector thereby inserting the foreign gene into the progeny virus.

The linear baculovirus genomes were produced by digestion of the DNA of an AcNPV derivative (AcRP23lacZ) with Bsu36I. The use of this derivative, in which the E.coli lacZ gene, encoding the B-galactosidase protein, has been inserted into the wt AcNPV genome at the polyedrin locus, provides an efficient method for isolating recombinant baculoviruses since the unique Bsu36I cleavage site is located within the lacZ gene.

The presence of any parental AcRP23lacZ plaques on Sf cell monolayers can be detected following the addition of a chromogenic substrate (X-gal) that produces a blue

product when cleaved by B-galactosidase. In contrast, in recombinant viruses the lacZ gene has been replaced by the gene of interest and easily distinguished white plaques are produced.

70% confluent monolayers of Sf cells in 35 mm petri dishes were co-transfected, using the calcium phosphate precipitation method (Graham and van der Eb, 1973), with Bsu36I linearised AcRP23lacZ DNA and modified transfer vector DNA containing the mutant UL9 genes. After three days at 28°C, the cells and medium were harvested and the virus titrated as described in Materials and Methods. After 4 days at 28°C, the plaques were stained with Neutral Red stain containing X-gal at 28°C and examined for the presence of viral plaques the following day.

In each case, approximately 60% of the viral progeny of co-transfected cells produced white plaques. Several white plaques were picked using sterile pasteur pipettes.

The progeny from white plaque were used to infect Sf cells in Linbro wells. 50 hpi the cells were harvested and proteins analysed by SDS-PAGE in parallel with mock-infected cells and cells infected with AcUL9 which expresses the wt UL9 gene (Stow, 1992). UL9 related polypeptides were detected by coomassie brilliant blue and silver staining (data not shown). Nine recombinant viruses (AcMAI581, AcMAI591, AcMAI630, AcMAI652, AcMAI668, AcMAI719, AcMAI799, AcMAD50 and AcMAD62), expressing the mutant UL9 proteins were identified and large scale working stocks prepared. These stocks were titrated on Sf cell monolayers at 28°C and the titres obtained ranged from $3x10^8$ to $9x10^8$ pfu/ml. Western blot assays and ^{35}S-labelling of expressed proteins were subseq uently performed to confirm expression of UL9-related proteins .

3.2.4 Protein Synthesis By Recombinant Baculoviruses

(a) Western Blot Analysis

Western blot assays were performed using a rabbit antibody raised against a peptide corresponding to the C-

terminal 10 amino acids of the UL9 protein in conjunction with the IgG/alkaline phosphatase detection system described earlier (see section 3.1.3). As this antibody recognises the C-terminal ten amino acids of the UL9 protein, Western blot analyses could only be carried out with the insertion mutants (**Figure 34a and b**).

Protein extracts were obtained from cells which had been either mock infected (**lane 1; a and b**), or infected with the parental AcRP23lacZ virus (**lane 2; a and b**), AcUL9 (**lane 3; a and b**) or one of the seven recombinants expressing the UL9 insertion mutants (**lanes 4 - 7 in Figure a; lanes 4 - 6 in figure b**). The presence of a novel protein band with the expected apparent molecular size of approximately 93 kDa (indicated by an arrow) in all extracts except from mock-infected and AcRP23lacZ infected cells indicated that the recombinant baculoviruses expressed the mutated UL9 proteins. The amounts of mutant UL9 protein expressed by several of the recombinant viruses was slightly lower than the level of wt UL9 produced by AcUL9 and, mutant AcMAI668 expressed very low levels. These differences probably reflect poor infections since ^{35}S- radiolabelling of proteins subsequently demonstrated levels of expression almost indistinguishable from wt (See **Figure 35**).

(b) ^{35}S-labelling of Proteins

Monolayers of Sf cells in 35 mm petri dishes were infected with either wt AcNPV, AcUL9 or one of the nine recombinant baculoviruses carrying mutated UL9 genes at a multiplicity of 20 pfu/cell. At 40 hpi, infected cells were labelled in TC100 salts (Calder and Stow, 1990) containing 25 uCi/dish of [^{35}S]-L-methionine for 20 hours at 28^{o}C.

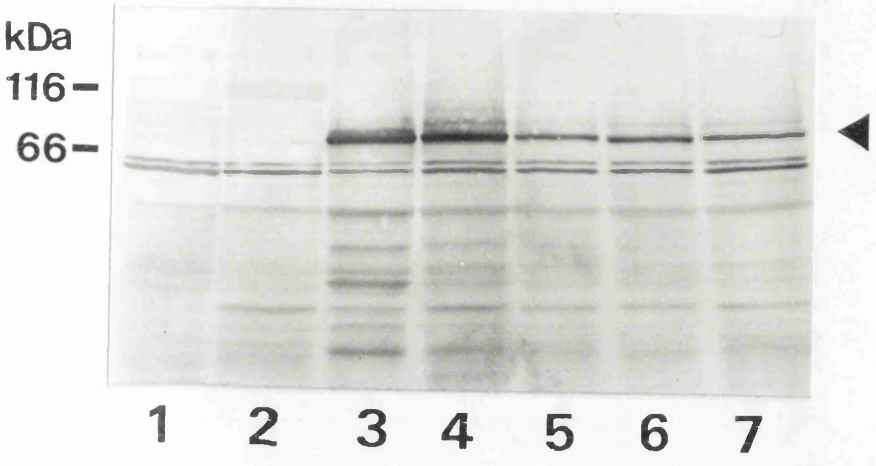
The cells were harvested in boiling mix and proteins resolved by SDS-PAGE on 8.5% gels prior to autoradiography (**Figure 35**).

The presence of a polypeptide the same size as UL9 protein produced by AcUL9 (**lane 2**), (indicated by arrow), in all the samples except from the wt baculovirus

Figure 34. Western Blot Analysis of Baculovirus Expressed Insertion Mutants

Infected cells were harvested, the proteins resolved on a 9% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane as described in the materials and methods. Proteins were detected using an anti-UL9 antibody and the Promega IgG/alkaline phosphatase protein detection kit. Extracts were from cells infected with the following recombinant baculoviruses: **Figure a**: lane 1, mock-infected cells; lane 2, AcRP23lacZ; lane 3, AcUL9; lane 4, AcMAI581; lane 5, AcMAI591; lane 6, AcMAI630; lane 7, AcMAI651. **Figure b**: lane 1, mock-infected cells; lane 2, AcRP23lacZ; lane 3, AcUL9; lane 4, AcMAI668; lane 5, AcMAI719; lane 6, AcMAI799.

a)



b)

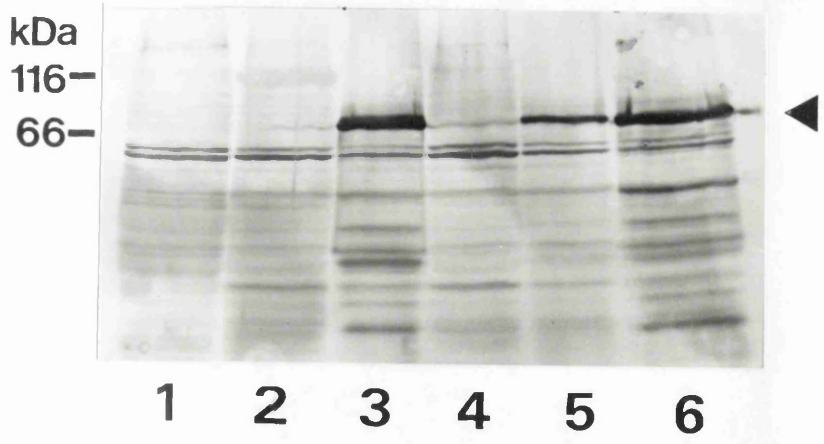
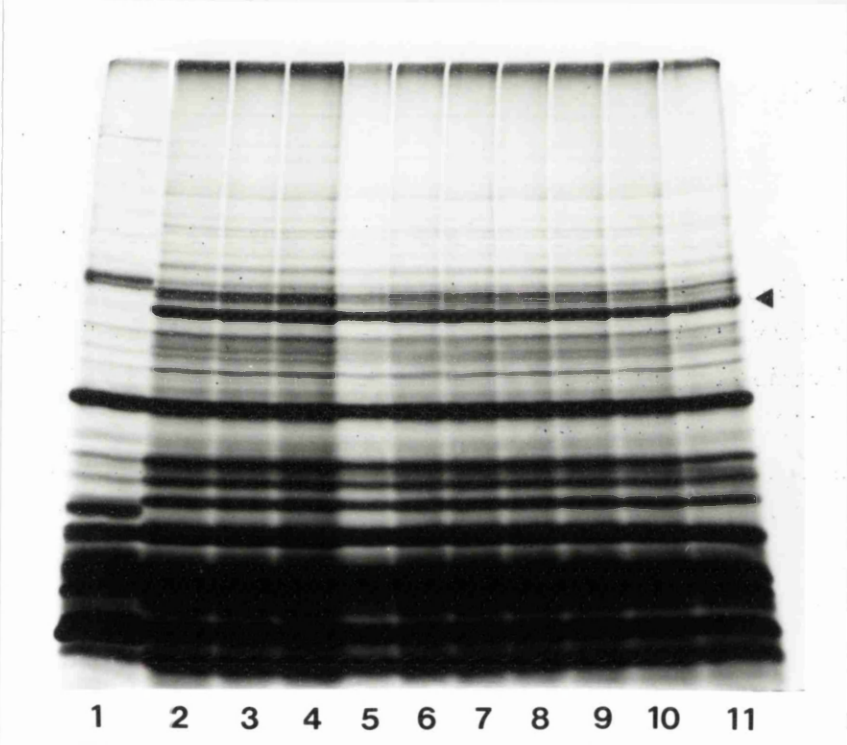


Figure 35. ^{35}S-labelling of Foreign Proteins Expressed in Recombinant Baculoviruses

The figure shows SDS-PAGE analysis of ^{35}S-labelled polypeptides prepared from Sf cells infected with either wt AcNPV (lane 1), wt AcUL9 (lane 2) or the following individual recombinant viruses AcMAI581, AcMAI591, AcMAI630, AcMAI652, AcMAI668, AcMAI719, AcMAI799, AcMAD50, and AcMAD62, (lanes 3 to 11 respectively). The overexpressed UL9 polypeptides are indicated by the arrow.

The data for this experiment was kindly provided by Dr G. McLean.


1
2
3
4
5
6
7
8
9
10
11

infected cells (**lane 1**) confirmed the expression of stable UL9 insertion mutant proteins (**lanes 3 - 9**) and also stable deletion mutant proteins (**lanes 10 and 11**).

3.2.5 Analysis of Replicative ability of Mutant UL9 Proteins Expressed By Recombinant Baculoviruses

It was reported by Stow, (1992) that seven baculoviruses expressing the seven essential HSV-1 DNA replication proteins can, in a mixed infection, support HSV-1 origin dependent replication of a co-transfected plasmid.

To determine whether the UL9 mutations affected origin-dependent replication similar replication experiments were performed in which the wt AcUL9 virus was substituted with each of the nine viruses expressing mutated UL9 polypeptides.

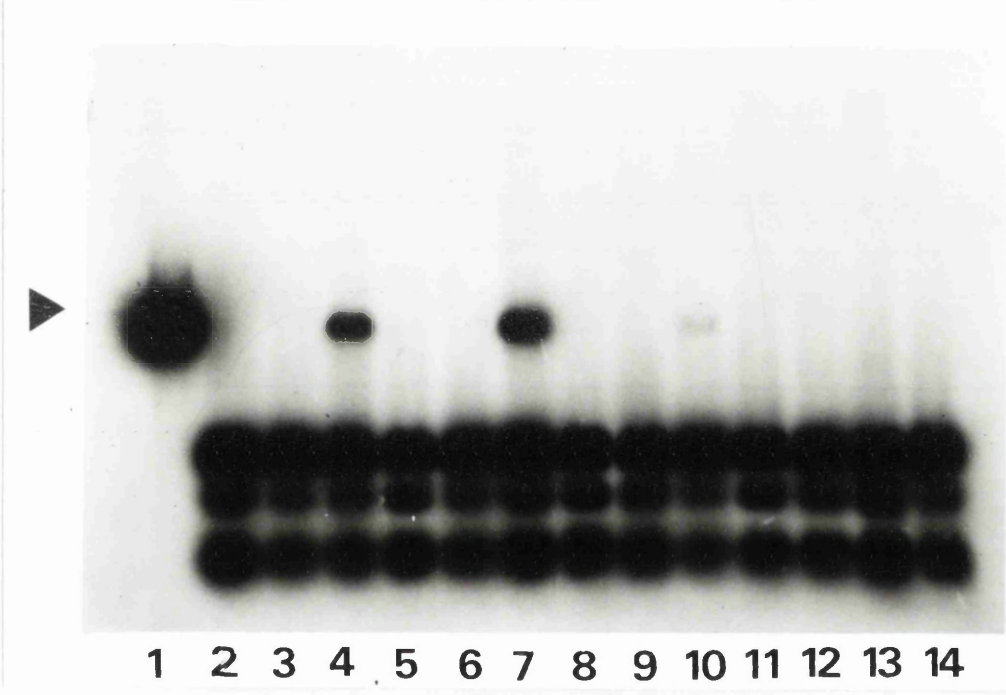
Stocks of seven recombinant baculoviruses, each expressing a functional wt copy of one of the seven essential replication genes under the control of the polyhedrin promoter were provided by Dr N.D. Stow.

Sf cells in Linbro wells were transfected with plasmid pEC8, which contains a functional copy of the HSV-1 ori_S replication origin using a liposome-mediated transfection procedure. After 4 hours at 28°C, the Sf cells were subsequently superinfected with a mixture of viruses expressing the seven replication proteins (AcUL5, AcUL8, AcUL29, AcUL30, AcUL42, AcUL52 plus AcUL9 or one of the recombinants expressing a mutated UL9 protein). Total cellular DNA was prepared from the cells at 50 hpi and examined for the presence of replicated (DpnI-resistant) plasmid sequences by cleaving with EcoRI and DpnI. The resulting fragments were resolved on 1% agarose gels, transferred to nylon membranes and hybridised to ^{32}P-labelled pAT153 DNA. The results are shown in **Figure 36.**

A band corresponding to replicated input plasmid DNA, which co-migrated with EcoRI-cleaved pEC8 (**lane 1**), was detected in the samples from cells infected with all seven wt viruses (**lane** 4), the six wt viruses plus AcMAI630 (**lane 7**), and the six wt viruses plus AcMAI719

Figure 36. Replication of an Ori_S-containing Plasmid

Sf cells were transfected with plasmid pEC8 (containing a functional ori_S) and either mock-infected (lane 2) or super-infected with a mixture containing 5 pfu of each of the six recombinant baculoviruses AcUL5, AcUL8, AcUL29, AcUL30, AcUL42 and AcUL52 (lane 3), all six viruses plus AcUL9 (lane 4) or all six viruses plus one of the AcUL9 mutants: lane 5, AcMAI581; lane 6 AcMAI591; lane 7 AcMAI630; lane 8 AcMAI652; lane 9, AcMAI668; lane 10, AcMAI719; lane 11, AcMAI799; lane 12, AcMAD50; lane 13, AcMAD62; lane 14, mock infected. One third of the DNA recovered from the infected cells at 50 hpi was digested with EcoRI and DpnI and the fragments resolved on a 1% agarose gel alongside linearised pEC8 (lane 1). Replicated plasmid molecules were detected by Southern blotting using ^{32}P-labelled plasmid pAT153 as a probe. An autoradiograph of the washed filter is shown. The position of DpnI resistant EcoRI-cleaved molecules is indicated by an arrow. The smaller fragments detected are DpnI cleavage products of unreplicated input plasmid DNA.


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

(**lane 10**). The degree of DNA replication observed with AcMAI630 was slightly higher than with wt AcUL9 while the infection including AcMAI719 shows a much reduced level of plasmid amplification. Replicated plasmid DNA was not detected in any of the remaining samples.

Of the mutations tested in this assay those amino acid insertions which abolished DNA binding of the UL9 fusion proteins also abolish the ability of the full length mutant protein to function in origin dependent DNA replication. Of the mutations tested which showed positive binding of UL9 fusion proteins to ori_S sequences, an insertion at amino acid 799 in the full length UL9 protein also failed to produce a protein functional in DNA replication. Insertions at two further positions (mutants AcMAI630 and AcMAI719) however, generated products which were functional in the replication assay, and resulted in either greater than or less than wt levels of DNA replication respectively.

CHAPTER 4: DISCUSSION

4.1 FUSION PROTEIN EXPRESSION IN E.COLI

Weir et al., (1989) had previously expressed fragments of the UL9 polypeptide fused to Staphylococcus aureus protein A in E.coli, and demonstrated that the C-terminal 317 amino acids specify all the structural information required for sequence-specific recognition and binding. The E.coli expression system used in these experiments provided a very convenient source of DNA binding activity. Bacterial extracts were simple to prepare and because DNA binding activity remained stable at -70°C for a few months, this system appeared to be ideal for use in a mutagenic analysis of the UL9 DNA binding domain.

In the work of Weir et al., (1989), fusion proteins were expressed under the control of the bacteriophage lambda P_R promoter and synthesis was induced by temperature upshift of an E.coli host containing a ts bacteriophage lambda repressor. This method ensured that protein expression could be controlled and minimalised any toxic effect the foreign protein may have had on its host.

In preliminary experiments I examined the expression of origin-binding activity by another E.coli host (DH5) lacking the lambda repressor, which had been transformed with the same fusion protein expressing plasmid, pB1. Extracts were prepared and their ori_S binding activity compared with the heat induced activity from the E.coli strain K12ΔHIΔtrp carrying the lambda repressor, in a gel retardation assay.

Efficient constitutive expression of UL9 fusion proteins was demonstrated in DH5 cells. As this method of expression was much more convenient to use since a temperature shift stage was not needed and did not appear to have a detrimental effect on the host, DH5 cells were chosen for all subsequent experiments with fusion proteins.

Another advantage of expressing the DNA binding

domain fused to protein A was that it also provided an easy tag for identifying the expressed proteins in Western blot assays. Initially, because of a lack of good antibodies against the HSV-1 UL9 protein, detection of the fusion protein proved to be difficult. The only suitable antibody available at the time was a rabbit antibody which had been raised against a peptide corresponding to the C-terminal 10 amino acids of UL9 protein (Olivo et al., 1988). Unfortunately, the antibody was of low affinity and produced a high non-specific background in Western blot assays. In addition, it was in short supply being a gift from Dr M.Challberg. Also, any mutation eliminating the antibody's epitope would render it useless in detecting the mutant protein. An alternative method was therefore developed which did not rely on a primary UL9-specific antibody for detection. In this method, fusion proteins were specifically detected due to the interaction of their protein A moiety with the Fc region of the antibody conjugate. This method provided a very specific detection system with no background problems and was suitable for all the mutants isolated. In addition, because the primary antibody incubation step was omitted, it also proved to be quicker and more convenient.

This method should be generally applicable and be of particular use when no specific antibody to the protein under study is available.

4.2 MUTATIONAL ANALYSIS OF THE UL9 DNA BINDING DOMAIN

Although Weir et al., (1989) had demonstrated that the UL9 DNA binding domain was contained within the C-terminal one third of the protein (amino acids 535 - 851), at the start of this study it was unclear as to which residues within this region were important for sequence-specific DNA binding. During the course of this work Deb and Deb (1991) narrowed the DNA binding domain to within a 269 amino acid segment (amino acids 564 - 832). My aim was to carry out a mutational study of the DNA binding domain in order to identify the structurally

important regions required for UL9/origin interactions.

By analysing a series of mutants with C-terminal deletions of varying lengths a C-terminal boundary for the DNA binding domain was defined, (See section 4.3).

It was also desired to examine the effect of mutations within the DNA binding domain which, in principle, could be introduced at pre-selected or random locations. Although sequences conserved within UL9 homologues may have provided a useful target for mutagenesis, it was also of interest to determine whether poorly conserved regions of the C-terminal domain were essential for its function. For this reason a series of linker insertion mutations were introduced essentially at random throughout the region.

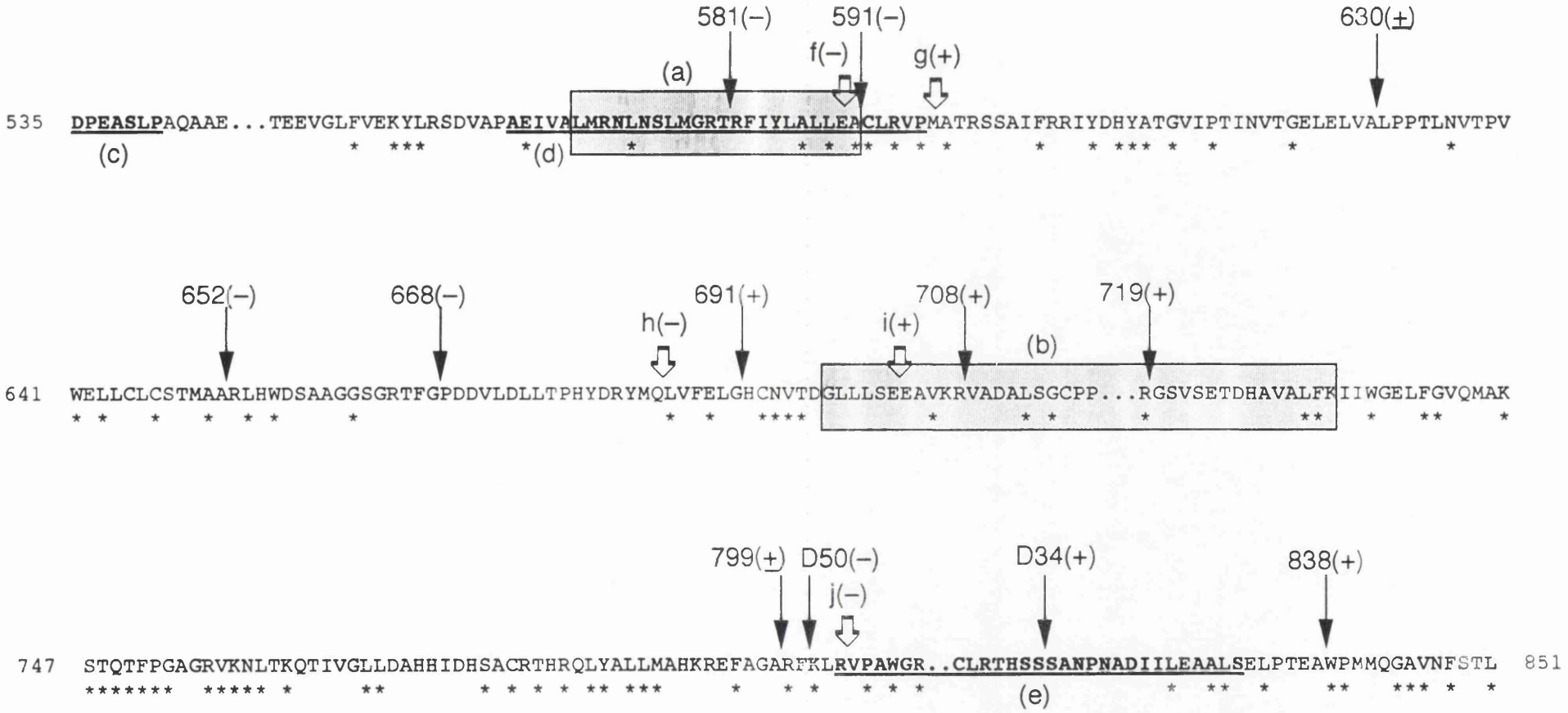
4.3 THE UL9 DNA BINDING DOMAIN

DNA sequence motifs identical to those recognised by HSV-1 UL9 have been identified within the known or presumed replication origins of other members of the alphaherpesvirus subfamily (Stow and Davison, 1986; Baumann et al., 1989; Nicolson et al., 1990). These too share a similar location either adjacent to or within an A + T-rich palindromic region. DNA sequence analysis thus far has revealed the presence of UL9 gene homologues within the genomes of varicella zoster virus (VZV) and equine herpesvirus type 1 (EHV-1), (genes 51 and 53 respectively). Like UL9, the VZV gene 51 product has been shown to bind specifically to DNA sequences within its own origin. Indeed, Stow et al., (1990) demonstrated that the C-terminal 322 amino acids of the VZV counterpart was able to bind specifically to the conserved UL9 recognition sequence.

A comparison of the aligned amino acid sequences of all three origin binding proteins revealed that 86 of the 317 residues within the UL9 DNA binding domain were conserved in both the VZV and EHV-1 homologues. The positions of these residues are indicated on the amino acid sequence of the UL9 DNA binding domain shown in **Figure 37,** which also summarises the results from

Figure 37. Summary of Results

Amino acids 535 - 851 of UL9 are shown with the positions at which identical residues are present in the VZV gene 51 and EHV-1 gene 53 products indicated by asterisks. The positions of insertion and deletion mutants described in this thesis are shown above the sequence with their binding ability indicated in parentheses. The positions and phenotypes of mutants described by Deb and Deb (1991) (f, h and j) and Martinez et al., (1992) (i and g) are similarly indicated . The N-terminal boundary of the DNA binding domain as identified by Deb and Deb (1991) lies within either region c or d (the intervening amino acids are not essential) and their C-terminal boundary within region e. The boxed sequences a and b indicate the pseudo-leucine zipper and helix-turn-helix motifs proposed by Deb and Deb (1991).


581(−)
591(−)
630(±)
f(−)
g(+)
(a)
535 DPEASLPAQAAE...TEEVGLFVEKYLRSDVAPAEIVALMRNLNSLMGRTRFIYLALLEACLRVPMATRSSAIFRRIYDHYATGVIPTINVTGELELVALPPTLNVTPV
(c)
*(d)
652(−)
668(−)
691(+)
708(+)
719(+)
h(−)
i(+)
(b)
641 WELLCLCSTMAARLHWDSAAGGSGRTFGPDDVLDLLTPHYDRYMQLVFELGHCNVTDGLLLSEEAVKRVADALSGCPP...RGSVSETDHAVALFKIIWGELFGVQMAK
799(±)
D50(−)
D34(+)
838(+)
j(−)
747 STQTFPGAGRVKNLTKQTIVGLLDAHHIDHSACRTHRQLYALLMAHKREFAGARFKLRVPAWGR..CLRTHSSSANPNADIILEAALSELPTEAWPMMQGAVNFSTL 851
(e)

mutagenesis experiments from two other laboratories (Deb and Deb, 1991; Martinez et al., 1992).

Deb and Deb, (1991) reported that the N-terminal boundary of the DNA binding domain is most probably located between residues 565 and 596 (although the presence of essential amino acids between positions 535 and 541 was not excluded by their experiments). The presence of inactivating mutations within this N-terminal boundary region (mutants I581 and I591 described in this thesis) indeed supports their conclusion.
Deb and Deb (1991) previously referred to the sequence spanning amino acids 570 - 591 as a pseudo-leucine zipper (LMRNLNSLMGRTRFIYLALLEA) in which the underlined residues form the proposed heptad repeat). This region is clearly important for sequence-specific DNA binding since the insertions in mutants I581 and I591 inactivates binding. Two observations however suggest it is unlikely to act as a traditional leucine zipper. First, the heptad repeat residues are not well conserved in the alligned VZV and EHV-1 sequences (VFKALACPIEQPRLVNTAILGA and LLVELNSPIVREQFVNVAVLGA, respectively), and second, amino acids involved in UL9 dimerisation are located outside the DNA binding domain (Elias et al., 1992).

The results from the gel retardation assays demonstrate that a deletion of up to 33 amino acids from the UL9 C-terminus can be tolerated without affecting the protein's ability to bind specifically to ori_S. Deletions of 50 amino acids and greater however completely abolished this binding activity. My study therefore places the C-terminal boundary of the DNA binding domain between residues 802 and 818 (defined by mutants D50 and D34) indicating that this region must contain essential sequences required for sequence-specific DNA recognition. This finding is in agreement with and further refines the mapping of the C-terminal boundary between amino acids 833 and 805 (Deb and Deb, 1991). Interestingly, the sequences close to the C-terminus (amino acids 839 - 851) represent one of the more conserved regions within the C-terminal one third of the protein (**Figure 37**). The

presence of conserved residues suggests that the C-terminus of UL9 may be involved in a function other than origin binding.

Gel retardation analysis of the insertion mutants demonstrated that four amino acid insertions introduced at amino acid positions 691, 708, 719 and 838 did not affect sequence-specific binding of UL9 to ori_S. Insertions at amino acid positions 581, 591, 652 and 668 however abolished this interaction. Two further insertions at positions 630 and 799, generated proteins which exhibited reduced ori_S binding activities.

Binding experiments carried out at an increased temperature (37°C) revealed that the weak binding of mutant I630 was ts. In addition, binding of the I630 protein to the complete ori_S fragment produced a single retarded complex with a higher mobility than that observed for the wt product specified by pB1. Binding of the other mutants to the complete ori_S also resulted in the production of this higher mobility complex as a minor band together with a major complex of the same mobility as that produced with the wt UL9 fusion protein. The most likely explanation is that the lower mobility major complex represents DNA molecules with protein bound to both sites I and II while the higher mobilty complex represents molecules with protein bound to only a single site.

Mutant I799 also exhibited reduced binding to ori_S sequences, particularly to the lower affinity site II. Indeed, binding to the complete ori_S fragment produced a major retarded complex whose electrophoretic mobility was intermediate between the major and minor complexes produced with the other mutants (**Figure 29a and b, lane 11**). The explanation for this is unclear. It is possible that the mutant protein has an altered conformation which may effect the mobility of the complex in polyacrylamide gels. This explanation however seems unlikely since a corresponding increase in electrophoretic mobility was not observed for the complex with the site I oligonucleotide (**Figure 27a and b, lane 11**). An

alternative explanation is that the I799 protein exhibits slightly weaker binding than the other mutants and tends to dissociate from and reassociate with the probe during electrophoresis. This may also explain the streaking effect observed with I799 protein in the presence of site I oligonucleotide.

Three of the insertion mutants which were unaffected in their interactions with sites I and II (I691, I708 and I719) are grouped near the centre of the DNA binding domain and identify a region of the protein which appears relatively tolerant of change. Two of these insertions (I708 and I719) lie within a helix-turn-helix motif (amino acids 698 - 733) predicted by Deb and Deb (1991) using the method of Chou and Fasman (1978). An independent mutant with a two amino acid insertion within this region was unimpaired in its replicative function (Martinez et al., 1992). It therefore seems very unlikely that the sequences comprising the predicted helix-turn-helix motif are involved in DNA binding.

In order to investigate whether the mutations which had abolished ability to bind ori_S had altered the structure of the proteins, the secondary structure of the DNA binding domains of wt UL9 and the 10 UL9 insertion mutants were predicted using the method of Chou and Fasman, (1978). In general the insertion of 4 amino acids did not appear to have much effect on the predicted secondary structure of the mutants compared with wt. Indeed the mutant which exhibited the most significant change to its predicted structure, was I719 whose binding ability was not significantly altered by the mutation.

The picture which emerges from these studies of the UL9 C-terminal DNA binding domain is that sequence-specific binding does not appear to be associated with a single discrete stretch of amino acids. Rather, residues toward either end, separated by at least 200 amino acids appear to contribute. It is not known whether the inactivating mutations affect parts of the protein which interact directly with the DNA target or whether they operate indirectly through changes in protein

conformation. Since the UL9 protein and its VZV and EHV-1 counterparts recognise identical seuences, it is tempting to speculate that the most conserved parts of the DNA binding domain would participate in sequence-specific recognition. Unfortunately, mutations within two particularly conserved segments (amino acids 746 - 762 and 777 - 790) have not yet been examined. It is nevertheless interesting to note that one mutant, I799, with an insertion close to one of these regions appears more greatly impaired in its binding to site II than to site I suggesting that this region may in fact be important for sequence recognition.

4.3.1 Conserved Structures Found in Protein DNA Binding Domains

Analysis of predicted amino acid sequences and X-ray crystallography studies of many sequence-specific DNA binding proteins have identified three types of conserved structural motifs which proteins use for recognition of DNA or in dimerisation; i) Helix-turn-helix motif, ii) Leucine zipper motif, iii) Zinc finger motif (Anderson et al., 1981; Landshulz et al., 1988). Much of this work has arisen from the extensive study of DNA binding proteins such as gene activator and repressor proteins.

(a) Helix-turn-helix motif

The helix-turn helix motif is characterised by two successive alpha helices juxtaposed at an angle of approximately 90^{o} by a turn of 4 amino acids (Steitz et al., 1982). Some bacterial gene regulatory proteins use this motif for dimerisation on the target sequence where one alpha helix from each monomer is arranged in such a way to fit into successive major grooves of DNA. These are then held in position due to hydrophobic interactions. Helix-turn-helix motifs are also found in many eukaryotic homeo domains.

When statistical procedures based on known helix-turn-helix DNA-binding motifs (Brennan and Mathews, 1989) were used on the predicted amino acid sequence of UL9, no

part of the DNA binding domain scored very highly (Weir, 1990, Ph.D. thesis). Thus, if UL9 utilises this type of motif to bind DNA it is likely to be substantially different from other helix-turn-helix structures. Moreover as discussed above, the properties of insertion mutants with alterations in the helix-turn-helix region postulated by Deb and deb, (1991), suggest that this region (what ever its actual structure) is not important for DNA binding.

(b) Leucine Zipper Motif

The leucine zipper was first described by Landschultz et al., 1988, as a motif involved in sequence-specific DNA binding and dimerisation. This motif contains 4 - 5 leucine residues separated from each other by six amino acid residues [ie. $(L\ X\ X\ X\ X\ X\ X)_{4-5}$] within a sequence which can form a stable alpha helix. Adjacent to this region lies a stretch of basic amino acid residues. It has been proposed that during dimerisation, the leucine side chains of one alpha helix interdigitate with those of a matching helix from a second polypeptide to form a stable non-covalent interaction. This leads to a particular positioning of the basic regions of the two polypeptides in a manner suitable for sequence-specific recognition of DNA (Landschultz et al., 1988).

Within the UL9 amino acid sequence, a motif containing four leucine repeat elements is located between residues 150 - 170. This region however does not lie adjacent to a basic segment and the leucine residues do not appear to reside within a predicted stable alpha helix. These points and the fact that it also lies outwith the DNA binding domain suggest that it does not play a significant role in DNA binding. However this motif may be important in UL9 dimerisation since the whole protein but not the isolated C-terminal DNA binding domain exists as a dimer in solution. It has also been suggested that the leucine zipper may allow the formation of heterodimers so long as each subunit contains such a motif (Landschultz et al., 1988). Thus, this motif may

enable interaction of UL9 with other replication proteins.

As described above, a second leucine repeat is located within the DNA binding domain of UL9 from positions 570 - 593, but again this region does not appear to be a traditional leucine zipper motif as there are no adjoining basic residues and 8 residues lie between the second and third leucine.

(c) Zinc Finger Motif

Many eukaryotic gene regulatory proteins contain zinc finger motifs, (Berg, 1986) which comprise a repeating motif of cysteine and histidine residues. Studies have shown that the cysteine/histidine motif complexes with a zinc ion. The resulting tetrahedral coordination of this complex is known to impart a stable conformation which in some instances is associated with DNA sequence-specific binding of the protein (Diakun et al., 1986). No such motif occurs within the UL9 sequence.

It is clear from studies made thus far that the UL9 protein does not contain any obvious counterpart of structural motifs identified in other sequence-specific DNA binding proteins and that a more detailed understanding of sequence specific recognition is needed. Hopefully this will be achieved using techniques such as specific mutagenesis of single amino acids within the DNA binding domain to pin point essential residues involved in these interactions. In addition, chemical cross linking of the protein to DNA followed by protease digestion could identify the peptides protected by binding and ultimately single amino acids involved. Purification of the protein would also enable the interactions to be examined by X-ray crystallography.

4.4 THE EFFECT OF MUTATIONS ON UL9 FUNCTION DURING HSV-1 ORIGIN DEPENDENT DNA SYNTHESIS

Although it was convenient to study the effect of mutations on the ability of UL9 to bind to HSV-1 origin

sequences by expressing the DNA binding domain as part of a fusion protein, it was not possible to directly relate these results to the overall function of UL9 during HSV-1 origin dependent DNA synthesis. Several C-terminal mutations were therefore introduced into the whole UL9 gene and the mutated proteins expressed by recombinant baculoviruses in order to investigate whether the alterations had any effect on the replicative function of the intact protein.

This system is routinely used in the lab and was chosen because it would allow high level expression of mutant UL9 proteins which would facilitate future biochemical studies and also the screening of mutant proteins for ability to participate in a transient origin dependent DNA replication assay (Stow, 1992).

Mutations were introduced into the full length gene by inserting the appropriate C-terminal fragments into a specially constructed baculovirus transfer vector containing the polyhedrin promoter and the N-terminal two thirds of the UL9 gene flanked by baculovirus DNA sequences.

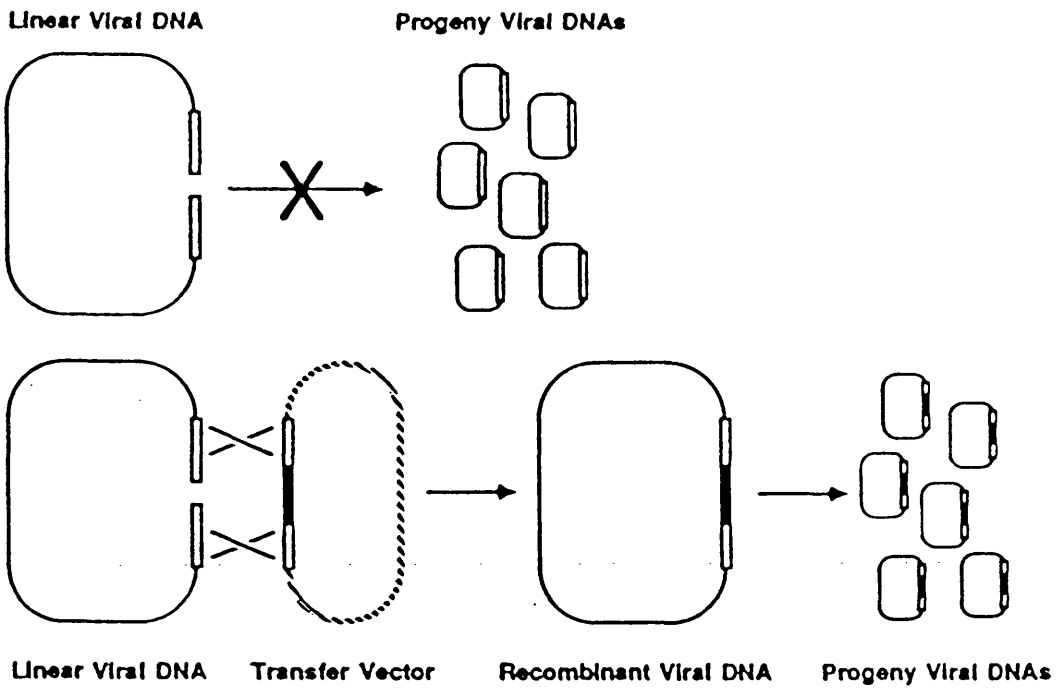
The proportion of recombinants generated within insect cells co-transfected with transfer vector and infectious circular AcNPV genomes is usually very low which makes the identification of recombinant baculoviruses against a high background of parental virus very difficult. In view of this problem I used a method developed by Kitts et al., (1990) for increasing the percentage of recombinants among the progeny virus. This method is based on the fact that viral DNA linearised within the non-essential polyhedrin locus is of extremely low infectivity. Recombination events between the linearised genome and circular transfer vector to generate circular infectious AcNPV molecules are therefore strongly selected (**Figure 38**). Such events concommitantly insert the gene of interest into the AcNPV genome and as a result the proportion of recombinants in the progeny is greatly increased.

As a parental virus I used an engineered derivative

Figure 38. Rescue of Linear Viral DNA by Recombination with a Transfer Vector

Upper panel: linear DNA cannot replicate because the replication apparatus of AcNPV is designed to work on the native viral DNA which is circular.

Lower panel: the circularity of the viral DNA can be restored by recombination with a transfer vector carrying DNA homologous to the viral sequences on either side of the break. A double crossover generates a recombinant viral DNA molecule which, being circular, is competent for replication (reproduced from Kitts et al., 1990).


Linear Viral DNA
Progeny Viral DNAs
Linear Viral DNA
Transfer Vector
Recombinant Viral DNA
Progeny Viral DNAs

of AcNPV, AcRP23lacZ (Possee and Howard, 1987), which contains the E.coli lacZ gene encoding B-galactosidase, inserted into the viral DNA at the polyhedrin locus. The virus contains a unique Bsu36I within the lacZ gene. Since recombination between linear parental DNA and transfer vector deletes the lacZ gene, recombinants can be recognised by their failure to express a functional B-galactosidase product. Using a chromogenic substrate, recombinant viruses can be easily identified since, in contrast to the parental virus, they form white plaques. White plaques may also arise as a result of recircularisation of parental DNA by processes that disrupt DNA sequences around the double strand break and result in the generation of a mutated lacZ gene.

It was therefore necessary to confirm the expression of UL9 polypeptides by recombinant viruses using SDS-PAGE, Western blot assays and radiolabelling of proteins. As discussed earlier, the only antibody available for Western blot assays was of low affinity, and non-specifically detected a large range of proteins. It nevertheless allowed expression of the seven UL9 polypeptides containing insertions mutations to be unambiguously demonstrated. The two recombinant viruses carrying deletion mutations could not be tested on Western blot assays using the UL9 antibody because the antibody epitope had been deleted. However, by radiolabelling the proteins expressed by the recombinant baculoviruses, expression of all the mutant UL9 proteins, including the C-terminal truncations, was demonstrated.

Using an in vitro replication assay developed by Stow, (1992) I confirmed that multiple infection of Sf insect cells with seven recombinant baculoviruses expressing the seven essential HSV-1 DNA replication proteins facilitated replication of a co-transfected HSV-1 ori_S-containing plasmid. Using the same assay and by substituting the wt UL9 recombinant baculovirus with each of the mutant UL9 viruses it was possible to investigate the effect of the alterations on UL9 function in origin-dependent DNA replication.

Each of the mutations which had previously been shown to abolish DNA binding activity also prevented replication of the HSV-1 ori_S-containing plasmid suggesting that the interaction of UL9 with the origin is essential for the process of DNA replication. Of the mutant proteins which retained their DNA binding activity (AcMAI799, AcMAI630, AcMAI719), one failed to promote replication of the origin-containing plasmid (AcMAI799) while the others showed slightly higher than wt (AcMAI630) or diminished levels of activity (AcMAI719).

The mutation in AcMAI799, although not abolishing UL9 DNA binding activity, may have altered the structure of part of the protein which is not required for binding but has some other essential function in DNA replication. This might be an interaction with either other HSV-1 DNA replication proteins or perhaps cellular proteins which may play a role in replication. Interestingly this mutant also exhibits a reduced binding activity towards ori_S binding site II. It is possible that due to its reduced binding, appropriate protein/protein interactions between UL9 molecules bound to binding sites I and II may not be able to occur.

The diminished levels of replication observed with mutant AcMAI719 whose cognate fusion protein exhibited binding activity indistinguishable from wt may also be the result of the insertion altering an important site of interaction with other essential proteins or perhaps even interactions between UL9 molecules.

Mutant AcMAI630 exhibited a somewhat surprising result: although its ability to interact with the origin was impaired by the introduced insertion mutation, replication of the ori_S-containing plasmid was greater in its presence than in the presence of the wt UL9 protein. The reason for this unexpected result is unclear. One possibility may be that the insertion alters the protein structure in such a way that although its affinity for the origin is decreased, its affinity for one of the other components of the replicative machinery or a cellular protein important for DNA replication is

enhanced. It is also possible that UL9 may act in a negative regulatory manner during HSV-1 DNA replication. The mutation at amino acid 630 may affect this role by decreasing the ability of UL9 to down regulate replication resulting in levels of replication greater than wt.

Clearly the production of these mutations has provided the basis for a number of future experiments. It is firstly important to repeat the origin binding assays using the intact mutant UL9 polypeptides to determine whether they exhibit identical origin binding properties to those observed with the Protein A/UL9 fusion proteins. Because the mutant UL9 proteins can be expressed in large quantities using the baculovirus system, they can be tested for various biochemical activities including nuclear localisation and helicase activities. By performing helicase assays, one could investigate whether the sequence-specific DNA binding function of UL9 is necessary for helicase activity of the protein.

The in vitro replication assay described earlier could also be used to investigate whether different defective mutants are able to complement one another. This is of particular interest since the possibility of intragenic complementation is suggested by observations that UL9 exists as a dimer in solution. Finally, it will be interesting to perform similar experiments to determine whether UL9 homologues encoded by other alpha herpesviruses can substitute for the UL9 protein in HSV-1 origin-dependent DNA replication.

4.4.1 Comparison of Two Assays Used To Study In vitro HSV origin Dependent DNA Synthesis

The ability of mutant UL9 proteins to support replication has been analysed by other groups using a transient replication complementation assay (Zhu and Weller, 1992a). This assay is also based on the observation that a HSV origin-containing plasmid can be amplified in a transient transfection experiment if all the necessary trans-acting functions are provided either

by superinfection with HSV or by co-transfection with plasmids encoding the set of essential replication proteins.

Weller and collegues used a viral null mutant in which the lacZ gene had been inserted within the UL9 coding sequence. This virus is completely defective in viral growth and DNA synthesis, and is propagated on a complementing cell line which expresses wt UL9 protein.

Their assay involves the introduction of two plasmids into cells by transfection, one of which contains the HSV-1 origin, ori_S, while the other expresses the mutant UL9 product under the control of the UL39 promoter (Zhu and Weller, 1992a). These cells are subsequently super-infected with the UL9 null mutant virus which provides the other six replication genes and viral factors which trans-induce the UL39 promoter. Only if the mutation within the transfected UL9 gene does not disrupt replicative function will amplification of the ori_S-containing plasmid and complementation of the mutant virus occur.

Both this approach and the baculovirus system therefore provide a general method of assaying the effect of introduced amino acid changes on the ability of the replication proteins to function in viral DNA replication. There are however various advantages and disadvantages to both systems.

The assay described by Zhu and Weller (1992a), is convenient because mutations generated by site-directed mutagenesis of a UL9 containing plasmid can be assayed immediately without the time consuming generation of recombinant viruses. This system may also more closely resemble what happens in vivo as the other six essential replication genes are expressed from their own promoters within the HSV-1 genome.

A possible disadvantage of the baculovirus system is that the overexpressed replication proteins may not be present in the optimum amounts or ratios for efficient DNA replication. However, a major advantage to expressing mutant polypeptides using recombinant baculoviruses is

that in addition to assaying for the ability of the protein to function in viral replication, amounts of protein suitable for biochemical examination can be obtained.

4.4.2 Functions of UL9 other than DNA Binding

The sequence of the UL9 gene has been determined and the amino acid sequence of the encoded protein predicted (McGeoch et al., 1988a;b;). **Figure 39** shows the predicted amino acid sequence of the HSV-1 UL9 protein (amino acids 1 - 851) and indicates the locations of several interesting features which have been noted during the analysis of the sequence.

(a) Dimerisation

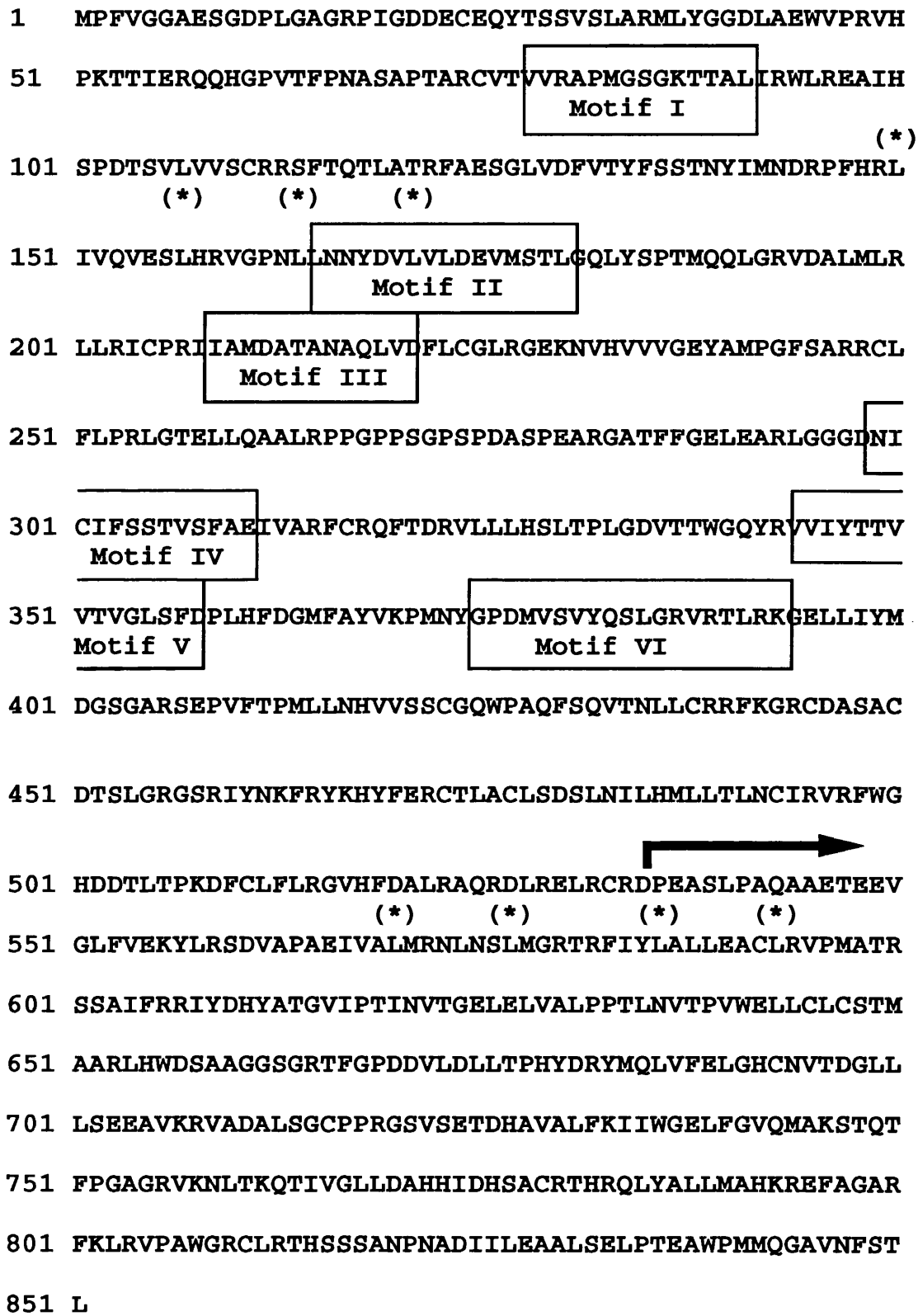
UL9 contains a putative leucine zipper motif towards its N-terminus (between amino acids 150 and 170). As mentioned earlier these motifs are common to several DNA binding proteins and are believed to signal regions of protein/protein interactions. Results with mutations producing alterations to the leucine residues of the leucine zipper motif within the N-terminus of UL9 suggest that these leucines are essential for UL9 function (Martinez et al., 1992). Whether the leucine zipper motif represents a site for dimerisation or for the interaction of UL9 with other members of the replicative machinery however remains to be elucidated. Indeed Elias et al., (1992) reported that the structural elements required for the cooperative binding of the HSV UL9 protein to ori_S reside in the N-terminal part of the protein.

(b) UL9 as a helicase

The UL9 protein has been shown to exhibit a 3′ - 5′ intrinsic DNA helicase activity (Fierer and Challberg, 1992; Bruckner et al., 1992)). The finding that UL9 exhibited a helicase activity suggested that the protein may act to initiate DNA synthesis after binding to the origin by catalysing the unwinding of the two parental strands. Studies have revealed that UL9 is capable of

Figure 39. Primary Structure of the UL9 Polypeptide

The predicted amino acid sequence (residues 1 - 851) of the HSV-1 UL9 protein is shown in the single letter amino acid code. The six conserved helicase motifs predicted by Gorbalenya et al., 1989 (motifs I - VI) are highlighted within boxes. Motif I and II contain the NTP binding sites predicted by Gorbalenya and Koonin, (1989). Leucine residues within potential leucine zipper motifs have been indicated by asterisks (*) above the sequence. The C-terminal DNA binding domain (amino acids 535 - 851) has been outlined but a more detailed picture can bee seen in **Figure 37.**


1 MPFVGGAESGDPLGAGRPIGDDECEQYTSSVSLARMLYGGDLAEWVPRVH
51 PKTTIERQQHGPVTFPNASAPTARCVTVVRAPMGSGKTTALIRWLREAIH
Motif I
(*)
101 SPDTSVLVVSCRRSFTQTLATRFAESGLVDFVTYFSSTNYIMNDRPFHRL
(*) (*) (*)
151 IVQVESLHRVGPNLLNNYDVLVLDEVMSTLGQLYSPTMQQLGRVDALMLR
Motif II
201 LLRICPRIIAMDATANAQLVDFLCGLRGEKNVHVVVGEYAMPGFSARRCL
Motif III
251 FLPRLGTELLQAALRPPGPPSGPSPDASPEARGATFFGELEARLGGGDNI
301 CIFSSTVSFAEIVARFCRQFTDRVLLLHSLTPLGDVTTWGQYRVVIYTTV
Motif IV
351 VTVGLSFDPLHFDGMFAYVKPMNYGPDMVSVYQSLGRVRTLRKGELLIYM
Motif V
Motif VI
401 DGSGARSEPVFTPMLLNHVVSSCGQWPAQFSQVTNLLCRRFKGRCDASAC
451 DTSLGRGSRIYNKFRYKHYFERCTLACLSDSLNILHMLLTLNCIRVRFWG
501 HDDTLTPKDFCLFLRGVHFDALRAQRDLRELRCRDPEASLPAQAAETEEV
(*) (*) (*) (*)
551 GLFVEKYLRSDVAPAEIVALMRNLNSLMGRTRFIYLALLEACLRVPMATR
601 SSAIFRRIYDHYATGVIPTINVTGELELVALPPTLNVTPVWELLCLCSTM
651 AARLHWDSAAGGSGRTFGPDDVLDLLTPHYDRYMQLVFELGHCNVTDGLL
701 LSEEAVKRVADALSGCPPRGSVSETDHAVALFKIIWGELFGVQMAKSTQT
751 FPGAGRVKNLTKQTIVGLLDAHHIDHSACRTHRQLYALLMAHKREFAGAR
801 FKLRVPAWGRCLRTHSSSANPNADIILEAALSELPTEAWPMMQGAVNFST
851 L

unwinding long segments of duplex DNA in the presence of the HSV-encoded ss-DNA binding protein (Fierer and Challberg, 1992). Unfortunately, origin-specific unwinding by UL9 has yet to be demonstrated. It is possible that the optimum experimental conditions to detect origin unwinding have not been found or that other factors such as a cellular component may be needed.

Nevertheless, strong evidence exists that the helicase activity is essential for viral DNA replication since insertion mutations within the six conserved helicase domains present within the N-terminal one third of the protein rendered the protein inactive for DNA replication.

(c) Nuclear Localisation

Nuclear localisation studies have shown that the UL9 protein is capable of entering the cell nucleus without the aid of other components of the replicative machinery (Calder et al., 1992). The sites to which UL9 localises resemble the pre-replicative sites to which the mDBP localises when DNA synthesis is blocked. Whether these sites are functionally equivalent however remains to be determined. In view of these observations it appears likely that the UL9 polypeptide contains a nuclear localisation signal although no obvious candidate has yet been identified.

(d) Interaction with other HSV-1 Replication Proteins

None of the other replication proteins have yet been shown to interact with UL9, but by analogy with other DNA replication initiator proteins it is likely that such interactions do occur.

(e) Interaction with cellular proteins

Studies of the interactions between UL9 and ori_S have demonstrated the existence of a cellular factor(s) which is capable of binding to the HSV-1 ori_S (Dabrowski and Schaffer, 1991). This factor(s) was also shown to enhance complex formation between the UL9 protein and site I DNA,

suggesting that the cellular factor(s) may facilitate initiation of HSV-1 DNA synthesis by enhancing the UL9/origin interaction (Dabrowski and Schaffer, 1991). The identification of this factor and a detailed study of its interaction with UL9 and the origin would be of considerable interest.

Cellular transcription factors have been shown to be involved in the initiation of bovine papilloma virus, adenovirus and SV40 virus DNA synthesis (Yang et al., 1991; Guo et al., 1989). It is interesting to note that in HSV-1, both ori_L and ori_S lie close to or within viral promoter regions and it is possible that cellular transcriptional activators binding to these sequences may be involved in initiation of DNA synthesis. For example, HSV-1 ori_S is surrounded by multiple Sp1-binding sites (Jones and Tjian, 1985) generating the possibility of a significant interaction between OBP and Sp1 at the origin.

Further biochemical characterisation and isolation of additional mutations across the UL9 gene are required for a more complete understanding of the domain structure of this multifunctional protein.

4.5 THE ROLE OF UL9 IN THE INITIATION OF HSV-1 DNA SYNTHESIS

The events following the interaction of UL9 with HSV-1 origin sequences which lead to initiation of viral DNA synthesis are not yet well understood.

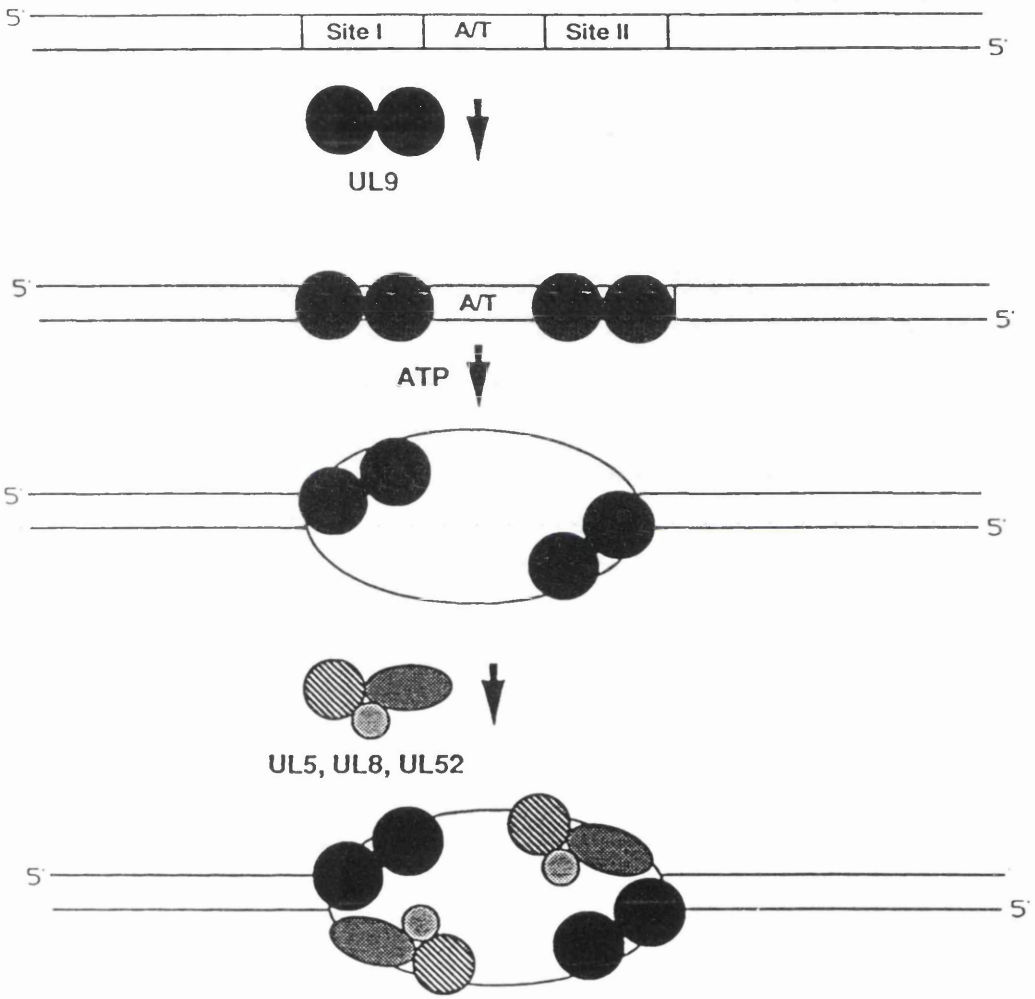
In vitro studies have demonstrated that UL9 exhibits several interesting properties which include the ability to form dimers in solution, a DNA-dependent nucleoside 5'-triphosphatase activity, cooperative origin specific DNA-binding activity and a DNA helicase activity.

A prelude to the initiation of daughter strand synthesis is the unwinding of the two parental strands. By analogy with other prokaryotic and eukaryotic initiator proteins, it has been suggested that the binding of UL9 to ori_S or ori_L is just the first step in

Figure 40. A Model for the Initiation of HSV DNA Replication

A model for the initiation of HSV-1 DNA replication is outlined opposite. As shown, a putative local unwinding event at the origin mediated by the binding of UL9 could be followed by the entry of the helicase/primase complex (UL5/UL8/UL52). This complex could function to enlarge the unwound region, prime nascent strands at the origin, and then continue unwinding the duplex along the lagging strand template as semidiscontinuous DNA synthesis proceeds (reproduced from Olivo and Challberg, 1989)

It should be noted that this model could not be used for the initiation of VZV as its OBP binding sites are all located on one side of the origin palindrome.


5'
Site I
A/T
Site II
5'
UL9
5'
A/T
5'
ATP
5'
5'
UL5, UL8, UL52
5'
5'

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