CHARACTERIZATION OF THREE DELETION VARIANTS OF HERPES SIMPLEX VIRUS TYPE-1 (HSV-1): SEQUENCE, LATENCY AND VIRULENCE ANALYSIS

by

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Dedication

In the memory of my loving father

Late Mohammed Umer Junejo

SUMMARY

The aim of the work described in this thesis was to further characterise three spontaneously derived deletion variants of herpes simplex virus type-1 (HSV-1) strain 17 syn^+, designated as 1704, 1705 and 1706 (MacLean and Brown, 1987b). The characterization included, (1) sequencing across the end points of deletions by the dideoxy chain termination reaction method , to (a) investigate the relationship of the variants to each other, since they had arisen from a single recombination experiment (b) determine the extent of the deletions with respect to the location of the latency associated transcripts (LATs) and the LAT promoter region and (2) to analyse the pathogenic and latency phenotype of the three variants in the mouse model system.

The characterisation of the variants 1704, 1705 and 1706 was carried out by restriction enzyme digestion of virus DNA, selective oligonucleotide hybridisation and more precisely by sequencing across the end points of the deletion by the dideoxy chain termination reaction method.

In the variant 1704, the deletion in U_L/IR_L is 3758bp in length, starting at nucleotide position (np) 116502 and ending at np120260. The deletion removes 655bp of U_L and 3103bp of IR_L. The UL56 gene and 799bp of the 5' end of the latency associated transcripts (LATs) are deleted including the LAT promoter region. In TR_L the deletion is 942bp in length extending from np7202 to 8144 and is confined entirely within TR_L. The 5' end of the LAT is not affected but the LAT promoter region is deleted.

Sequencing analysis of the variant 1705 showed that the deletion in U_L/IR_L is 4735bp in length, extending from np 115453 to np120188. This deletion is 183bp and 694bp downstream from the 3'

ends of the IE2 and IE1 genes respectively and removes the genes UL55 and UL56. One copy of the LAT coding region plus the LAT promoter region is deleted. The variant 1705 is not deleted in TR_L.

Sequencing analysis of the variant 1706 showed that it has a 1807bp deletion at the right hand end of U_L which has been replaced by 4754bp from the left end. The deletion starts just 80bp downstream from the 3' end of the IE2 gene and terminates at the U_L/IR_L junction. The deletion therefore completely removes the UL55 and UL56 genes. The deleted sequences are replaced by sequences from the left end of U_L containing the genes UL1, UL2, UL3, UL4 and a partial copy of UL5 in an inverted orientation.

To study the biological properties of the variants, a baseline was established from which to evaluate pathogenicity. Nine individual plaques were picked from the elite stock of 17 syn^+; restriction enzyme analysis of the DNA from each of the nine plaque stocks showed no differences in the size of fragments or distribution of the sites. These plaques were inoculated intracranially into three week old BALB/c mice and showed no differences in their LD_{50} values compared to the parental 17 syn^+ stock.

Inoculation of the variants, 1704, 1705 and 1706 into 3 week old BALB/c mice showed that 1705 was not different in pathogenicity from the wild type following intracranial, footpad and intraperitoneal inoculations. Therefore, despite the deletion, 1705 consistently behaved as wild type. On the other hand 1704 and 1706 compared to wild type were 20 fold and 460 fold less virulent respectively following intracranial inoculation and failed to kill any animal following footpad inoculation, even at doses of 10^7 pfu/mouse. In *in vivo* replication experiments in the peripheral nervous system (DRG of the spinal cord) of mice 1704 and 1706 grew very poorly .

Latency analysis of the variants showed that the three variants established, maintained and reactivated from latency. The kinetics of reactivation of 1705 and 1706 were similar to the parent 17 syn^+, in which reactivation occured 5-6 days post explantation, but 1704 reactivated with delayed kinetics i.e on the 12th day post explantation. Since 1704 has deleted both copies of the LAT promoter region and one copy of the LAT coding region in IR_L, it was concluded that the LATs play a part in latency reactivation of 1704 from DRG (dorsal root ganglia of spinal cord) in the mouse model.

Restoration of the deleted sequences in the variant 1704 by marker rescue with the wild type *BamHI b* fragment resulted in a wild type genotype. This virus was designated 1704R. Latency studies on 1704R revealed that the rate and frequency of reactivation was intermediate between 17 syn^+ and 1704, suggesting a secondry undetected mutation affecting latency phenotype. Isolation of $1704LP^-$ in which both copies of the promoter region of the LAT are deleted and reactivation of this virus from latency with delayed kinetics confirms that the LATs paly a role in reactivation from latency.

ABBREVIATIONS

A	adenine
AIDS	acquired immunodeficiency syndrome
APS	ammonium persulphate
ATP	adenosine triphosphate
BHK	baby hamster kidney cells
BMV	bovine mammilitis virus
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CCV	channel catfish virus
Ci	curies
CIP	calf intestinal phosphatase
cm	centimeter
cpe	cytopathic effect
dATP	deoxyadenosine triphosphate
DBP	DNA binding protein
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
ddNTP	dideoxynucleoside triphosphate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DR	direct repeat
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E	early

EBV	Epstein-Barr virus
E.Coli	*Escherichia coli*
EDTA	sodium ethylene diamine tetra-acetic acid
EHV	equine herpes virus
Fc	crystalisable fragment of immunoglobulin
G	guanine
g	gram(s)
h	hour
HCMC	human cytomegalovirus
HHV	human herpes virus
HSV	herpes simplex virus
HVS	herpes virus siamiri
ICP	infected cell polypeptide
IE	immediate early
IgG	immunoglobulin G
IPTG	isopropyl-D-thiogalactoside
IR_L	inverted long repeat
IR_S	inverted short repeat
k	kilodalton(s)
kb	kilobase(s)
L	late
LATs	latency associated transcripts
LD_{50}	50% lethal dose
M	molar
mA	milliamps
mCi	millicuries
MDB	major DNA binding protein
MDV	Marek's disease virus
mg	milligram
min	minute

ml	millilitre
mM	millimolar
mm	millimeter
moi	multiplicity of infection
Mr	molecular weight
mRNA	messenger ribonucleic acid
m.u.	map units
NP40	nonidet P40
OD	optical density
ORF	open reading frame
ori	origin of viral DNA replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
pfu	plaque forming units
PRV	pseudorabies virus
RF	replicative form
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RR	ribonucleotide reductase
RT	room temperature
SDS	sodium dodecyl sulphate
syn^+	non-syncytial
T	thymine
TIF	trans-inducing factor
TK	thymidine kinase
TK^-	thymidine kinase negative
TK^+	thymidine kinase positive
ts	temperature sensitive

TR_L	terminal long repeat
TR_S	terminal short repeat
U_L	long unique
U_S	short unique
Vmw	molecular weight of viral-induced polypepetide
v/v	volume per volume
VZV	varicella zoster virus
W	watt
w/v	weight per volume
w/w	weight per weight
x	times
^oC	degree centrigrade
uCi	microcurie
ug	microgram
ul	microlitre
uM	micromolar
%	percentage
<	less than
>	higher than

CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1 OBJECTIVES.

This project involved the further characterization of three spontaneously derived deletion variants (1704, 1705 and 1706) of herpes simplex virus type-1 (HSV-1) strain 17 syn^+. The characterization included (1) finding the precise end points of the deletions by dideoxy sequence analysis (2) determining the relationship if any of the variants to each other (3) analysing the latency and pathogenic phenotypes of the variants in the mouse model system.

The aim of this introduction is to provide a general overview of HSV emphasising the areas related to the project, in particular, genome structure and latency.

1.2 CLASSIFICATION OF THE FAMILY HERPESVIRIDAE.

At least 80 viruses, which comprise the family herpesviridae have been isolated from a wide variety of vertebrates and invertebrates (Roizman, 1982). The virion is 150-200 nm in diameter and contains a double stranded linear DNA genome, which is enclosed in an icosahedral capsid, containing 162 capsomeres. The capsid is surrounded by a lipid envelope (Wildy *et al.*, 1960). The other structural elements of herpes viruses are (i) the core, an electron opaque fibrillar spool around which double stranded linear DNA is wrapped and (ii) the tegument, an electron dense amorphous layer distributed asymmetrically around the capsid. Viruses of this family replicate in the nucleus and acquire their envelope by budding through the nuclear membrane (Wildy *et al.*, 1960).

It is difficult to classify herpesviruses merely on the basis of their morphology (Fenner, 1976). They can be differentiated by their biological and pathogenic properties, including host range,

duration of lytic cycle, cytopathology, characteristics of latent infection (Mathews, 1982), immunological cross reactivity, size, base pair composition and structure of their genomes (Roizman, 1982).

1.2.1 Classification on the basis of biological properties.

On the basis of biological properties, the members of the family herpesviridae have been classified into three sub-families, α-herpesvirinae, β-herpesvirinae and γ-herpesvirinae.

1.2.1.a Sub-family α-herpesvirinae.

Although members of this sub-family have a narrow host range in nature, some, for example, HSV can infect a variety of experimental animals and tissue culture cells. This family usually causes an acute, self-limiting disease in their natural host. They also have the capacity to establish a primary infection followed by latent infection, typically in the dorsal root ganglia of the spinal cord. HSV-1 is the prototype example of this family. Primary infection can be inapparent but sometimes manifests as acute gingivo-stomatitis (cold sores), occasionally ocular keratitis and in very rare cases, acute necrotising encephalitis. HSV-2 is another member of this sub-family, which is closely related to HSV-1 and causes genital lesions in man and is venereally transmitted. Another α-herpesvirus is varicella zoster virus (VZV) which causes varicella or chicken pox, usually in childhood and zoster or shingles, after latent virus reactivation in adults. The other members include bovine mammilitis virus (BMV), pseudorabies virus (PRV), and equine herpes virus-1 (EHV-1).

1.2.1.b Sub-family β-herpesvirinae.

Members of this sub-family are characterized by

restricted host range and a long replicative cycle. Their growth in tissue culture progresses slowly and infected cells become enlarged. Latent virus has been demonstrated in secretory glands, lymphoreticular cells, kidneys and other tissues. This sub-family consists of human cytomegalo virus (HCMV) and murine cytomegalovirus (MCMV) . Although most infections with HCMV are symptomless, HCMV is a major cause of congenital disease. This virus can also cause severe generalised disease in immunocompromised patients, principally those undergoing transplant surgery (Alford and Britt, 1985) and more recently those with Acquired Immune Deficiency Syndrome (AIDS).

1.2.1.c Sub-family γ-herpesvirinae.

Members of this group are lymphoproliferative viruses. They normally exhibit a narrow range *in vivo*. *In vitro*, viruses can infect lymhoblastoid cells which are usually non permissive or semipermissive for virus replication. Viruses are generally specific for T or B lymphocytes. Although they usually have restricted growth in lymphoblastoid cells, many viruses in this sub-family productively infect fibroblastic cells. This sub-family contains herpes virus saimiri (HVS), Epstein-Barr virus (EBV), Marek's disease virus (MDV) and herpes virus ateles.

Classification into various families is somewhat arbitrary and subjective, and as a consequence some herpes viruses have been incorrectly assigned, for example, MDV. In general, however, the classification system has proved to be reasonably satisfactory.

1.2.2 Classification on the basis of genomic structure.

Using genomic structure, members of the family

herpesviridae have been classified into five major groups (Roizman., 1982). They differ considerably in their base pair composition (32-75% G+C) , the size of their genome (80 to 150 million molecular weight) and the arrangement of the reiterated sequences (Figure 1.1).

Group A.

In this group, the genome is characterized by a set of reiterated sequences, located in the same orientation at the termini. The DNA is present as only one isomer. This group is represented by channel catfish virus (CCV) (Chousterman et al., 1979).

Group B.

This group is represented by HVS and their genomes contain multiple reiterations of the same set of sequence, present as a direct repeat at both termini in the same orientation. The DNA is present as only one isomer (Bornkamm *et al.*, 1976).

Group C.

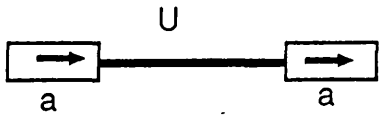
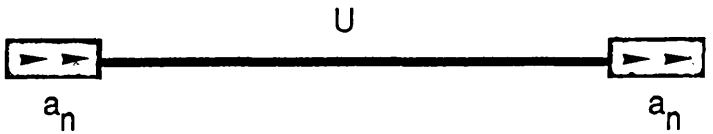
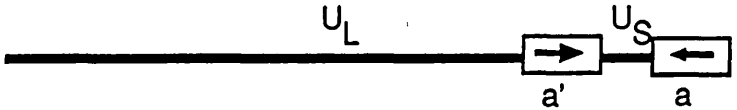
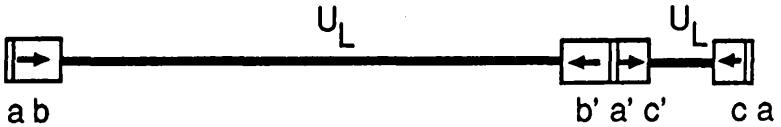
The genome of the members of this group contain multiple reiterations of one set of sequence present at both termini in the same orientation as a direct repeat and internal tandem reiteration of a second set of sequences. The DNA is present as a single isomer. This group is represented by EBV (Raab-Traub *et al.*, 1980).

Group D.

Group D genomes are characterized by the presence of two regions of unique sequence, one of which is flanked by inverted repeats, which allow inversion of the short unique sequence leading to the presence of two isomers. This group is represented by PRV

Figure 1.1

The genomic layout of channel catfish virus (CCV), herpes virus siamiri (HVS), Epstein-Barr virus (EBV), pseudorabies virus (PRV) and herpes simplex virus (HSV). Unique sequences are denoted by solid lines and repeat sequences by boxes. The arrows indicate relative direction of reiterated sequences. Letters a_n and b_n signify multiple tandem repeat sequences. The small terminal direct and internal inverted repeats of HSV (the 'a' sequences) are indicated. The type of genome and numbers of isomers are also indicated.

Genome type	Isomer		
A	1	CCV	
B	1	HVS	
C	1	EBV	
D	2	PRV	
E	4	HSV	

(Ben-Porat *et al.*, 1979).

Group E.

Group E has been divided into two subgroups:E1 comprises a group of viruses whose genomes contain two unique sequences, each flanked by inverted repeats, which share no homology. VZV is a member of this group.

The second subgroup, E2, represented by herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), have their genomes with two unique regions, flanked by inverted repeats which share a short region of DNA directly repeated at the termini and indirectly repeated at the junction between the internal inverted repeats. This type of genome results in four isomers, which are present in equal populations in a virus stock.

1.3 HUMAN HERPESVIRUSES.

Man is the natural host for six herpesviruses; which are, HSV-1, HSV-2, Varicella-zoster virus (VZV), Epstein-Barr virus (EBV), Human cytomegalovirus (HCMV) and Human herpes virus-6 (HHV-6). The first five are relatively well characterized with respect to biology and pathogenesis (Roizman, 1982). In contrast HHV-6 was first isolated from persons suffering from AIDS or other lymphoproliferative disorders (Salahuddin *et al.*, 1986). It has also been isolated from children affected by exanthema subitum, a transient childhood illness and on account of this, the virus has been proposed as the possible causative agent of the disease (Yamanishi *et al.*, 1988). It has been reported that more than 80-90% of the human adult population have developed anti-body to the virus early in life (Saxinger *et al.*, 1988). Recently, there has been a report of a seventh human herpesvirus, designated HHV-7 (Frenkel

et al., 1990).

1.4 STRUCTURE OF THE HERPES SIMPLEX VIRUS GENOME.

1.4.1 General properties of the HSV genome.

The genome of HSV-1 strain 17 syn^+ is a linear double stranded DNA molecule containing 152,260 bases in each strand (Perry and McGeoch, 1988). Like all herpes viruses there is heterogeneity between HSV-1 strains, so this number refers only to strain 17 syn+. HSV-1 has a base composition of 68.3% G+C which is not uniform throughout the genome, the short repeat region for example has a base pair composition of 79.5% G+C (McGeoch *et al.*, 1986) and the short unique region is 64.3% G+C (McGeoch *et al.*, 1985). The genome of HSV DNA is unmethylated (Low *et al.*, 1969). Each terminus has an overhanging residue with its 3' hydroxyl (OH) group free and lacking a complementary residue on the opposite strand (Mocarski and Roizman, 1982b).

HSV-1 DNA consists of two covalently linked components, long (L) and short (S) containing 82% and 18% of the total DNA respectively (Roizman, 1979). The long region consists of a long unique sequence (U_L) flanked by a pair of inverted repeat sequences at the terminus (TR_L) and the joint (IR_L). Similarly the short region consists of unique sequences (U_S) and repeat sequences, TR_S and IR_S. The TR_L/IR_L sequences flanking the U_L segment are designated as a, b and b', a' while IR_S/TR_S flanking U_S are designated as a', c' and c, a, respectively. With the exception of a 400bp direct repeat at the genome termini known as the 'a' sequence , the sequences of R_L and R_S are distinct. The 'a' sequence is present as one copy at the terminus of the S component and in one to numerous copies at the L component terminus and at the junction between the L and S

components (Wagner and Summer, 1978). The repeat sequence in TR_L and IR_L, excluding the 'a' sequence is known as the 'b' sequence. A characteristic of HSV-1 DNA is that the L and S components invert. As a consequence, viral DNA extracted from infected cells consists of four equimolar populations that differ in the relative orientation of the two components (Figure 1.2). One specific orientation is designated P (prototype), the others are designated I_L (L inverted with respect to P), I_S (S inverted with respect to P) and I_{SL} (both L and S inverted with respect to P).

In the HSV genome there are groups of short tandem reiterated sequences ranging from 10 to 100bp (McGeoch ,1989). The copy number of these repeats varies among different virus isolates (Rixon *et al.*, 1984). Serial passaging and recloning of the same isolates leads to variation in the number of such repeats (Davison and Wilkie, 1981; Watson *et al.*, 1981a, Murchie and McGeoch, 1982, Perry and McGeoch, 1988). The short tandem reiterations may serve to promote genetic exchange between the repeats thus maintaining homology (Umene,1987) or they could promote a high degree of recombination, but the function of such reiterations in the genome is basically unknown.

1.4.2 Organisation of HSV genes.

The genome of HSV-1 Glasgow strain 17 syn^+ has been sequenced and the genetic organisation has been analysed (McGeoch *et al.*, 1985, 1986, 1988; Perry and McGeoch, 1988). There are over all 72 recognised genes encoding 70 distinct proteins (Figure 1.3). There are 56 genes in UL from UL1 to UL56, 12 genes in US from US1 to US12 and the genes present in the repeats are diploid (designated as IE1 and IE3). Another gene (ICP34.5) has been postulated in HSV1 strain F (Chou and Roizman, 1986; Ackermann

Figure 1.2

Diagramatic representation of the HSV-1 genome. The genome is divided into L and S regions bounded by terminal redundant sequences, TR_L and TR_S, which are repeated in an internal inverted form, IR_L and IR_S that joins L to S. The redundant sequences of L and S flank two unique sequences U_L and U_S. The 'a' sequence is present as a direct repeat at the genomic termini and as an inverted repeat at the L-S junction. The remainder of R_L is known as b and b' and R_S as c and c'. Intermolecular or intramolecular recombination events in the redundant sequences can generate inversion of the U_L and/or U_S leading to four equimolar isomers. These are designated P (prototype), I_L (inversion of the long segment), I_S (inversion of the short segment) and I_{LS} (inversion of the long and short segments).

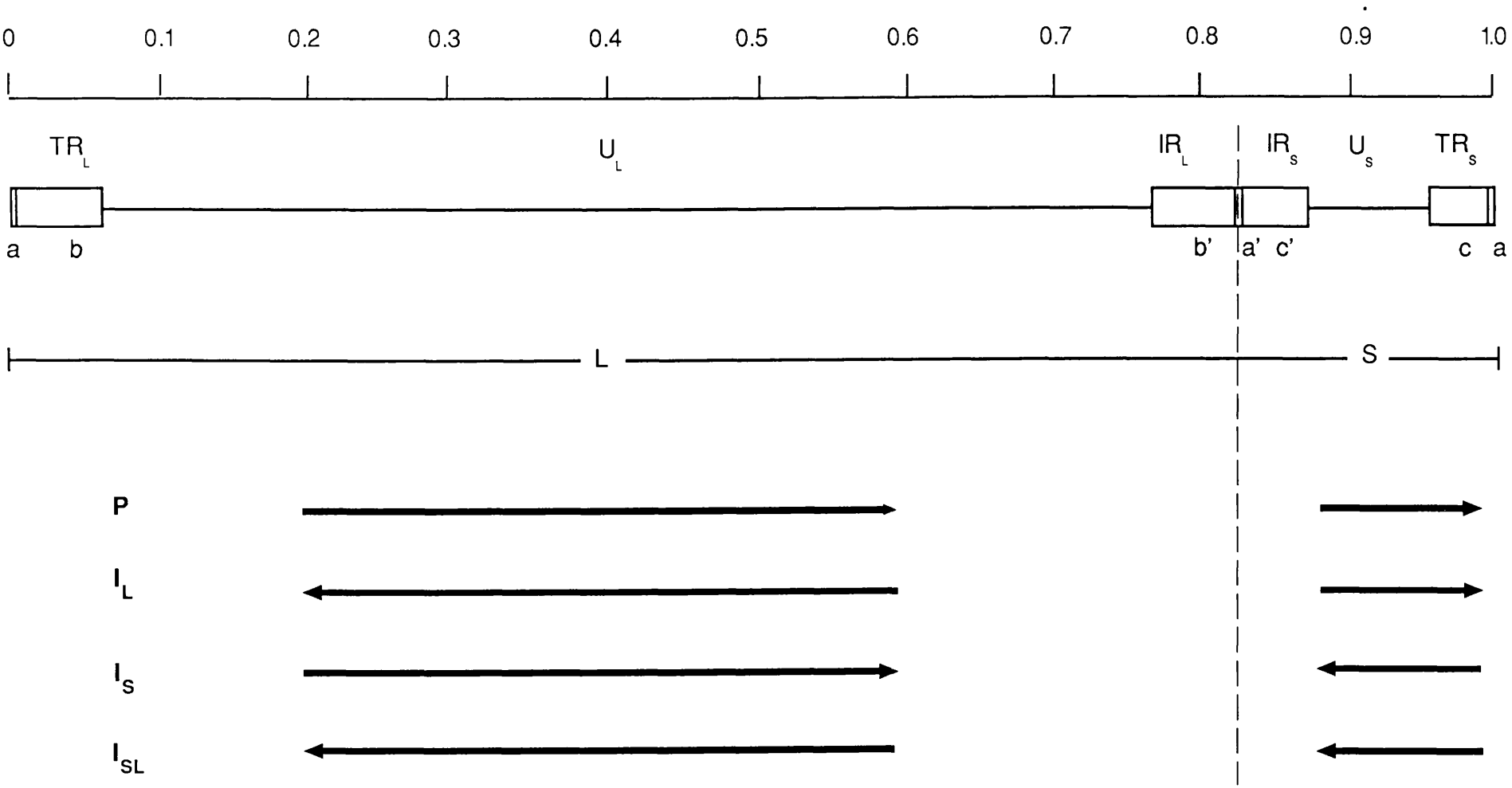
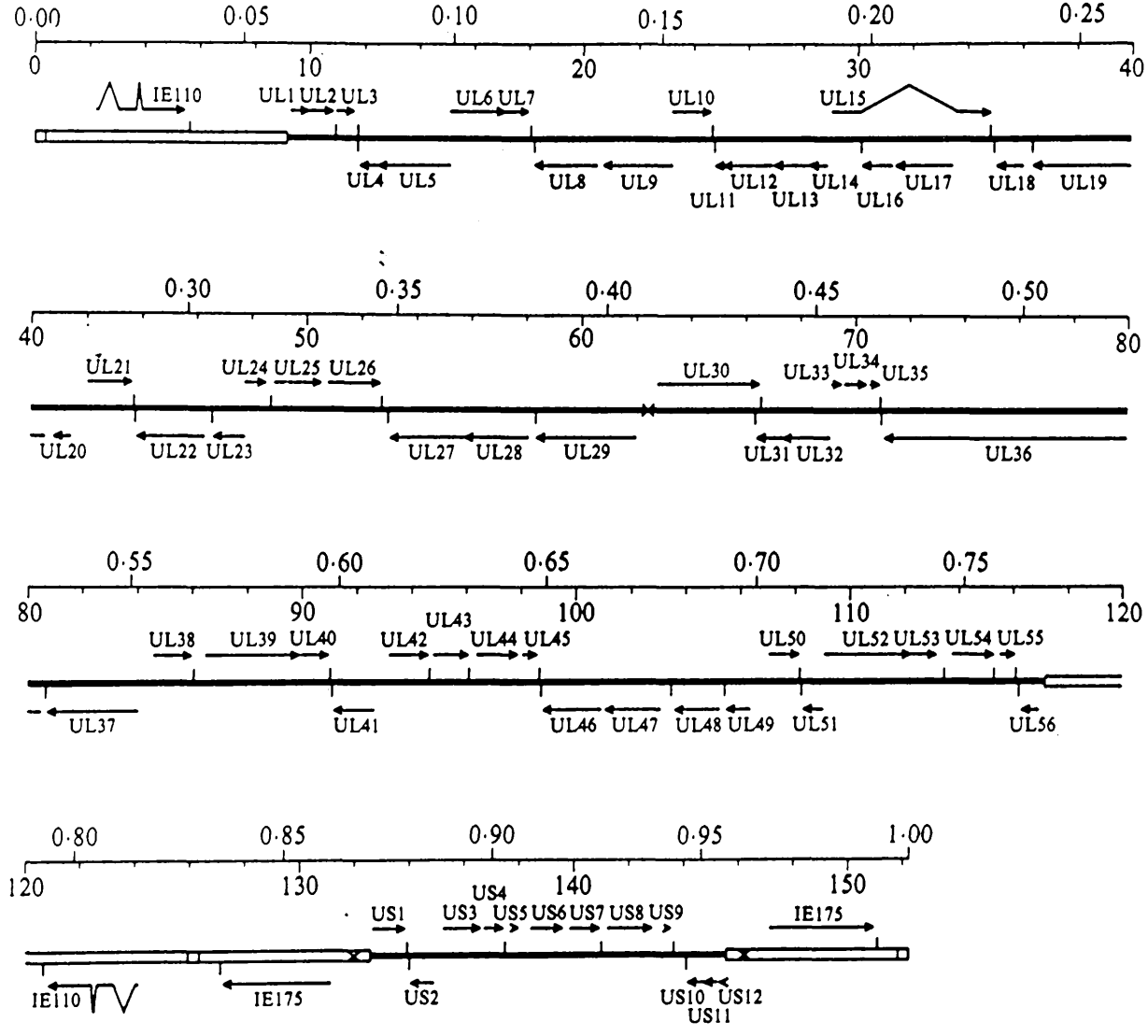

0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
TR L
U L
IR L
IR S
U S
TR S
a
b
b'
a'
c'
c
a
L
S
P
I L
I S
I SL

Figure 1.3

Origanization of the genes of HSV-1, represented on four successive lines. Location of HSV-1 open reading frames are indicated by arrows above or below the genome representing genes transcribed rightward or leftward respectively. Repeat sequences are represented as open boxes. Origins of DNA replication are indicated by crosses and location of probable polyadenylation sites are marked by small vertical lines. The upper scale is map units and lower is Kbp (From McGeoch *et al.*, 1989)


0·00
0·05
0·10
0·15
0·20
0·25
0
10
20
30
40
IE110
UL1 UL2 UL3
UL6 UL7
UL10
UL15
UL4 UL5
UL8 UL9
UL12 UL14 UL17
UL11 UL13 UL16
UL18 UL19
0·30
0·35
0·40
0·45
0·50
40
50
60
70
80
UL21
UL24 UL25 UL26
UL30
UL33 UL34 UL35
UL20
UL22 UL23
UL27 UL28 UL29
UL31 UL32
UL36
0·55
0·60
0·65
0·70
0·75
80
90
100
110
120
UL43
UL38
UL39 UL40
UL42
UL44 UL45
UL50
UL52 UL53 UL54 UL55
UL37
UL41
UL46 UL47 UL48 UL49
UL51
UL56
0·80
0·85
0·90
0·95
1·00
120
130
140
150
US4
US1
US3 US5 US6 US7 US8 US9
IE175
IE110
IE175
US2
US10 US12
US11

et al., 1986) upstream of IE1 in the same orientation but this assignment has been disputed (Perry and McGeoch, 1988). It has also been demonstrated that a part of the genome expresses the latency associated transcripts (LATs) found in latently infected animal and human ganglia (Stevens *et al.*, 1987; Krause *et al.*, 1988). It is thought not to be translated into a protein product, as no such protein has been demonstrated and analysis of the proposed transcript does not support its existence (Perry and McGeoch, 1988). Genes which encode proteins with known functions or properties are listed in Table 1.1.

There is no difference in the synthesis and processing of virus specific RNAs from eukaryotic RNA. Host encoded RNA polymerase II is utilised and transcription occurs in the nuclei of infected cells (Wagner and Roizman, 1969; Ben-Zeeve and Becker, 1977; Costanzo *et al.*, 1977). Most HSV genes possess upstream and downstream regulatory regions similar to those of host cell genes (McKinght, 1980). These include 5' end promoter sequences such as a TATAA box and a CAAT box motif and a 3' pre mRNA polyadenylation signal, AATAAA (Benoist *et al.*, 1980; Zarkower *et al.*, 1986). There is another motif, the YGTGTTYY (where Y represent C or T) sequence found downstream from the polyadenylation site (McLauchlan and Clements, 1983) which has been shown to be required for efficient processing of the 3' end of mRNA (McLauchlan *et al.*, 1985). One aspect in which HSV differs from host, is the degree of gene splicing. There are relatively few genes spliced for example IE1, UL15, 1E4,IE5 and mRNA of UL44 (Rixon and Clements, 1982; Watson *et al.*, 1981b; Perry *et al.*, 1986; McGeoch *et al.*, 1988; Frink *et al.*, 1983). LATs also appear to be spliced (Wechsler *et al.*, 1988; Wagner *et al.*, 1988). Recent construction of a recombinant virus containing an intronless IE gene

Table: 1.1 Properties of HSV-I encoded polypeptide.

Gene	Function or properties.
IE110	Immediate-early transcription regulator (Perry *et al.*, 1986).
UL2	Uracil-DNA glycosylase (Mullaney *et al.*, 1989).
UL5	DNA replication (McGeoch *et al.*, 1988; Heillbronn *et al.*, 1990).
UL6	Virion protein (McGeoch *et al.*, 1988).
UL8	DNA replication (Wu *et al.*, 1988; McGeoch *et al.*, 1988).
UL9	DNA replication; origin binding protein (Weir *et al.*, 1989).
UL12	Alkaline nuclease (Weller *et al.*, 1990).
UL13	Putative protein kinase (Smith and Smith, 1989).
UL18	Capsid protein (Rixon *et al.*, 1990).
UL19	Major capsid protein (Costa *et al.*, 1984).
UL22	Virion glycoprotein H (Gompels and Minson, 1986).
UL23	Thymidine kinase (McKnight, 1980; Wagner *et al.*, 1981).
UL25	Virion protein (Addison *et al.*, 1984)
UL26	Capsid protein (Preston *et al.*, 1983).
UL27	Virion glycoprotein B (Bzik *et al.*, 1984).
UL28	Capsid protein (Addison *et al.*, 1990)
UL29	DNA replication; major DNA binding protein (Conley *et al.*, 1984; Quinn and McGeoch, 1985).
UL30	DNA repliation; DNA polymerase (Chartrand *et al.*, 1979; Quinn and McGeoch, 1985).
UL32	Locus of immune cytolysis resistance mutation (Coen *et al.*, 1984).
UL34	Virion protein (Marsden *et al.*, 1978)
UL36	Virion protein (Batterson *et al.*, 1983)
UL37	Viral replication; DNA binding protein (Shelton *et al.*, 1990).

Table:1.1 (continued)

UL38	Virion protein (Rixon *et al.*, 1990)
UL39	Large sub-unit of ribonucleotide reductase (Preston *et al.*, 1984).
UL40	Small sub-unit of ribonucleotide reductase (Preston *et al.*, 1988).
UL41	Virion host shutoff protein (Fenwick and Everett, 1990a).
UL42	DNA replication; sub-unit of DNA polymerase (Gottlieb *et al.*, 1990).
UL44	Virion glycoprotein C (Frink *et al.*, 1983).
UL47	Tegument protein (McLean *et al.*, 1990).
UL48	Major tegument protein; activator of IE genes (Campbell *et al.*, 1984; Dalrymple *et al.*, 1985).
UL50	Deoxyuridine triphosphatase (Preston and Fisher, 1984).
UL52	DNA replication (Challberg, 1986; Wu *et al.*, 1988).
UL54	IE transcriptional regulator (Watson *et al.*, 1979).
UL55	IE transcriptional regulator? (Block *et al.*, 1991).
IE175	IE transcriptional regulator (Preston, 1979).
US1	IE protein (McGeoch *et al.*, 1985).
US3	Protein Kinase (McGeoch and Davison, 1986).
US4	Virion glycoprotein G (McGeoch *et al.*, 1985, 1987).
US5	Putative glycoprotein (McGeoch *et al.*, 1985).
US6	Virion glycoprotein D (Watson *et al.*, 1982).
US7	Virion glycoprotein I (McGeoch *et al.*, 1985; Longnecker and Roizman, 1987).
US8	Virion glycoprotein E (McGeoch *et al.*, 1985).
US9	Tegument phosphoprotein (Frame *et al.*, 1986).
US10	Virion protein (Rixon and McGeoch, 1984).
US12	IE protein (Murchie and McGeoch, 1982).

by site-directed deletion mutagenesis, revealed no distinguishable differences in the characterization of the mutants from the parent. Using transfection assays, the loss of both intron sequences resulted in the elimination of the ability of a plasmid-encoded IE1 to activate gene expression, implying that in certain situations, the introns in the IE1 gene may contribute to the efficient expression of the VmwIE110 polypeptide (Everett, 1991).

1.5 HERPES SIMPLEX VIRUS 'a' SEQUENCE.

1.5.1 General properties of the 'a' sequence.

The HSV genome contains specific sequences called the 'a' sequence varying in length from 250-550bp in a directly repeated orientation at the termini of the L and S components and in inverted orientation at the L-S junction (Wadsworth *et al.*, 1975, 1976). The terminus contains only a single copy of the 'a' sequence but the number of copies varies at the L-S junction and the L terminus. Variation in the size of the 'a' sequence varies both within and between strains (Wagner and Summers., 1978; Locker and Frenkel, 1979; Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982b; Mocarski *et al.*, 1985; Varmuza and Smiley, 1985).

1.5.2 Structure of the 'a' sequence.

The structure of HSV-1 strain F is shown in Figure 1.4 (Mocarski and Roizman, 1982b). The 'a' sequence consist of:

(1) **DR1**: a 17-21 bp element present as a direct repeat at the ends of the 'a' sequence
(2) **Ub**: a unique sequence located towards the b sequence
(3) **DR2**: a 12bp element present in 1 to at least 22 copies.

Figure 1.4

The structure of HSV-1 strain F in the prototype orientation (top line). An expansion of the 'a' sequence in the orientation found at the L-S junction is shown below the top line (Mocarski and Roizman, 1982).

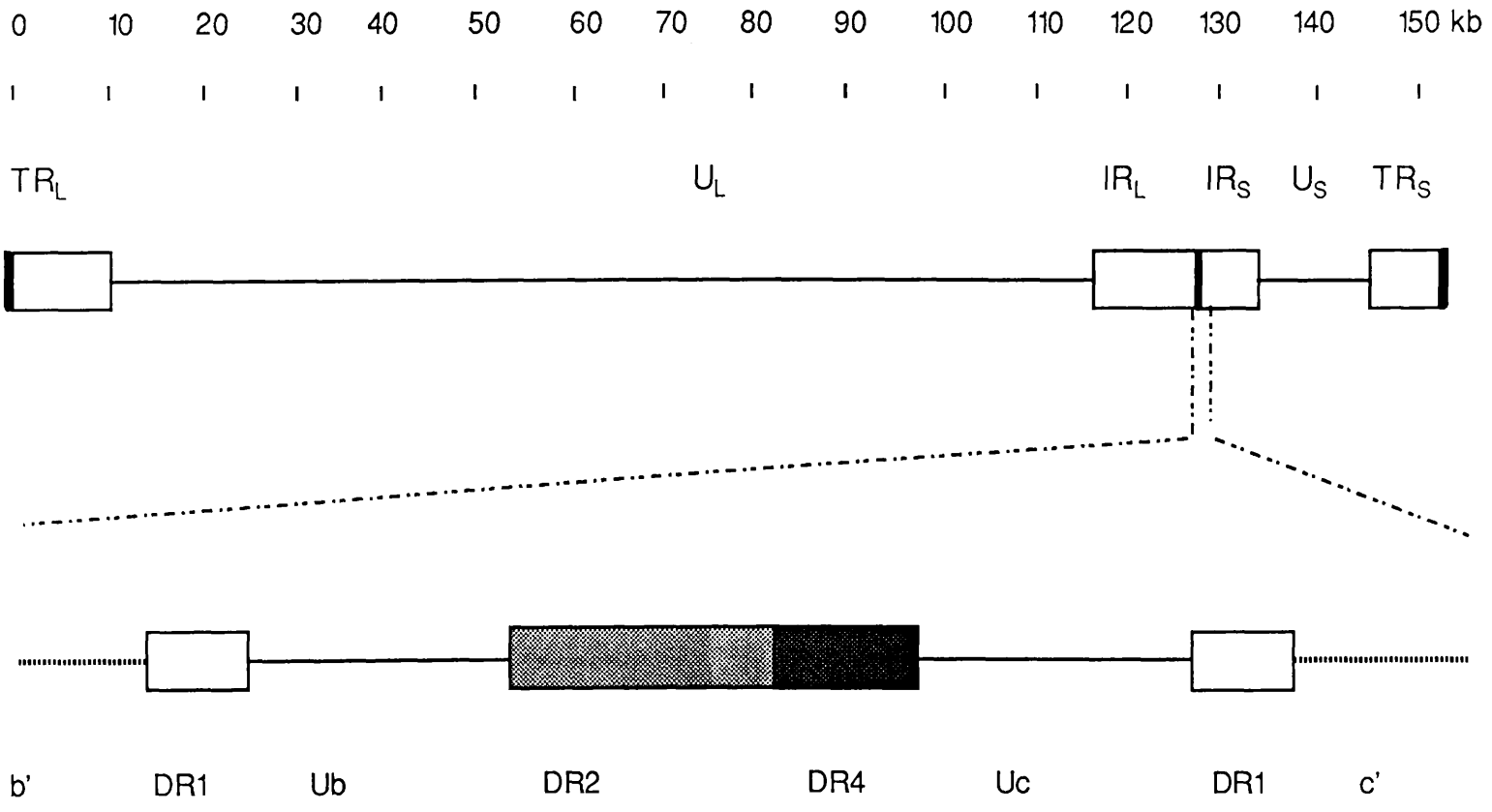
DR1: 20bp element present as a direct repeat at the ends of the 'a' sequence.

Ub: a unique sequence of 64bp located towards the b' sequence.

DR2: a 12bp repeat element present in 22 copies.

DR4: a 37bp repeat element present in 3 copies.

Uc: a unique sequence of 58bp towards the c' sequences.


0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150 kb
TRL
UL
IRL
IRS
US
TRS
b'
DR1
Ub
DR2
DR4
Uc
DR1
c'

(4) **DR4**: one to three repeats of a 37bp sequence containing 11 of the 12 nucleotides of DR2.

(5) **Uc**: a unique 58bp sequence located towards the c sequence.

(6) **DR1**: a second copy of DR1.

1.5.3 Functions of the 'a' sequence.

1.5.3.a Circularisation of the genome.

Linear virion HSV DNA circularises soon after infection. This is believed to be mediated by the 'a' sequence (Davison and Wilkie, 1983b; Poffenberger *et al.*, 1983; Poffenberger and Roizman, 1985). It is likely that circularization takes place by ligation of the two termini aided by the complementary single base at the 3' end overhang (Mocarski and Roizman, 1982b).

1.5.3.b Isomerisation of the genome.

HSV-1 DNA contains four equimolar amounts of isomers. The existence of four isomers was subsequently demonstrated by restriction enzyme analysis (Hayward *et al.*, 1975; Clements *et al.*, 1976). Studies on intertypic recombination between HSV-1 and 2 demonstrated that inversion of the L and S segments was specifically dependent upon the 'a' sequence (Davison and Wilkie, 1983b). Detailed analysis was carried out by deletion in the 'a' sequence (Chou and Roizman, 1985). It was found that deletion in DR2 resulted in a low frequency of inversion (Varmuza and Smiley, 1985); deletion of DR4 sequences resulted in impairment of genomic inversion, whilst deletion of DR2 and DR4 together resulted in completely abolishing inversion indicating the presence of cis-acting signals for recombination and inversion in the DR2 and DR4

sequences. Harland and Brown (1989) reported an approximately 13.5kb deletion in HSV-2 strain HG52 across the L-S junction with loss of the 'a' sequence and complete loss of IR_L and half of the IR_S region, resulting in a fixed prototype orientation of the L segment. However, both the IR_S and TR_S fragments were present albeit, in unequal proportions indicating that the 'a' sequence is not necessary for the isomerisation of HSV. They postulated that the inversion of the S segment is taking place through homologous sequences in TR_S and the remainder of IR_S. This indicates that the recombinational sequences are dispersed throughout the 15kb L-S junction region (Varmuza and Smiley, 1985)

1.5.3.c Cleavage and packaging of the genome.

It is accepted that HSV replicates by a rolling circle mechanism and newly replicated viral DNA molecules consist of large head to tail concatamers (Stow et al., 1983; Stow, 1985). Cleavage of concatameric DNA into unit lengths is mediated by site-specific signals found in the 'a' sequence (Davison and Wilkie, 1981; Varmuza and Smiley, 1985). Cleavage of the DNA is thought to be coupled to encapsidation (Deiss and Frenkel, 1986) and the *cis*-acting signals responsible for both events are all believed to be within the 'a' sequence (Stow, 1985). These signals are now found to be within a 179bp fragment containing Uc-DR1-Ub from the junction of the two tandem 'a' sequences (Nasseri and Mocarski, 1988).

1.5.3.d Promoter activity.

It has been found in HSV-1 strain F that the Ub region of the 'a' sequence contains a promoter for a gene encoding a polypeptide ICP 34.5 (Chou and Roizman, 1986). The 5' end of the transcript is in DR1 and the coding region is in the long repeat in

the same orientation as IE1 (Ackermann *et al.*, 1986). The sensitivity to phosphonoacetic acid suggests it belongs to the late gene category. The promoter is atypical of HSV genes in that there is no TATA consensus in the normal position i.e around -25, but there is TATA homology i.e TTTAAA at around -15 position. A similar ORF has not been found in the corresponding region of HSV-1 strain 17 syn^+ (Perry and McGeoch, 1988).

1.5.3.e Protein binding to the 'a' sequence.

The 'a' sequence encodes *cis*-acting signals which are necessary for circularization ,cleavage-packaging, inversion and promoter activity. A small polypeptide attaching to the L-S junction of the virus DNA has been observed under electron microscopy (Wu *et al.*, 1979). Some late polypeptides (21KDa and 22KDa) encoded by the gene US11 (Rixon and McGeoch, 1984; Johnson *et al.*, 1986)) have been shown to interact with the 'a' sequence of HSV-1 *in vitro* (Dalziel and Marsden, 1984) and are strong DNA binding proteins (MacLean *et al.*, 1987). The function of these proteins remain unclear since US11 deletion mutants are viable in tissue culture (Umene, 1986; Brown and Harland, 1987). Further more two proteins (>250k and 140K) and virus specific DNAses which form complexes with both *PacI* (signal specific site I for cleavage and packaging of DNA located in the Uc) and the DR1 region of the 'a' sequence have been reported (Chou and Roizman, 1989).

1.6 HERPES SIMPLEX VIRUS GENE EXPRESSION.

1.6.1 Temporal gene expression.

HSV-1 encodes at least 70 predicted proteins (McGeoch *et al.*, 1988). The expression of HSV genes is tightly regulated and systematically ordered in cascade fashion. According to their order

of synthesis the genes are classified into three broad groups: immediate early (IE), early (E) and late (L) genes.

1.6.1.a Immediate early (IE) gene expression.

There are five immediate early genes named IE1, IE2, IE3, IE4 and IE5 encoding VmwIE110, VmwIE63, VmwIE175, VmwIE68 and VmwIE12 polypeptide products respectively (Clements *et al.*, 1979; Preston, 1979; Easton and Clements, 1980; Wagner, 1985).

IE RNAs are first detected 1h post-infection, reach their peak at 4-6 h post infection and then decrease. Although IE RNA can be detected at late infection times (Harris-Hamilton and Bachenheimer, 1985; Godowski and Knipe, 1986), the transcription of these genes is independent of de novo protein synthesis in newly infected cells (Honess and Roizman, 1974; Harris-Hamilton and Bachenheimer, 1985).

The UL48 gene product (Vmw65) has been shown to trans-activate IE gene expression (Campbell *et al.*, 1984). Additionally IE gene expression is enhanced by functional IE1 (VmwIE110) (O'Hare and Hayward, 1985)and IE3 (Vmw175) gene products and repressed by IE3 (IE175) (Preston, 1979; Paterson and Everett, 1990)). The IE5 gene has been shown to be non-essential for growth in tissue culture (Umene, 1986; Brown and Harland, 1987). Four out of five IE genes are in the repeats and three of the five IE transcripts are spliced. IE4 and IE5 are derived from identical promoters. The splice sites of their RNAs are in the short repeat region while their coding regions are different (Watson *et al.*, 1981b; Rixon and Clements, 1982). The transcripts of IE2 and IE3 genes are unspliced and are transcribed entirely within U_L and IR_S/TR_S respectively (Rixon *et al*,. 1982).

A highly conserved 5'-TAATGARATTC-3' (R= a purine residue) sequence present upstream of the mRNA cap site present in

all five IE genes is important for interaction with the major tegument protein (Vmw65 TIF) (Post *et al.*, 1981; Mackem and Roizman, 1982a, 1982b; Whitton *et al.*,1983). This motif appears to be unique to IE genes and is crucial for IE1 stimulation by Vmw65 (Preston *et al.*, 1984). Mutation in the Vmw65 TIF gene abolishes its ability to stimulate IE gene expression (Ace *et al.*, 1989). This motif is also conserved in HSV-2 (Whitton *et al.*, 1983; Whitton and Clements, 1984). Detailed analysis of the Vmw65 protein in transactivation and protein complexes with the cellular protein OCT-1 was carried out by detailed analysis of deletions within the Vmw65 polypeptide (Greaves and O'Hare, 1990). It was found that amino acid residues 49-75 from the amino-terminal end are essential for complex assembly and transactivation. Single amino acid substitution in this region abolishes the function of Vmw65.

1.6.1.b Early (E) gene expression.

After the IE genes the next genes to be expressed are the E genes. They appear after functional IE gene products reach their peak, i.e., 4-6 h post infection, after which they decline with time (Honess and Roizman,1974). Some E polypeptides require DNA synthesis for maximal expression and gD is an example of such a protein (Johnson *et al.*, 1986).Early gene expression is regulated by a number of factors. In short term transfection assays, VmwIE175 is capable of inducing E gene expression. Transactivation by VmwIE110 is less specific than by VmwIE63 or VmwIE175. A scheme of nomenclature has been proposed where E genes are subdivided into 1 (major DNA binding protein and large subunit of ribonucleotide reductase) and 2 (thymidine kinase and DNA polymerase) according to the time they are first expressed (Mavromara-Nazos *et al.*, 1986).

1.6.1.c Late (L) gene expression.

These are expressed during late times of infection, their gene products being detected 3 h post infection and reaching their peak by 10-16 h post infection (Roizman,1979). The L genes are divided into two classes gamma$_1$ (the leaky late) which are expressed to some extent in the absence of virus DNA replication and gamma$_2$ (true late) which have an essential requirement for virus DNA replication (Holland *et al.*,1980). Leaky late genes are exemplified by the major capsid protein (MCP) encoded by the gene UL19 and gB encoded by the gene UL27 while true late genes are gC (UL44), 21K/22K (US11) and 82KDa & 81KDa proteins encoded by the gene UL47 (McLean *et al.*, 1990).

The promoters of L genes are mediated by the functional VmwIE110 and VmwIE175 and true late genes have a stringent dependence on viral DNA synthesis for their expression, unlike leaky lates whose expression is reduced but not abolished(Silver and Roizman, 1985). The role of the IE2 gene product (VmwIE63) affecting the L genes is that it not only stimulates gamma$_1$ genes but is also required for gamma$_2$ induction (Rice and Knipe, 1990). Ts mutants in the IE3 gene show a profound reduction in the level of L proteins at the non-permissive temperature indicating an important role of VmwIE175 in regulation (DeLuca *et al.*, 1984).

1.7 LYTIC INFECTION BY HERPES SIMPLEX VIRUS.

1.7.1 Virus adsorption, penetration and uncoating.

Virus particles attach to specific cell receptors initially weakly and then by irreversible binding of the virion to the cell surface (Rosenthal *et al.*, 1984). The nature of the cellular receptors

is not yet known but appears to be different for HSV-1 and 2. Neomycin blocks the receptor binding of HSV type 1 but not type 2, implying that aminoglycosides have a role in the type 1 virus receptor interaction. Analysis of intertypic recombinants show that the region encoding HSV-1 adsorption to the cell receptor is between 0.580-0.687 map coordinates (Langeland *et al.*, 1990). Certain host factors like the fibroblast growth factor receptor also facilitate virus entry into the cell (Kaner *et al.*, 1990).

Following attachment the virus penetrates into the cell cytoplasm by membrane fusion rather than phagocytosis and by tranferring virus envelope glycoproteins to the cell membrane (Para *et al.*, 1980). At least three glycoproteins (gC, gB and gD) of the seven known HSV-1 glycoproteins are able to form a complex with cellular surface structures (Kuhn *et al.*, 1990). Glycoprotein gD appears to be essential for virus penetration (Johnson et al., 1990).An essential epitope for glycoprotein gD has been identified and substitution of leucine at position 25 by proline renders the virus incapable of entry into the cell (Campadelli-Fiume *et al.*, 1990). Various deletion mutants have been analysed in order to map the essential domains of gD. This resulted in distortion of the peptide structure or loss of its antigenic characteristics. In contrast Muggeridge (1990) has reported another domain in gD in which deletion of residues 234-244 has only a localised effect on antigenicity but results in loss of infectivity by preventing the virus from making a complex with the cell receptor. gB has been implicated in virus fusion to the cell surface (Johnson *et al.*, 1984; Cai *et al.*, 1988).

After penetration the virus capsid is degraded and transported to the nucleus via the cytoplasm where the viral DNA is released (Batterson and Roizman, 1983). The DNA enters the nucleus

through the nuclear pores (Batterson *et al.*, 1983).

1.7.2 Suppression of host cell macromolecular synthesis.

During lytic infection HSV directs the host cell metabolic machinery to facilitate its own genomic replication. Synthesis of host macromolecules typically declines within 2-4 h post infection, depending on the virus strain and cell type (Fenwick and McMenamin, 1984). As a result there is a decline in host cell DNA, RNA and protein synthesis. The components of infectious virus mediate disaggregation of polyribosomes. There is a specific appearance of RNA polymerase in extracts of herpesvirus infected cells due to infection induced disruption of mitochondrial membranes, followed by release of the enzyme into the cytosol (Tsurumi and Lehman, 1990) As a consequence of this, host protein synthesis is inhibited (Sydiskis and Roizman, 1966; Fenwick and Walker, 1978; Nishioka and Silverstein, 1978).

The mechanism of host shut-off differs in HSV-1 and 2 (Hill *et al.*, 1983). Some strains of HSV-2 produce strong and rapid inhibition of host proteins (Pereira *et al.*, 1977, Schek and Bachenheimer, 1985). The virion function involved in shut-off of host protein synthesis has been mapped to the region between 0.52-0.59 map units on the HSV-2 strain HG52 genome (Morse *et al.*, 1978; Fenwick *et al.*, 1979) and to a 265bp fragment spanning map coordinates 0.604-0.606 of the HSV-1 strain KOS genome (Kwong *et al.*, 1988) which corresponds to the product of the UL41 gene (McGeoch *et al.*, 1988). The protein encoded by this gene has not been identified but it is presumed to be a non-essential virion structural protein (Fenwick and Everett , 1990a). This protein is also responsible for early degradation of host mRNA *in vitro* (Kirkorian and Read, 1991). Transfer of the UL41 gene from the strong

shut-off HSV-2 strain G to the weak shut-off HSV-1 strain 17 syn^+ results in restoration of efficient early shut-off of host protein synthesis (Fenwick and Everett, 1990a, b; Everett and Fenwick 1990).

1.7.3 DNA replication.

Very little is known about the mechanism of viral DNA synthesis. Electron microscopy analysis shows that viral DNA molecules circularise after infection (Friedmann *et al.*, 1977; Hirsch *et al.*, 1977). This happens due to direct ligation of the terminal 'a' sequence (Jacob and Roizman, 1977; Davison and Wilkie, 1983a). At the onset of replication virus DNA molecules showing 'eyes' and 'D' loops at or near one end of the DNA were observed during electron microscopy. Late in infection, large head to tail concatemers lacking termini appear. These are generated by a rolling circle mechanism from which unit length genomes are cleaved within the 'a' sequence (Davison and Wilkie, 1981) and packaged (Jacob *et al.*, 1979; Kaerner *et al.*, 1981; Vlazney and Frenkel, 1981). *In vitro* DNA synthesis of HSV-1 also supports the idea of a rolling-circle molecule (Rabkin and Hanlon, 1990).

1.7.3.a Origin of DNA replication.

Evidence for at least two *cis*-acting signals (origin of replication) that could mediate HSV DNA replication came from the studies using defective virus DNA (Frenkel *et al.*, 1975) and electron microscopy (Friedmann *et al.*, 1977; Hirsch *et al.*, 1977). Subsequently greater accuracy was achieved by characterization of those origins in a plasmid replication system (Stow and McMonagle, 1983; Weller *et al.*, 1985).

One located close to the center of U_L (Ori_L) is present as a

single copy between divergent promoters of the genes encoding DNA polymerase (UL30) and the major DNA binding protein (UL29) (Weller et al., 1985). The other (Ori_S) is in both copies of R_S and is therefore diploid being situated between the divergent promoters of IE3 and IE4/5 in R_S (Stow, 1982). In HSV-1 strain 17 syn^+ Ori_S contains a nearly perfect 45bp palindromic sequence featuring 18 centrally located AT motifs surrounded by GC residues (Stow and McMonagle, 1983).

Ori_L contains a perfect 144bp large palindrome (Weller *et al.*, 1985; Gray and Kaerner, 1984; Quinn and McGeoch, 1985) which is presumably responsible for Ori_L deletion during cloning in plasmid vectors (Spaete and Frenkel, 1982).

There is a high degree of homology including an AT rich region between Ori_L and Ori_S (McGeoch, 1987). Sequence analysis of HSV-2 Ori_L shows a strong homology, to that of HSV-1 especially in the palindrome (Lockshon and Galloway, 1986). The significance of three origins of replication in HSV-1 remain unsolved. Mutant viruses lacking either one copy of Ori_L (Polvino-Bodnar *et al.*, 1987) or Ori_S (Longnecker and Roizman,1986; Brown and Harland, 1987) are viable in cell culture. Hubenthal-Voss *et al* (1987) have reported that the Ori_S of HSV-1 is contained within a transcribed ORF, which could encode a 34 KDa protein. The interpretation of that ORF remains questionable (McGeoch *et al.*,1988).

1.7.3.b Proteins involved in DNA replication.

During the course of infection several viral specific functions are involved in DNA replication and metabolism. Some of them are associated with the virus particles and others are identified in virus infected cells.

(1) A HSV encoded *polymerase* which is distinguishable from the

host cell polymerase by having an associated 3'-5' exonuclease, a 5'-3' exonuclease (RNase H) and DNA polymerase catalytic activities (Keir *et al.*, 1966; Knopf, 1979; Haffey *et al.*, 1990). It is mapped to gene UL30 (Quinn and McGeoch,1985) and been shown to be essential for viral DNA replication (Hay and Subak-Sharpe 1976; Chartrand *et al.*, 1980). There is increasing evidence that the product of the gene UL42 acts as an accessory sub unit of DNA polymerase. Both genes are required for viral replication and the combined action of both products results in increased processivity of polymerisation (Gottlieb *et al.*, 1990).

(2) HSV-1 encodes *thymidine kinase* (pyrimidine deoxyribonucleotide kinase) which has been mapped to the UL23 gene and sequenced (McKnight, 1980; Wagner *et al.*, 1981). This enzyme is dispensable for virus growth (Jamieson *et al.*, 1974), but Tk negative mutants show reduced pathogenicity (Field and Wildy, 1978).

(3) The *alkaline exonuclease* activity associated with HSV infected cells was first reported by Keir and Gold (1963). Later it was found that they not only exhibit 3' to 5' exonuclease activities but also endonuclease activity (Hoffman and Cheng, 1979; Hoffman, 1981). In HSV-2 it has been mapped between 0.145-0.185 m.u (Moss *et al.*, 1979; Preston and Cordingley, 1982) and has been shown to be essential for DNA synthesis (Francke *et al.*, 1978; Moss *et al.*, 1979; Moss, 1986). Recent evidence indicates that in HSV-1 the alkaline exonuclease encoded by the gene UL12, is not essential for viral DNA synthesis but may play a role in the processing and packaging of viral DNA into infectious virions (Weller et al., 1990).

(4) Viral encoded *uracil-DNA glycosylase* involved in DNA repair

(Lindahl, 1979) is responsible for removing uracil residues created by deamination of cytosine (Caradonna *et al.*, 1987). Recently it has been shown that in HSV-1 UL2 encodes uracil-DNA glycosylase which is dispensable in tissue culture (Mullaney *et al.*, 1989).

(5) Virally induced *DNA topoisomerase, helicase and primase* activities (Muller et al., 1985) ; may be involved in DNA replication, transcription and recombination (Gellert, 1981). The products of UL5 and UL52 genes form a holoenzyme and are associated with DNA-dependant ATPase, DNA-dependant GTPase, DNA helicase and DNA primase activities (Dodson and Lehman, 1991).

(6) *Deoxyuridine triphosphatase* which catalyses the conversion of dUTP to dUMP and pyrophosphate is encoded by the UL50 gene (Preston and Fisher, 1984). This enzyme is dispensable in tissue culture (Preston and Fisher, 1984; Williams, 1988).

(7) The viral encoded *ribonucleotide reductase* catalyses the reduction of ribonucleotides to deoxyribonucleotides (Thelander and Reichards, 1979). The enzyme consists of two subunits RR1 (large unit) and RR2 (small unit) encoded by the gene UL39 (Preston *et al.*, 1984; Nikas *et al.*, 1986) and the gene UL40 (Preston *et al.*, 1988) respectively. The RR1 and RR2 form a holoenzyme which is essential for its activity (Frame et al.,1985; Bacchetti *et al.*,1986; Nikas *et al.*, 1990). This complex can be inhibited by targeting synthetic oligopeptides against the carboxy terminus of RR2 (Frame *et al.*, 1985). Although this enzyme is dispensable in tissue culture (Goldstein and Weller, 1988) it is essential *in vivo* in the mouse model (Jacobson *et al., 1989).*

(8) *Protein kinases* encoded by the gene UL13 (Smith and Smith, 1989) and the gene US3 (McGeoch and Davison, 1986a) are homologous with members of the protein kinase family of *eukaryotes*. Although US3 (Longnecker and Roizman, 1987) and UL13 (L.J Coulter, personal communication) are dispensable *in vitro*, their role in HSV infection is yet to be decided.

(9) The major DNA binding protein (mDBP), which preferentially binds to single stranded DNA (Bayliss *et al.*, 1975) and is encoded by the gene UL29 in HSV-1. The mDBP mutants have altered sensitivity to the inhibitors of virus DNA polymerase, suggesting a functional interaction between these proteins (Chiou *et al.*, 1985). Functional mDBP is essential for virus DNA replication (Conley *at al.*, 1981).

(10) An origin binding protein assigned to UL9 has been shown to play an essential role in DNA replication (Elias *et al.*, 1986; Elias and Lehman, 1988; Olivo *et al.*, 1988; Weir *et al.*, 1989; Weir and Stow, 1990). Heilbronn *et al* (1990) reported that the UL9 gene is dispensable for SV40 origin of virus replication. A newly recognised DNA binding protein of 120KDa encoded by the gene UL37 may be involved in late events of viral replication (Shelton *et al.*, 1990).

(11) An essential 65K DNA binding protein ($65K_{DBP}$) (Bayliss *et al.*, 1975; Powell and Purifoy, 1976) encoded by UL42 gene (Parris *et al.*, 1988) and distinct from the 65k virion polypeptide (Marsden *at al.*, 1987) is essential for DNA replication (McGeoch, 1987). The $65K_{DBP}$ has been shown to be strongly associated with DNA polymerase (Vaughan *et al.*, 1985; Gottlieb *et al.*, 1990).

HSV-1 mutants and the use of plasmid amplification assays for HSV-1 origin dependent DNA replication demonstrated that the

products of genes UL5, UL8, UL52 (Challberg, 1986, Wu *et al.*, 1988), UL9 (Olivo *et al.*, 1988; Weir *et al.*, 1989; Weir and Stow, 1990), UL 29 (Conley *et al.*, 1981; Quinn and McGeoch 1985), UL30 (Chartrand *et al.*, 1979; Quinn and McGeoch, 1985) and UL 42 (Parris *et al.*, 1988) are involved, necessary and sufficient for viral DNA replication.Three of these genes UL29, UL30 and UL42 encode for the major DNA binding protein, viral DNA polymerase and $65K_{DBP}$ respectively.

1.8 VIRUS ASSEMBLY AND MATURATION.

Mature DNA which has been replicated from a circular spool and forms concatemers, is cleaved into unit length molecules in the nuclei of infected cells (Stow *et al.*,1983). Cleavage-packaging and encapsidation occur at the 'a' sequence (see section 1.5.3.c). There are at least 15-33 different structural polypeptides, including those of the nucleocapsid, the tegument and the glycoproteins (Spear and Roizman, 1980). The nucleocapsid of HSV-1 is considered to be composed of seven structural proteins (Gibson and Roizman, 1972; Heilman, 1979; Cohen *et al.*, 1980). There is a striking similarity of the structural polypeptides from the different herpes viruses (Dargan, 1986), which reflects the rigid structure of herpes virion architectural restraint on the size and number of the proteins which make up the nucleocapsid. Although several viral genes important for the formation of nucleocapsids have been identified, the way in which virion proteins are assembled into the virus particle is still poorly understood. Analysis of different ts mutants demonstrated that structural polypepide p40 (Vp22a) (Preston *et al.*, 1983) and some other unidentified polypeptides play a role in DNA packaging (Addison *et al.*, 1984). Lack of these polypeptides is associated with empty capsids (Preston *et al.*, 1983; Rixon *et al.*, 1988). Viruses

acquire their envelopes by budding into the cytoplasmic vacuoles of the golgi membrane (Nii, 1971). Preston *et al* (1983) showed that a mutant containing a temperature sensitive lesion in gene UL26 is defective in processing VP22a. It has been found that the gene UL26 transcribes two mRNAs, translation of which give rise to 635 and 329 amino acids (Liu and Roizman, 1991). Subsequently it was found that the UL28 gene product is important for mature capsid formation (Addison *et al.*, 1990). Furthermore, two more capsid protein products encoded by the genes UL18 and UL38 were found by direct amino acid sequencing (Rixon *et al.*, 1990). Recently Al-Kobaisi *et al* (1991) showed that the product of UL33 gene is required for the assembly of full capsids. A group of four genes, UL10, UL20, UL43 and UL53 whose product proteins were assigned to be membrane-inserted (McGeoch *et al.*, 1988), have recently been characterised by MacLean *et al* (1991). They revealed that any mutation in UL20 and UL53 is lethal for the virus, however changes in the ORFs of UL10 and UL43 barely affect the viability of the genome. Furthermore, products for the gene UL10 and UL20 have also been detected.

Very little is know about the nature of the tegument but it is estimated that there are at least 15 non-glycosylated polypeptides, which the virion acquires during envelopment (Dargan, 1986). One of the major tegument components is Vmw65. This protein is involved in transactivation of IE genes (Campbell *et al.*, 1984).

The glycoproteins form part of the envelope and are among the structural proteins, which has been extensively studied. Apart from the envelope, structural glycoproteins have been detected in both the nuclear and cytoplasmic membranes of infected cells (Spear *et al.*, 1970). HSV-1 encodes at least seven glycoproteins. These include gB, gC, gD, gE, gG, gH and gI (Spear 1976; Marsden *et al.*,

1978; Bauke and Spear, 1979; Roizman *et al.*, 1984; Frame *et al.*, 1986; Longnecker *et al.*, 1987; McGeoch, 1987) encoded by the genes UL27, UL44, US6, US8, US4, UL22 and US7 respectively (McGeoch *et al.*, 1988). Clustering of glycoproteins in U_S with US4, 6, 7 and 8 which encode of gG, gD, gI and gE is an interesting feature to note. There may have been gene duplication at one point (McGeoch *et al.*, 1988). Among the cluster of the genes there is one ORF (US5) with the potential for an additional glycoprotein in the HSV-1 genome (McGeoch *et al.*, 1985) but this remains to be identified.

Several glycoproteins are dispensable in tissue culture (Hoggan and Roizman, 1959; Heine *et al.*, 1974; Cassai *et al.*, 1975; Holland *et al.*, 1984; Zezulak and Spear, 1984; Longnecker and Roizman, 1986, 1987; Harland and Brown, 1988). Only three glycoproteins gB, gD and gH are essential for infectivity (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Weller *et al.*, 1983; Buckmaster *et al.*, 1984; Gompels and Minson, 1986; McGeoch and Davison, 1986b, Desai *et al.*, 1988; Ligas and Johnson, 1988). The functions of these glycoproteins are described briefly in Table 1.2.

Egress of viruses from the infected cell occurs by reverse phagocytosis (Katsumoto *et al.*, 1981). Apart from virus components a number of cellular factors play a part in the egress of virus. The mouse L-cell mutant gro29 survives HSV-1 infection due to a defect in propagation of the virus. It has been revealed that the maturation of virus and glycoprotein expression on the cell surface is normal but the gro29 cells harbour a lesion that inhibits the egress of virus from the cell, indicating an active role of some unidentified cellular components in this process (Banfield and Tufaro, 1990). Use of brefeldin A, a fungal metabolite which causes redistribution of Golgi into endoplasmic reticulum, arrested the maturation and egress of herpes simplex virus particles during infection by inducing

Table: 1.2 Known properties of the HSV-1 encoded glycoproteins

Glycoprotein	Gene	Properties	
		Biological	Immunological
gB	UL27	Required for cell fusion and infectivity Essential for replication in tissue culture.	Provokes helper T-lymphocytes and humoral immunity.
gC	UL44	Involved in adsorption, penetration and cell fusion.	Induces humoral and delayed cytotoxic T-cell immunity. Binds to C3b component of complement
gD	US6	Required for adsorption and cell fusion.	Induces humoral and delayed type of hypersensitivity.
gE	US8	Required for adsorption but not essential for replication in tissue culture.	Binds to Fc portion of IgG.
gG	US4	Not determined.	Induces neutralising anti-bodies, delayed hyper sensitivity and react with Fc portion of IgG.
gH	UL22	Required for egrees, cell to cell spread and replication of virus in tissue culture.	Induces neutralising antibodies.
gI	US7	Dispensible for replication in tissue culture.	Induces neutralising antibodies and interact with Fc portion of IgG.

retrograde movement of molecules from Golgi complex to the endoplasmic reticulum early in infection, idicating that brefeldin A causes changes in cellullar factors affecting the progress of the virus (Cheung *et al.*, 1991).

1.9 HERPES SIMPLEX VIRUS LATENCY.

1.9.1 Introduction.

At the start of the twentieth century herpes zoster lesions were documented coincidental with herpetic lesions; trigeminal ganglionitis and pnueumonitis (Head and Campbell, 1900). At the same time Cushing (1905) observed a sequellae after removing the trigeminal ganglia (an operation performed for the treatment of trigeminal neuralgia) in that the patients treated had no herpetic eruptions on the same side but did on the opposite side of the face innervated by the nerve.

Two decades after the disease was known to be infectious and virally induced, the crucial role of the nervous system in the pathogenesis of infection was firmly established by inducing herpetic keratitis in a rabbit (Goodpasture and Teague, 1923).

Following primary infection and active replication at peripheral sites, the virus attaches to the sensory nerve terminals, (Vahlne *et al.*, 1978), enters them and travels centripetally via neural routes to sensory ganglia (Stevens and Cook, 1971; Cook and Stevens, 1973). It is within the neurons of the sensory ganglia that the virus becomes latent (Cook *et al.*, 1974; Stevens, 1975). During this phase of the virus, it cannot be isolated from homogenised ganglionic tissue inoculated into cell culture.

1.9.2 Animal models for latency.

Several animal model systems have been developed for HSV latency. The earliest one is the rabbit eye model. The finding of the histological lesions in the trigeminal ganglia of rabbits infected on the cornea was observed by Friedenwald (1923) and further extended by Goodpasture (1925, 1929). Latency induced in animal models like rabbits, mice, guinea-pigs and rats resembles the human disease in many respects though spontaneous recurrences have only been observed in mice inoculated in the ear flap (Hill *et al.*, 1975), in guinea-pigs inoculated intravaginally and in the foot-pad (Scriba, 1975; Donnenberg *et al.*, 1980). In guinea-pigs spontaneous reactivation is very common (Scriba, 1976; Stanberry *et al.*, 1985).

The pioneering work in the mouse model latency system was described by Stevens and Cook in 1971. The pattern of establishment of latency is similar to other animal models like the rabbit eye model (Stevens *et al.*, 1972), the mouse ear model (Hill *et al.*, 1972) and the guinea-pig for genital HSV infection (Scriba, 1976). Typically inoculation in the mouse rear foot-pad causes local cutaneous lesions followed by centripetal movement of virus through the peripheral and central nervous system. Viral replication ends either with an outcome of complete recovery or permanent paralysis of the posterior root of the spinal cord and sometimes death . During acute infection which usually lasts up to ten days the virus can be recovered from the sciatic nerve, DRG, posterior root, spinal cord and brain. However in mice recovered after 3 weeks, the virus was not present in homogenates of the nervous system but could be reactivated by co-cultivation of the DRG with indicator cell monolayers (Stevens and Cook., 1971). This demonstrates that the virus was in a latent state in nervous tissue.

1.9.3 Sites of latency.

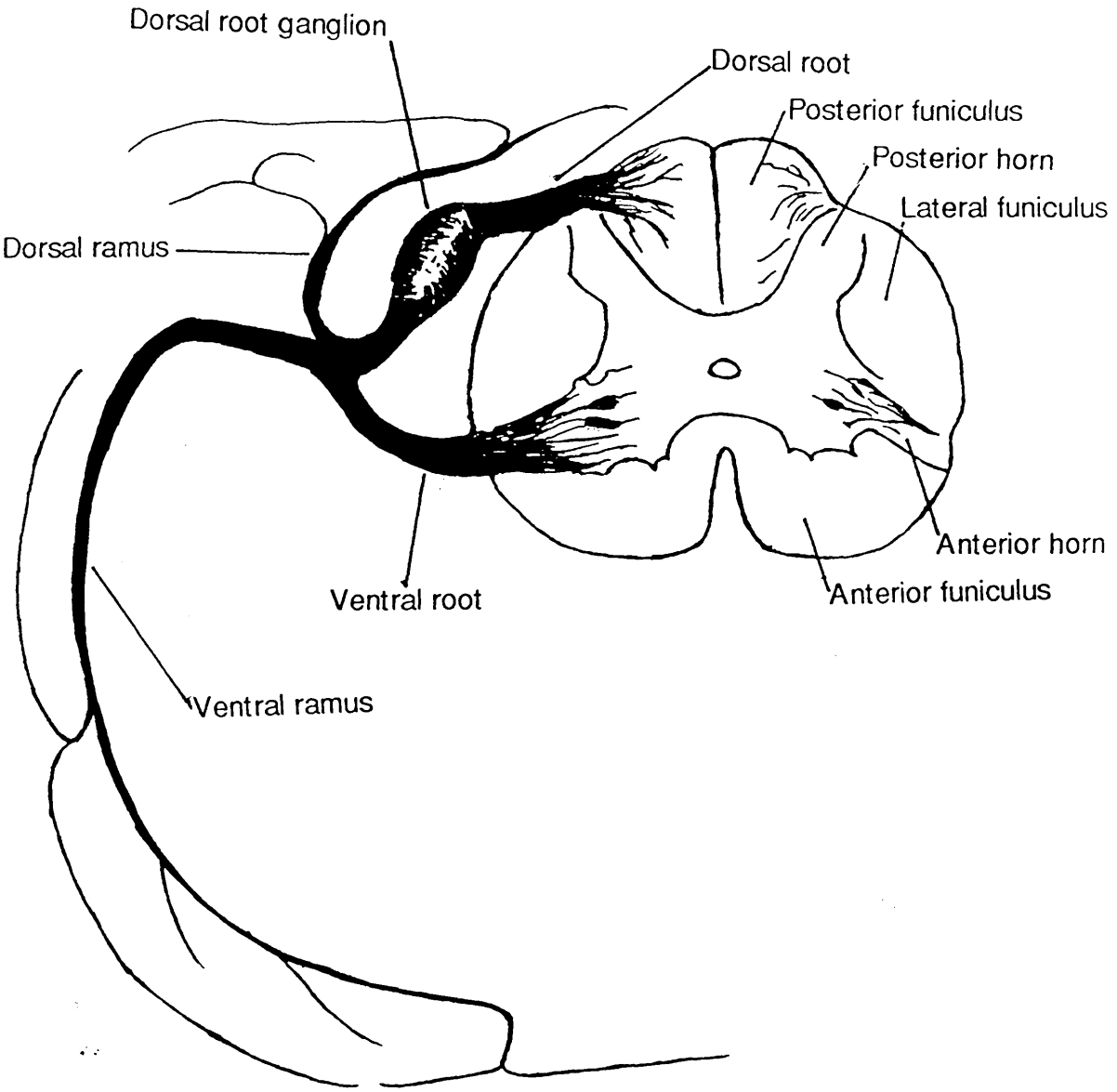
Clinical and histological evidence that nervous tissue is the site of herpes latency arose much earlier (Cushing, 1905; Goodpasture and Teague 1923). Reactivation of infectious HSV from sensory ganglia following explantation of nervous tissue and organs (spleen and adrenals etc.) cultured in the presence of indicator monolayers gave direct evidence of sites of latency in mice, rabbits and guinea pigs (Stevens and cook,1971; Stevens *et al.*, 1972; Knotts *et al.*, 1973; Baringer and Swoveland, 1974; Walz *et al.*,1974; Scriba, 1975, 1976). More importantly isolation of HSV from human trigeminal ganglia was first reported by Bastian *et al* (1972). Subsequent studies have shown that HSV could be routinely isolated from trigeminal, cervical, vagus and sacral ganglia (Plummer, 1973; Baringer and Swoveland, 1973; Rodda *et al.*, 1973; Brown *et al.*, 1979; Lonsdale *et al.*, 1979, Warren *et al.*, 1979) (Figure 1.5). Signals of HSV DNA present inside nervous tissue was not only reported in animal models, (Walz *et al.*, 1976; Puga *et al.*, 1978; Cabrera *et al.*, 1980; Fraser *et al.*, 1984; Stroop *et al.*, 1984) but also in human brain tissue (Sequiera *et al.*, 1979; Fraser 1981).

1.9.4 Latency in non-neuronal tissues.

Evidence is accumulating which suggests that peripheral non-neuronal sites of herpes virus latency in human and experimentally infected animals also exists. The phenomenon of virus shedding in the abscence of clinical disease was seen in body secretions of humans or rabbits (Kaufman *et al.*, 1967; Douglas and Couch, 1970).In the guinea pig model spontaneous reactivation of HSV is common (Scriba, 1977). Nerve section in such animals after inoculation of the footpad could not prevent spontaneous recurrent infection at the site of inoculation. Although the incidence of such occurrence was very low, indicating further evidence of peripheral

Figure 1.5

Diagram of a typical spinal nerve, transection of spinal cord at the level of 12th thoracic vertebra and dorsal root ganglia of humans (adapted from Cunningham's Manual of Practical Anatomy).


Dorsal root ganglion
Dorsal root
Posterior funiculus
Posterior horn
Lateral funiculus
Dorsal ramus
Anterior horn
Anterior funiculus
Ventral root
Ventral ramus

non-neuronal latency of HSV. However, treating such an animal with phosphonoacetic acid and acycloguanosine eliminated HSV in peripheral tissues but that did not eliminate the ability of latent HSV to reactivate in ganglia following explantation (Scriba, 1980). Latent HSV-1 has also been recovered from the anterior chamber of the mouse eye including uvea, the tissue most susceptible to productive infection (Claoue *et al.*, 1990). HSV-1 and 2 have both been recovered from the footpad as well as from the dorsal root ganglia of latently infected mice (Al-Saadi *et al.*,1983; Clements and Subak-Sharpe, 1988; Subak-Sharpe *et al.*, 1984a, 1984b, Al-Saadi *et al.*, 1988). The presence of HSV specific RNA in the mouse foot-pad further confirmed the evidence of non-neuronal latency by HSV. Using in situ hybridization techniques, viral RNA was detected in basal cells, root sheet cells of hair follicles, epithelial cells of the sebaceous glands and cells within the epidermis (Clements and Jamieson, 1989). Recent evidence after using phase contrast mircoscopy of dorsal root ganglion cells shows that specific latency-associated transcript of HSV-2 have also been detected in non-neuronal ganglion cells (Tenser *et al.*, 1991) indicating non-neuronal latency of HSV.

HSV has been reactivated from cultured cells of explanted corneal tissue of rabbits (Cook *et al.*, 1987) and mice (Openshaw, 1983). In humans, attempts to recover latent HSV from peripheral sites have been unsuccessful (Rustigian *et al.*, 1966) apart from the isolation from human corneas explanted prior to corneal transplantation due to chronic stromal keratitis (Shimeld *et al.*, 1982).

1.9.5 Nerve cells harbouring the virus.

The soma (nerve cell body) has been presumed to harbour latent herpes virus. This was supported by the evidence in mice and

rabbits that virus was regularly reactivable from ganglia which contain cell bodies but not from nerve roots which are axons (Baringer and Swoveland, 1973; Cook *et al.*, 1974). Ultrastructural and immunofluorescent studies have demonstrated acute HSV replication inside the Schwann, satellite and intercellular connective tissue in mice (Dillard *et al.*, 1972; Cook and Stevens, 1973; Knotts *et al.*, 1974; Lascano and Berria, 1980).

1.9.6 Factors affecting latency.

1.9.6.a Physiological state of neurons.

After the neonatal period, no cell division occurs, so that individual neurons must live throughout the life of the animal. Many functions of the cell, particularly those concerned with cell division are repressed so that turnover of DNA is very low. Therefore cellular DNA synthesis occurs only as a consequence of DNA repair mechanisms and large areas of the genome remain untranscribed (Sanes and Okun, 1972; Ishiwaka *et al.*, 1978; Blyth and Hill, 1984). It was postulated that latency is the natural relationship between virus and neurons (Blyth and Hill, 1984). Many of the treatments directly damage neurons of the latently infected ganglia and will inevitably induce cellular repair. In turn this would require transcription of regions of cellular DNA that are normally silent. If viral DNA was associated with such regions, the repair might reactivate virus replication (Watson, 1974; Grafstein, 1975). Other factors which break that natural relationship are immunological factors, electrical stimuli to the trigeminal ganglia *in vivo* (Nesburn *et al.*, 1977; Green *et al.*, 1981), damage to neurons by neurectomy (Price and Schmitz, 1978) or by 6-hydroxydopamine (Price, 1979) can reactivate virus from the neurons.

1.9.6.b Immunity.

A variety of immune mechanisms might be playing a part in establishing the latent state of HSV other than the physiological state of the neurons. Suppression of productive infection was demonstrated *in vitro* by immunoglobulin (Costa *et al.*, 1977) and *in vivo* when ganglia from latently infected mice were implanted into recipient animals treated with anti- HSV serum (Stevens and Cook, 1974). Therefore it was suggested (Lehner *et al.*, 1975; Costa *et al.*, 1977) that immunoglobulins binding to the cell membrane of the neurons suppress viral replication *in vivo*. But the role of circulating anti-HSV antibodies became controversial after an experiment in which mice were passively immunised with anti-HSV anti-body and latently infected. Nine weeks later when anti-HSV antibodies had been eliminated, only 14% of the animals maintained latent infection and were found to be seropositive after 13 weeks. Cold stimuli at the primary site of inoculation (skin of lip) was followed by the appearance of anti-HSV anti-bodies in serum of 90% of the mice with no clinical sign or visible lesion at the skin of the lip (Sekizawa *et al.*, 1980). Reinfection of latently infected mice ganglia with homotypic and heterotypic HSV-1 and HSV-2 suggested that resistance to the establishment of a second latent infection in a gaglion is determined by the general immunity of the animal rather than immunity of the latently infected ganglion itself (Yirrell *et al.*, 1990).

In humans, there is no correlation between the level of neutralizing antibodies and the frequency of recurrent disease. Pre-existing antibodies to HSV-1 do not prevent recurrence of HSV-2 genital disease (Corey *et al.*, 1982; Reeves *et al.*, 1981). There is evidence about the correlation between recurrence and T-cell

immunity. Frequent recurrence has been found in humans (Shillitoe *et al.*, 1977) and in guinea pigs (Donnenberg et al., 1980) with impaired T-cell immunity. The role of interferon in protection against herpes labialis has also been discussed (Cunningham and Merigan, 1983).

Suppression of cell mediated immunity in latently infected immunocompetent animals by cyclophosphamide (Openshaw *et al.*, 1979) and x-irradiation (Hill *et al.*, 1981) induces reactivation. However in those cases the physiological state of the neurons cannot be ruled out because x-rays cause damage to the DNA and similar effects of cyclophosphamide are found if used in higher doses (100 to 200 mg/kg) (Ludlum., 1975).

1.9.7 Reactivation/Recurrence and recrudescence.

A variety of stimuli can cause reactivation in human beings, e.g fever, stress and sunburn (UV radiation) (Hill, 1985) and several chemical and physical stimuli can cause reactivation in animals . Several hypotheses proposed to explain the ability of external stimuli to cause reactivation are discussed below (Wildy *et al.*, 1982).

The *ganglion trigger* theory proposes virus reactivation from the ganglia following nonspecific stimuli like fever and menstruation and shedding of virus at the periphery with or without clinical lesions and virus can be isolated. This phenomenon is called recurrence.

The *ganglion and skin trigger* theory suggests reactivation in the ganglia and transfer of the virus to the end of the dermatome, following peripheral stimuli, where it replicates in epidermal tissue and causes clinical lesions. This is called recrudescence.

Skin trigger hypothesis proposes that local stimuli create changes feasible for HSV growth. As a result microfoci of HSV latency already present due to latent infection, or reactivation of the

latent infection in the ganglia, grow and cause clinical lesions at the periphery.

It is well documented that HSV can establish latency in peripheral tissue (see section 1.9.4). So any changes in the physiological environment could cause reactivation. Various stimuli at the peripheral site or primary site of inoculation (Table 1.3) like injury to skin by plucking the hair (Hurd and Robinson, 1977), UV light (Blyth *et al.*, 1976), application of cellophane tape (Hill *et al.*, 1978) and xylene (Harbour *et al.*, 1983) cause reactivation. Stripping of skin with cellophane, however causes several changes in the epithelium and its environment. Cellophane removes cornified epithelial layer, hairsare plucked (Hill *et al.*, 1978), there is increased multiplication of epithelial cells with short term release of histamine and 5-hydroxytryptamine and up to a 35 fold increase in prostaglandin E_2 in the tissue (Harbour *et al.*, 1983). Injection of prostaglandin E_2 also induces infectious virus in the skin (Blyth *et al.*, 1976).

Reactivation of HSV has been reported after treating skin and ganglia with DMSO. Methylation of cytosine bases in DNA is associated with transcriptional inactivity. DMSO causes hypomethylation of cellullar DNA (Christman *et al.*, 1977). There has been reactivation induction of HSV in ganglia treated with DMSO (Hill *et al.*, 1983; Harbour *et al.*, 1983). Furthermore a very small region of the HSV genome is transcribed during latent infection (Stevens *et al.*, 1987) and it was assumed that latency is maintained by extensive methylation of the HSV genome while demethylation reactivates it. There is also evidence for extensive methylation of the HSV genome in an *in vitro* latency system (Youssoufian *et al.*, 1982). But the report that the HSV genome *in vivo* is not extensively methylated (Dressler *et al.*, 1987) suggests

Table: 1.3 Reactivation of HSV-1 latent infection in ganglia *in vivo*

Site of inoculation	Stimuli	Site of reactivation
Footpad	Section of peripheral nerve	Dorsal root ganglia (Walz *et al.*, 1974)
Footpad	Intratracheal injection of mucin	Dorsal root ganglia (Stevens *et al.*, 1975)
Intraocular	Postganglionic neurectomy	Superior cervical ganglia (Price and Schmitz, 1978)
Cornea	Cyclophosphamide or X-ray	Trigeminal ganglion (Openshaw *et al.*, 1979)
Cornea	Prednisolone, antithymocyte serum or trauma to ganglion	Trigeminal ganglion (Hill *et al.*, 1981)
Lip	Dry ice on lip	Trigeminal ganglion (Openshaw *et al.*, 1979)
Skin of ear pinna	Cellophane tape stripping, DMSO, xylene or retinoic acid to ear.	Cervical dorsal root ganglion (Hill *et al.*, 1983)
Cornea	Cyclophosphamide, dexamethasone or U.V radiation.	Trigeminal ganglion (Shimeld *et al.*, 1990)

that hypomethylation of the cellular DNA initiates transcription of silent host genes thus reactivating latent HSV genomes.

1.9.8 Viral DNA during latency.

Virion HSV DNA is linear and after infection it circularises and initiates its replication (Poffenberger and Roizman, 1985). Transition from linear to circular DNA causes the disappearance of terminal fragments and the appearance of head to tail junction fragments. Terminal fragments are repeated internally while the L and S portions of the genome invert relative to each other, giving rise to four equimolar isomers in a population of virus DNA. This gives rise to four molecules of unique (1M), two (0.5M) of repeat and one (0.25M) junction fragments. Thus if DNA circularises this will decrease the unique and relatively increase the L-S junction fragments (Roizman and Sears, 1987).

Rock and Fraser (1983, 1985) analysed DNA from trigeminal ganglia and brains of latently infected mice and showed that joint fragments were present at an approximately 2:1 molar ratio with respect to unique sequences of HSV. These observations were confirmed by Efstathiou *et al* (1986). These findings indicate that latent HSV DNA is in a nonlinear endless form, i.e. either integrated, concatemeric or circular. In an attempt to distinguish between either possibility, Mellerick and Fraser (1987) examined the HSV-1 genome both in acutely and latently infected mice by cesium chloride density gradient centrifugation. Since they concluded that the majority of the latent HSV DNA exists in an extrachromosomal state in mouse ganglia, entrapment of viral DNA in the chromosomal band makes it difficult to decide whether it is episomal or in an integrated form.

1.9.9 TK⁻ Mutants.

Preliminary studies with TK⁻ mutants had suggested an essential role for TK in the establishment of latency (Tenser *et al.*, 1979). Several studies have suggested TK⁻ mutants are less pathogenic, appear to replicate at peripheral sites but could not be recovered by explantation of ganglia (Tenser and Dunstan, 1979; Tenser *et al.*,1979; Katz *et al.*, 1990). Using genetically engineered mutants in the TK gene it has been shown that neither acute nor latent infection establishment has been successful in mice (McDermott *et al.*, 1984; Tenser and Edris, 1987). Surprisingly Meignier *et al* (1988) reported a host cell specific determinant of TK⁻ mutant, that a well characterized TK⁻ with a 700bp deletion in the TK gene was latency noncompetent for mice but competent for rabbits. Recent reports (Efstathiou *et al.*, 1989; Leist *et al.*, 1989; Friedrich and Schnewis, 1990; Kosz-Vnenchak *et al.*, 1990) show HSV-1 and 2 TK⁻ mutants capable of establishing latent infection in mice. These findings conclude that although TK⁻ mutants are capable of establishing latency, they may play a role in reactivation from latency.

1.9.10 Deletion and insertion mutants.

After the discovery that certain viral genes are dispensable (Heine *et al.*,1974), an interest arose in using deletion and insertion mutants in the establishment, maintenance and reactivation from latency. Experiments using deletion and insertion mutants have shown that the ori_L region of the HSV genome (Polvino-Bodnar *et al.*, 1987), viral protein kinase (Meigneir *et al.*, 1988) IE Vmw68, TK (Sears *et al.*, 1985; Meigneir *et al.*, 1988), IE110 (Clements and Stow, 1989) and riboucleotide reductase (Katz *et al.*, 1990) are not needed for latency establishment. Although the deletion mutants in the

ICP4 (IE3) gene are severely impaired in replication they do reactivate from latently infected mice ganglia (Katz *et al.*, 1990).

Among the insertion mutants used was HSV-1 *in*1814 which has a 12 base pair insertion in the gene UL48 (Steiner *et al.*, 1990) and is capable of establishing a latent infection in mouse trigeminal ganglia. Since this mutant fails to produce a lytic infection it was interesting to observe that the mutant established a latent infection between 24-48 h without IE or L gene expression. Thus indicating that HSV-1 could establish latency without lytic infection and the block to productive replication during establishment of a latent infection by HSV occurs before or during the early expression of immediate early genes. This also indicates that the pathway leading to lytic and latent infection in neurons may diverge at an early stage of host-HSV-1 interaction and that the level of viral IE gene expression has a role in determining the outcome of infection (Ace *et al.*, 1989, Valyi-Nagy *et al.*, 1991)

1.9.11 *In vitro* latency systems.

Traditionally animal models have been used in order to investigate latency. In this system it is difficult to observe the events taking place in isolated cells from examination of the intact animals, so it would be much easier, if latency could be established in cell culture. Typically in; *in vitro* latency systems, cells are infected at superoptimal temperature ($42^{o}C$) in the presence of antiviral drugs or interferons in such a concentration that can inhibit viral lytic infection. Once the virus undergoes latency, the latent state can be maintained at $37^{o}C$. (Colberg-Poly *et al.*, 1979). The latent virus can be reactivated by superinfection with helper virus like, EBV, VZV and adenovirus or lowering the temperature. Colberg-Poly *et al* (1979) first reported reactivation of *in vitro* latent

HSV-2 by superinfecting it with CMV. This indicates that all the helper viruses carry a nonspecific inducer of latent viral genes. Russel *et al* (1987) have shown infection of human foetal lung cells with HSV-2 at the superoptimal temperature of 42°C results in a latent state which is stable on downshift of the culture to 37°C. The latent HSV-2 virus cannot be reactivated by superinfection with the HSV-1 deletion mutant *dl*1403 which has a deletion in the IE1 gene. An adenovirus recombinant expressing HSV-1 IE110 reactivated *in vitro* latent HSV-2 (Harris *et al.*, 1989; Zhu *et al.*, 1990) confirming the importance of the IE1 gene in the *in vitro* latency system.

The latent state of HSV in an *in vitro* system is different than *in vivo*. The genes or part of the genome expressed during *in vivo* latency (LATs) have not yet been reported *in vitro*. Moreover the state of the latent genome *in vitro* latency systems of Wigdahl is different than in animal models and appears to be linear (Wigdahl *et al.*, 1984). On the contrary Harris and Preston (1991) reported a HSV-1 mutant *in*1814 established latency *in vitro* and the DNA was found in a non-linear configuration similar to *in vivo*.

1.9.12 Molecular mechanism of latency.

The mechanisms of establishment, maintenance and reactivation from latency have been studied extensively (Wildy *et al.*,1982) but still remain poorly understood. Investigation of the molecular mechanism of viral genes expressed during latency has been difficult because only 1% of the neurons in a ganglion harbour the virus in the latent form. As neurons constitute 10-20% of the cells in a ganglion, approximately 0.1-0.2% of cells contain viral genomes that can be reactivated (Walz *et al.*,1976; Kennedy *et al.*, 1983). Various reports have shown HSV-1 and HSV-2 specific RNA

present in latently infected human ganglia (Galloway *et al.*, 1979; Steiner *et al.*,1988) and in mice ganglia (Stroop *et al.*, 1984; Deatly, 1987).

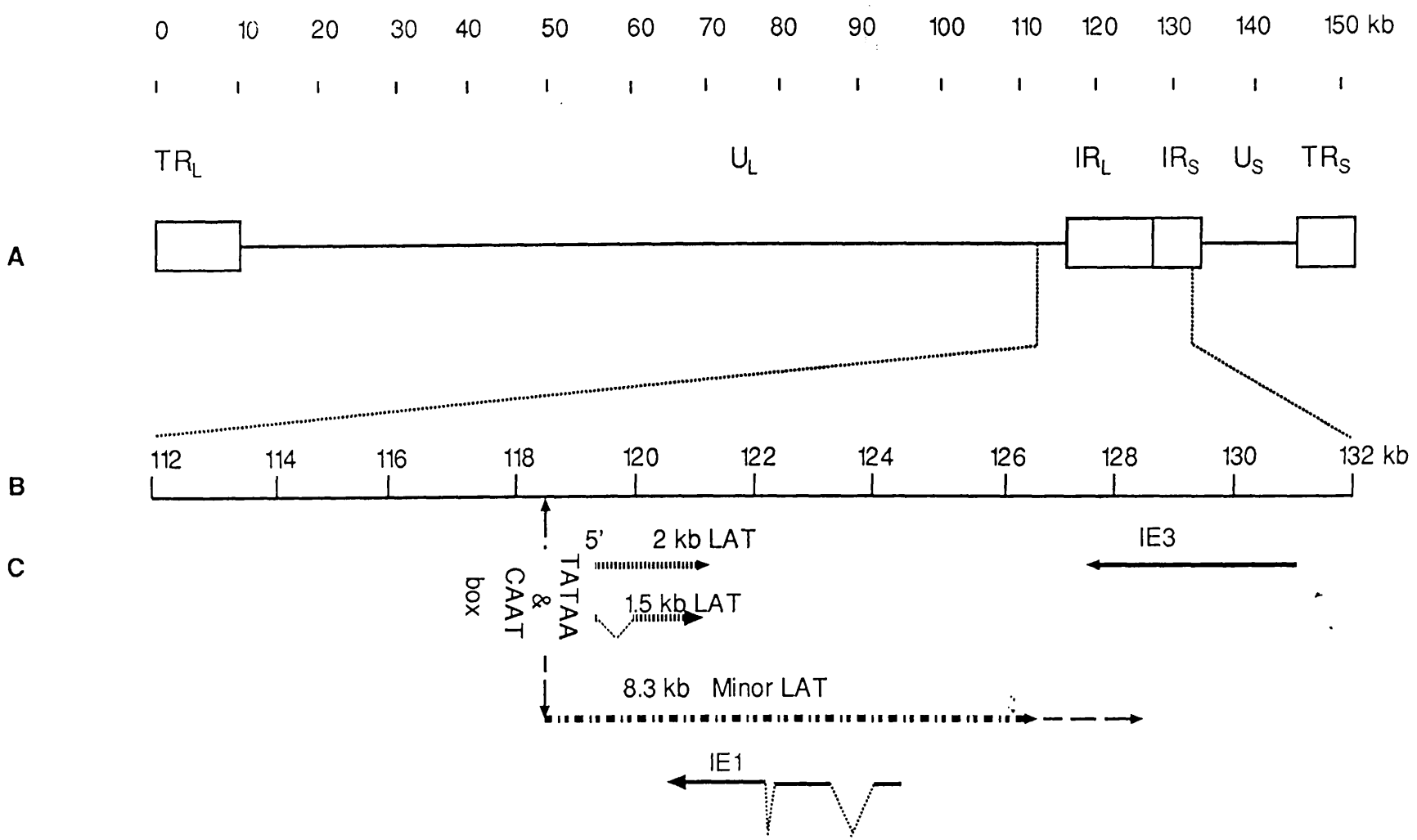
Recently work by Stevens *et al* (1987) defined the region of the HSV-1 genome expressing RNA detectable in sensory ganglia of latently infected mice. The molecular basis of this phenomenon has been studied in sensory ganglia of seropositive human cadavers and experimentally infected animals (mice and rabbits) (Croen *et al.*,1987; Rock *et al.*,1987a; Spivak and Fraser, 1987; Steiner *et al.*,1988; Deatly *et al.*, 1988; Stevens *et al.*, 1988). Similar transcripts have also been reported in HSV-2 (Mitchell *et al.*, 1990b, Burke *et al.*, 1991, Tensor *et al.*, 1991), bovine herpes virus and pseudorabies virus in different animal models (Rock *et al.*, 1987b, 1988). The area of the genome expressing the transcript encodes the IEVmw110 polypeptide (Stevens *et al.*, 1987 Puga and Notkins, 1987; Deatly *et al.*, 1987, 1988; Rock *et al.*, 1987a; Steiner *et al.*, 1988). The RNAs expressed during latency are diploid and are transcribed complementary to IE1 transcripts (Stevens *et al.*, 1987; Rock *et al.*, 1987b). This RNA is known as the latency associated transcripts (LATs). At least three transcripts, 2.0, 1.5 and 1.45 kb have been detected by Northern blot and *in situ* hybridisation and have been finely mapped (Spivak and Fraser, 1987; Wechsler *et al.*, 1988; Wagner *et al.*, 1988). These RNAs map within the HSV-1 *Bam*HI restriction *'b'* fragment and partially overlap the 3' terminus of IE1 on the opposite DNA strand (Spivak and Fraser , 1987). There are at least two LAT species; (1) major LATs (approximately 2, 1.3-1.5kb) which are in high abundance and hybridise to the HSV-1 BamHI b fragment (Wagner *et al.*, 1988, Rock *et al.*, 1987a) and (2) the minor LAT (approximtely 8.3kb), which is detected at less than 10% the abundance of the major one and hybridises to the HSV-1 *Bam*HI *k* fragment (Wagner *et al.*, 1988; Zwaagstra *et al.*, 1990) (Figure 1.6).

Figure 1.6

A. A diagramatic representation of the HSV-1 genome.

B. The region of the genome from 112 to 132 kbp map position has been expanded.

C. The location of the IE1, IE3 and LAT RNAs are shown. The location of TATAA and CAAT box is also indicated. Not all the copies of LAT are spliced. Larger 8.3kb minor LAT is represented by dot-dot-dash line.


0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150 kb
TR_L
U_L
IR_L
IR_S
U_S
TR_S
A
112
114
116
118
120
122
124
126
128
130
132 kb
B
C
5'
2 kb LAT
IE3
TATAA
box
&
CAAT
1.5 kb LAT
8.3 kb Minor LAT
IE1

The size of LAT transcripts reported by different authors varies. The variation in size of the 2kb transcript (Spivak and Fraser, 1987) and 2.6kb (Stevens *et al.*, 1987), might be due to differences in the transcripts expressed during latency in spinal and trigeminal ganglia in mice or may merely reflect technical variations.

The expression of LATs is regulated by an IE gene in cultured cells, since protein synthesis inhibition was shown to prevent the expression of the LAT gene. However this conclusion was not supported by Wagner *et al* (1988) who found expression of the LAT occurred despite protein synthesis inhibition. On the contrary Batchelor and O'Hare (1990) have recently shown that promoter activity of the LATs is regulated positively by VmwIE110 and very efficiently repressed by VmwIE175. The site of repression was located within a 55 bp region just downstream of a potential TATAA box. They further concluded that this region exhibited a high degree of homology with the IE3 gene cap site and may be a binding site for VmwIE175.

The sequence of the major LAT for HSV-1 strain 17 syn^+ and HSV-1 strain KOS is known by Northern blot hybridisation, using radioactively labelled probes from the *Bam*HI *b* fragment and synthetic oligonucleotides and more precisely by primer extension and S1 nuclease analysis. (Wechsler *et al.*, 1988; Wagner *et al.*, 1988).The homology between these strains is very high (Perry and McGeoch, 1988; Wagner *et al.*, 1988). The 5' end(the position of the 5' end is at 119461np in IR_L and 6910np in TR_L in HSV-1 strain 17 syn^+, TCCAGGTA, where the first G will be regarded as the nucleotide position 1) starts 1210 nucleotides downstream from the 3' end of IE1 mRNA. The 3' end overlaps IE1 by nearly 1000 nucleotides, which cannot be determined precisely as there are no polyadenylation sites close to the mapped end . The computer analysis by Wechsler *et al* (1988) in the search for the LAT promoter

in HSV-1 17 syn$^+$ sequences, however did not reveal any good consensus RNA transcriptional promoter element near the 5' end of the LATs . The first likely TATAA box found at -686, (TTTATAAAAGC) and first CAAT box is at position -817 (ATCAATCC). Three SpI binding sites or GC boxes were located beginning at the positions -886, -862 and -589. The combination of TATAA & CAAT boxes and several SpI binding sites makes this region a good transcriptional promoter for RNA polymerase II (Wechsler *et al.*, 1988; Wagner *et al.*, 1988; Zwaagstra *et al.*, 1990).

The chloramphenicol acetyletransferase (CAT) assay revealed that LAT promoter activity was decreased 3-12 fold in non-neuronal cells compared with neuronal derived cells indicating neuronal specificity. Furthermore it was found that transcription begins about 28 nucleotides from the first T of the TATA box which may indicate that transcription of the major LAT can start near the promoter region, but this needs to be confirmed *in vivo*. (Zwaagstra *et al.*, 1990). Potential splicing sites were located at the position predicted by hybridisation data (Wechsler *et al.*, 1988; Wagner *et al.*, 1988). It appears that at least one of the smaller transcripts is the spliced product of the larger one (Wechsler *et al.*, 1989). Some larger species were also detected in the same orientation as the major LAT. These species are unspliced and since they show very faint signals during hybridisation they were designated as the minor LATs (Mitchell *et al.*, 1990a; Zwaagstra *et al.*, 1990). The DNA of the region from which transcription occurs during latency has been sequenced and apart from IE1, does not contain any convincing protein coding sequences (Perry and McGeoch, 1988). Moreover there are no consensus polyadenylation signals in close proximity to the 3' end of the LATs (Perry and McGeoch, 1988; Wechsler *et al.*, 1988; Wagner *et al.*, 1988). However recently a protein of apparent

molecular weight of 80kDa has been recognised called a latency associated antigen (LAA) (Doerig *et al.*, 1991). This protein is yet to be identified in latently infected ganglia and its role in the machanism of latency is to be defined.

The role of LATs in the establishment, maintenance and reactivation from latency remains obscure. Deletion mutants apparently failing to produce LATs, established and maintained latency but reactivated with delayed kinetics (Dobson *et al.*, 1989; Leib *et al.*, 1989; Steiner *et al.*, 1989). However this is not supported by all LAT mutants (Ho and Mocarski, 1989; Block *et al.*, 1990). The other possibilities that can be envisaged are (i) LATs may function as an antisense regulatory factor for the IE1 gene. As the LATs are complementary to the 3' end of IE1 rather than the 5' end, and the IE1 gene plays a crucial part in the regulation of gene expression, it is possible to get antisense suppression by 3' end complementarity (Green *et al.*, 1981), (ii) Transcription of the LATs may have a cis-acting effect that physically prevents trancription of IE1 mRNA on the same strand of DNA (Rock *et al.*, 1987a), (iii) localization of the LATs to the nucleus during latency favours the idea that it may function as a trans-acting factor (Rock *et al.*, 1987a) and (iv) there are putative open reading frames within the LAT gene (Perry and McGeoch, 1988), therefore if LAT gene protein products are being translated, they might be involved in influencing latency.

The molecular basis of reactivation from latency and the virus host relationship during the reactivation process has recently been examined (Leib *et al.*, 1991). The LAT promoter region has a highly conserved 7-base consensus element (TGCGTCA) at -690 position, which is identical to the cAMP response element of the proenkephalin gene. The TGCGTCA motif of the cAMP response element is highly conserved and mutation of this motif leads to a loss

of inducible activity.

It was found that reactivation of wild type HSV-1 was significantly accelerated from dissociated latently infected murine trigeminal ganglia by the addition of cAMP analogs or adenylate cyclase activators. However, these agents did not accelerate reactivation of a deletion mutant in the LAT promoter region and LAT coding region, indicating a role for cAMP in triggering viral reactivation (Leib *et al.*, 1991).

1.10 PATHOGENICITY OF HSV.

HSV induces life threatening and fatal ecephalitis in humans. Although the incidence is rare, there is continued interest in defining the genes involved in pathogenicity. Various animal models such as mice, guinea pigs, rabbits, rats and monkeys have been used. Several factors controlling pathogenicity have been identified. Among the host factors, humoral immunity (McKendall *et al.*, 1979), cytotoxic immunity (Nash *et al.*, 1985), interferons (Lopez, 1985), state of skin and mucous membrane, age of host and route of inoculation (Sprecher and Becker 1986, 1987) effect the virulence. The degree of pathogenicity is also affected by virus strain, serial passage of the virus *in vivo* (Kaerner *et al.*, 1983), *in vitro* (Goodman and Stevens, 1986) and body temperature of the host (Thomson and Stevens, 1983).

HSV-1 thymidine kinase negative (tk^-) mutants are unable to grow in nondividing and serum-starved cells (Jamieson *et al.*, 1974) Neurons are among the cells of the body that do not regenerate. This led to the speculation that tk^- mutants might fail to grow in neurons, and could be avirulent. Various tk^- mutants of HSV-1 (Field and Wildy, 1978) and HSV-2 (Stanberry *et al.*, 1985) have been shown to be less virulent.

Like tk, ribonucleotide reductase is dispensable *in vitro* (Goldstein and Weller, 1988). The HSV-1 *ts* mutants in RR, ts 1207 and *ts* 1222 (Preston *et al.*, 1988) have reduced pathogenicity following intracranial and intraperitoneal inoculation (Cameron *et al.*, 1988).

The HSV-2 strain 186 grows poorly in the eyes and sensory ganglia of mice indicating that it is non-neuroinvasive following ocular infection (Oakes *et al.*, 1986). Marker rescue of that strain showed that the lesion was in the DNA polymerase gene and the rescued virus gained the wild type phenotype (Day *et al.*, 1987). Recently Lausch *et al* (1990) reported the failure of similar strains to grow in human lymphocytes indicating that the gene for HSV DNA polymerase can account for virulence

HSV-1 IE genes have a cis-acting regulatory element (TAATGARAT). This element responds to HSV-1 virion polypeptide Vmw65 transinducing factor (TIF) which initiates transcription from IE promoters (O'Hare and Hayward, 1987). Insertional mutagenesis in the TAATAGRAT element resulted in failure of transinduction of the IE gene expression and reduced pathogenicity following intraperitoneal and intracranial inoculation (Ace *et al.*, 1989).

The role of HSV glycoproteins in pathogenicity remains largely unexplored. The gC^- negative mutants of both HSV-1 and HSV-2 remain highly virulent for mice following intravaginal (Johnson *et al.*, 1986), intracranial and foot-pad inoculation (Dix *et al.*,1983; Sunstrum *et al.*, 1988). Therefore gC is not a virulence determinant in the mouse model. Similarly monoclonal antibody resistant (mar) gD or gB mutants have no considerable effect on pathogenicity (Kumel *et al.*, 1985). Intratypic recombinants of gB gene from HSV-1 strain KOS which has been shown to be apathogenic for mice following intraperitoneal inoculation (Schroder *et al.*, 1983) with HSV-1 strain ANG path, which is pathogenic for

mice when inoculated by the same route (Kaerner *et al.*, 1983) resulted in recombinant virus which was apathogenic for mice (Weise *et al.*, 1987) indicating some role of gB in pathogenicity. Some genetically engineered mutants in gG and gE showed 10^4 and 10^2 folds higher LD_{50} than the wild type respectively (Meignier *et al.*, 1988) showing involvement of those glycoproteins in pathogenicity.

There is growing evidence that the sequences in both copies of R_L between the 'a' sequence and the IE1 gene in HSV-1 and 2 carry a neurovirulence determinant. Loss of such sequences results in a non-neurovirulent phenotype. Taha *et al* (1989a, b) reported a 1488bp spontaneous deletion upstream of IE1 which eliminates neurovirulence in a variant of HSV-2 strain HG52 . Correction of the deletion restored the wildtype phenotype. Recently MacLean *et al* (1991) found that a deletion of 759bp upstream of IE1 in HSV-1 strain 17^+ also resulted in a non-neurovirulent virus. The above findings suggest that the sequences related to neurovirulence are conserved in HSV-1 strain 17 syn^+ and HSV-2 strain HG52. Both viruses have a common lesion in that they failed to replicate in mouse brain. These deletions in both variants removed one copy of the DR1 element of the 'a' sequence. The 5' end of the proposed $gamma_1$ gene ICP34.5 in HSV-1 strain F lies in the DR1 element of the 'a' sequence. Several other reports (Thompson *et al.*, 1989; Chou *et al.*, 1990; Jenkins and Martin, 1990) also support the above findings indicating a neurovirulent determinant in the R_L region of the HSV genome. The detailed study by Chou *et al* (1990) suggested the postulated $gamma_1$ protein designated as ICP34.5, though it is dispensable in tissue culture, is essential for virus replication in mouse brain cells. Although, no such gene was originally found in the HSV-1 strain 17 syn^+, there were protein coding sequences in

this region (Perry and McGeoch, 1988). Revised analysis of the clones containing the HSV-1 sequences upstream of the IE1 gene have shown that this region does contain a gene (Dr. D.J. McGeoch, personal communication).

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS

Cells

Baby hamster kidney clone 13 (BHK-21/C13) cells (MacPherson and Stoker., 1962) were used thoughout.

Viruses

Herpes simplex virus type-1 (HSV-1) Glasgow strain 17 syn^+ (Brown *at al.*, 1973) and the HSV-1 17 syn^+ deletion variants 1704, 1705 and 1706 isolated and characterized by MacLean and Brown (1987b) were used throughout.

Cell Culture Media

BHK-21 C13 cells were grown in modified Eagle's medium (Busby *et al.*, 1966) containing twice the normal concentration of vitamins and amino acids, 100 units/ml of penicillin, 100ug/ml streptomycin, 0.02 ug/ml amphotericin B, 0.002% (w/v) phenol red, 5% (v/v) tryptose phosphate broth and 10% (v/v) new born calf serum (ETC10). Variants of the media used during the work were

PIC

Phosphate free Eagle's medium containing 1% calf serum.

EHu5

Eagle's medium containing 5% human serum.

EMC5

Eagles medium containing 1.5% Methyl cellulose and 5% calf serum.

EMet/5C2

Eagle's medium containing one fifth the normal concentration of methionine and 2% (v/v) calf serum.

ECS50

Eagle's medium containing 50% foetal calf serum.

Agar and Bacterial Growth media.

2xYT Broth

85mM NaCl, 1%(v/v) Difco bactotryptone 1%(w/v)yeast extract.

L-Broth

170mM NaCl, 1% (v/v)Difco bactotryptone, 0.5% (w/v) yeast extract.

L-Broth Agar

L-Broth containing 1.5%(w/v) agar.

Top agar

1%(w/v) agar in water.

Bacteria

The bacteria used were *DH5 alpha* (Hanahan., 1985) and *JM101* (Messing ., 1979) strains of *E.Coli*. These were grown in L-broth with ampicillin or tetracycline, where appropriate.

Plasmids

pUC19 (Yanich-Perron *et al.*, 1985) was used as the cloning vector. The pGX 159 plasmid containing the HSV-1 17 syn$^+$ *Bam*HI *b* fragment cloned into pAT 153 (Twigg and Sherrat., 1980) was

kindly supplied by Dr V.G. Preston.

Experimental Animals.

Three week old BALB/c mice of both sexes obtained from commercial suppliers (Bantin and Kingman Ltd.) were used in this study.

Giemsa stain.

1.5% (w/v) Giemsa in glycerol, heated at 56°C for 120 min and diluted with an equal amount of methanol.

Enzymes

Restriction endonucleases, large fragment polymerase, T_4 DNA ligase and bovine serum albumin (BSA) were purchased from Bethesda Research Laboratories; T_4 DNA polymerase and T4 DNA kinase from Biolabs New England; calf intestinal phosphatase (CIP) from Boehringer Corporation and T_7 polymerase from Pharmacia Ltd.

Radiochemicals

All radiochemicals were obtained from Amersham Internationl plc at indicated specific activity.

[α-^{32}P] dNTPs	3000 Ci/mmol
[γ-^{32}P] ATP	5000 Ci/mmol
[^{35}S] Methionine	800 Ci/mmol
[^{32}P]-orthophosphate	200 mCi/mmol

Solutions and Buffers.

Phosphate buffered saline-A (PBS-A)

170mM NaCl, 3.4mM KCl, 1mM Na_2HPO_4,
2mM KH_2PO_4 pH 7.2

Phosphate buffered saline (PBS).

PBS-A supplemented with 6.8mM $CaCl_2$
and 4.9mM $MgCl_2$

PBS/Calf serum

PBS containing 5% calf serum.

Trypsin

0.25% (w/v) Difco trypsin dissolved
in Tris-saline

Tris-saline

140mM NaCl, 30mM KCl, 280mM Na_2HPO_4
1mg/ml glucose, 0.0015% (w/v) phenol red
25mM Tris-HCl (pH 7.4) 100 units/ml penicillin,
100ug/ml streptomycin.

Versene.

0.6mM EDTA dissolved in PBS
containing 0.002% (w/v) penol red.

Trypsin/Versene.

one volume of 0.25% of Trypsin
plus four volumes of versene.

Phenol saturation buffer.

10mM Tris-HCl pH 7.5 , 10mM EDTA and 100mM NaCl. phenol was saturated in phenol saturated buffer

Phenol-Chloroform (1:1).

A 1:1 mixture of phenol and chloroform.

Chloroform:isoamyl alcohol (24:1).

This is a 24:1 (v/v) mixture of chloroform and isoamyl alcohol.

TBE

89mM Tris-base, 89mM Boric acid 2mM EDTA pH 8.3.

TE

10mM Tris-HCl, 0.1mM EDTA pH 7.4.

1x E 35mM Tris-HCl, 29mM $NaH_2PO_4.H_2O$, 0.99 mM EDTA

Restriction enzyme buffers.

The buffers were used either as recommended by BRL or Maniatis *et al* (1982). These were prepared as 10x stock solutions and stored at -20°C.

Chemicals.

Most chemicals were supplied by BDH Chemicals UK or Sigma Chemical Co. and were analytical grade. M13 single stranded promoter, unlabelled nucleotides, 2' 3' dideoxyribonucleotide triphosphates were supplied by Pharmacia Ltd. Wacker silicone was

provided by Wacker Chemical Company Munich. Repelcote (2% solution of dimethyl dichlorosilane 1,1,1-trichloroethane) was supplied by Hopkin and Williams, England. DNA blotting membrane (Nytran) was purchased from Schleicher and Schuell.

METHODS

2.1 Growth of cells

BHK-21 C13 cells were propagated in 80oz roller bottles containing 150ml of ETC10 in the presence of 5% (v/v) CO_2 in air. The cells usually become confluent after three days and each confluent bottle contains $2\text{-}3x10^8$ cells. Confluent cells were harvested by washing twice with trypsin/versene and resuspending in a total volume of 20ml ETC10. Cells from one roller bottle are enough to seed ten further bottles. BHK-21 C13 cells were also used to seed flat bottom multiwell tissue culture microtitre plates at a density of $6x10^3$ cells in 0.15 ml of ETC10. Incubation was carried out at 37°C in a humidified incubator containing 5% (v/v) CO_2.

2.2 Cell storage.

Freshly harvested BHK-21 C13 cells were pelleted at 2000 rpm for 10 min at 4°C and resuspended in ETC10 containing 20% glycerol (v/v) and 20% FCS (v/v). Cells were aliquoted in 2 ml black cap vials at a concentration of 10^7/ml, frozen slowly to -70°C overnight and stored at -170°C. Cells were recovered by thawing quickly and resuspending in ETC10.

2.3 Growth of virus stocks.

Confluent BHK-21 C13 cells in 80oz roller bottles were infected at a moi of 0.003 pfu/cell. The infected cells were incubated at 31°C for 3-4 days or until extensive cpe appeared. Cells were harvested by shaking the cells into the medium or with the aid of glass beads and pelleted at 2000 rpm for 10 min. at 4°C. The supernatant was centrifuged at 12000 rpm for 2 hr at 4°C in a Sorval GSA rotor and the virus pellet resuspended in 5 ml of

supernatant and sonicated. This was termed supernatant virus. The cell pellet from the original centrifugation was resuspended in 5 ml of supernatant, sonicated thoroughly and the cell debris removed by centrifugation. The supernatant was cell associated virus.

2.4 Titration of virus stocks.

Ten fold serial dilutions of virus stocks were made in PBS/calf serum. After removing medium from nearly confluent BHK-21 C13 cells in 50mm petri-dishes, 0.1 ml of each dilution was added. Virus was allowed to absorb for an hour, the plates were overlaid by EMC5 and incubated at 37°C for 2 days or 31°C for 3 days or until plaques were visible. Monolayers were fixed and stained with Giemsa stain at room temperature (RT) for half an hour and plaques were counted using a dissecting microscope.

2.5 Sterlity checks on virus and cell stocks.

Cell or virus stocks were checked for bacterial contamination by streaking on blood agar plates in duplicate and incubating at 37°C and 31°C for at least 5 days. The cell and virus stocks were considered sterile if there was no contamination detected on the plates.

2.6 Preparation of virion DNA.

Virion DNA was prepared as described by Wilkie (1973) and Stow and Wilkie (1976). Confluent BHK-21 C13 cells in 80oz roller bottles were infected with virus at a moi of 1:300. Cells were harvested after the appearance of extensive cytopathic effect and pelleted at 2000 rpm for 10 min at 4°C. The supernatant was kept on ice and the cell pellet resusupended in RSB [10mM KCl, 1.5mM

$MgCl_2$, 10mM Tris-HCl and NP40 at a final concentration of 0.5%(v/v)]. Cells were incubated on ice for 10 min. To pellet the nuclei and debris, cells were centrifuged at 2000 rpm for 3 min and the pellet treated with RSB/NP40 as above. The two cytoplasmic supernatants were mixed with infected cell medium and centrifuged at 12000 rpm in a Sorvall GSA rotor for 2hr. After discarding the supernatant, the viral pellet was resuspended by sonicating in 8ml of NTE (10mM Tris-HCl pH 7.5 10mM NaCl and 1mM EDTA). Virion particles were lysed by adding SDS and EDTA to a final concentration of 2% (w/v) and 10mM respectively. Virion DNA was extracted with gentle mixing (2-3 times) with an equal volume of saturated phenol and once with chloroform:isoamyl alcohol (24:1 v/v) and precipitated by adding two volumes of absolute alcohol. DNA was stored at -20°C overnight and recovered by centrifuging at 2000 rpm for 10 min, washed once with 70% ethanol, dried in a vacuum desiccator and redissolved in water containing 50ug/ml RNase. The DNA was quantitated by agarose gel electorphoresis using a standard DNA of known concentration.

2.7 Virus particle counts.

A 5ul aliquot of virus stock was mixed thoroughly with an equal volume of 1% SST pH 7.00 and 5ul of latex beads (1.43×10^{11}/ml). A 5ul sample was spotted onto the mesh of a parlodium gird. After a few minutes excess was removed by filter paper and the grid visualized under the electron microscope. The number of virus particles and latex beads were counted and the concentration of particles in the virus stock was calculated by the following formula:

$$\text{Particle count} = \frac{\text{Number of virus particles}}{\text{Number of latex beads}} \times 1.43 \times 10^{11}$$

2.8 Purification of single plaque isolates.

Virus stocks were titrated as described (Section 2.4) and cell monolayers with the fewest plaques (5-10 plaques) were washed twice with PBS/calf serum. Individualy separated plaques were picked into 500ul PBS/calf serum, sonicated and stored at -70C. Individual plaques were purified an additional three times prior to further analysis. To grow a plate stock, a BHK-21 C13 monolayer was infected with 100ul of the plaque solution and incubated at 37°C until cpe was complete. The monolayer was harvested, sonicated and the virus stored at -70°C.

2.9 Virus growth properties *in vitro*.

One step growth experiments were carried out as described by Brown and Harland (1987). Confluent BHK-21 C13 cells in 35mm petri-dishes were infected at a moi of 5 pfu/cell and incubated at 37°C for 1 hr to allow the virus to absorb. Cells were washed twice with PBS/calf serum, overlaid with ETC5 and incubated at 37°C. Samples were harvested at 0, 2, 4, 6, 8, 12 and 24 hr post infection, sonicated and the virus yields titrated on BHK-21 C13 cell at 37°C.

2.10 Virus growth properties *in vivo*.

Virus growth properties *in vivo* were carried out by inoculating 1×10^5 pfu/cell into the left rear footpad of 3 week old female BALB/c mice. At 0, 24, 48, 72, 96, 120 and 144 hr post infection, two surviving mice from each time point were sacrificed and nine dorsal root ganglia (DRG) (last thoracic, six lumbar and two

sacral) were removed aseptically and homogenised in 500ul of PBS/calf serum, using a dounce homogeniser (Quick fit, England). The cell suspension was sonicated thoroughly and 0.1ml of the resulting suspension was titrated on BHK-21 C13 cells at 37°C as described (Section 2.4).

2.11 Animal inoculation.

Three week old BALB/c mice were anaesthetised with Halothane and 0.025ml of the appropriate virus dilution in PBS/calf serum was inoculated into the central region of the left cerebral hemisphere. Groups of four to eight mice were inoculated with a single dilution of each virus stock between 10^1-10^5 pfu/animal. The virus stocks were always titrated on BHK-21 C13 cells on the day of inoculation to determine the precise quantity of virus inoculated. Mice were observed daily for 21 days post inoculation and their clinical state recorded . The 50% lethal dose value (LD_{50}) was calculated according to the formula of Reed and Muench (1938), on the basis of deaths between days three and twenty one. Virulence studies were also carried out using the foot pad and the intraperitoneal routes of inoculation.

For the purpose of reactivation from latency, 0.025ml of appropriate virus dilutions in PBS/calf were inoculated with 10^5-10^7 pfu/mouse into the left rear footpad of 3 week old BALB/c mice. Six weeks later mice were sacrificed, DRG were explanted (see section 2.12) and procedure was carried out as described in the section 2.13.

2.12 Explantation of dorsal root ganglia (DRG).

Mice were killed by deep chloroform anaesthesia and fixed on a dissecting board in the dorso-ventral position. The back of the mouse was carefully washed with absolute alcohol. The skin was

dissected away from the body on either side and fixed by means of pins into the dissecting board. Two longitudinal incisions were made laterally on either side of the vertebral column so as to separate the column from the supporting muscles. A cut was made transversally through the vertebral column at the level of the last two ribs. The dorsal lamina of the vertebral column was separated and removed. The spinal cord was then removed and the dorsal root ganglia indentified under the dissecting microscope. Ganglia from the lower thoracic, six lumbar and two sacral vertebrae were dissected out. Each ganglion was placed separately in a round bottom microtitre plate well containing ECS50.

2.13 Virus reactivation from latently infected DRG.

Virus released into the supernatant of DRG cultures was detected by screening the supernatant on semiconfluent BHK-21 C13 cells grown in flat bottom microtitre plates at 37^{o}C. The screening procedure was carried out daily for the first week to avoid the risk of contamination and subsequently on alternate days, when the total supernatant was removed and plated on BHK-21 C13 cells. Virus was detected by the appearance of visible cytopathic effect in the indicator BHK-21 C13 cells. The microtitre plates were stained with Giemsa and scored +ve or -ve for virus. Fresh medium was added to the DRG tissue culture wells and incubation continued.

2.14 Preparation and isolation of ^{32}P labelled viral DNA *in vivo*.

This is a modification of the method of Lonsdale (1979). Nearly confluent BHK-21 C13 cells propagated in PIC medium in Linbro wells were infected at a moi of 10 pfu/ cell. One hr post infection unabsorbed virus was removed by washing twice and the

cells incubated at 31°C. After 2hr, 5uCi of ^{32}P-orthophosphate was added per well and incubation continued for 48 hr at 31°C. Cells were lysed by the addition of SDS at a final concentration of 2.5% (v/v) and incubated at 37°C for 10 min. DNA was extracted once with an equal volume of phenol, precipitated with two volumes of ethanol, dried at 37°C for 15 min and redissolved in 200ul of sterile H_2O. 20ul of DNA was used for appropriate restriction enzyme analysis and electophoresed on an agarose gel (0.5-1.2%) for approximately 16 hr at 40-50 V. Gels were air dried in a hot air oven at 80°C and autoradiographed using Kodak-Xomat S 100 film at room temperature.

2.15 Agarose gel electrophoresis.

Agarose gels (0.5%-1.2%) were prepared in 250ml of either 1xE or TBE buffer. The solutions were poured onto glass plates in which 12 to 15 teeth combs were placed. After setting gels at RT for an hr they were transferred to horizontal tanks containing the appropriate buffer. For non radioactive DNA samples the gels were stained with ethidium bromide. Gels were electrophoresed at 40-50 V overnight.

2.16 Elution of DNA fragments from agarose gels.

This is the method described by Maniatis *et al* (1982). The DNA was digested with a 5 fold excess of the appropriate restriction enzyme and run on an agarose gel in the presence of 5ug/ml Ethidium bromide . The gel was visualized under long wave UV light and the appropriate fragment identified and cut out with a sterile sharp scalpel. The isolated gel slice was immersed in 1 or 2 ml of 1x appropriate electrophoresis buffer in dialysis membrane preboiled in 0.1x TBE or E buffer for 10 min. The dialysis

membrane bag was electrophoresed at 200-300 V for 1-2 hr. DNA was collected from the membrane and purified by passing it through a DEAE-Sephacel column.

2.17 DEAE-Sephacel column purification of DNA.

This is modified from the method described by Maniatis *et al* (1982). The column was washed with sterile H_2O, 1M NaOH, 1M Tris pH 7.5 and H_2O respectively. The column was then packed with 0.5ml of sephacel and washed with 1.5 ml of NTE. DNA was loaded and washed with 2.5 ml of NTE again. DNA was eluted by TE plus 1M NaCl, phenol/chloroform extracted, precipitated with absolute alcohol, washed with 70% ethanol, dried in a vacuum desiccator, redissolved and quantitated by agarose gel electrophoresis against a known standard.

2.18 Transfection of virus DNA by Calcium phosphate precipitation/DMSO boost.

Transfection of viral DNA was performed as described by Stow and Wilkie (1976). 0.02-1 ug of viral DNA was mixed with 10ug of calf thymus DNA as carrier in HEPES buffer (130mM NaCl, 4.9mM KCl, 1.6mM Na_2HPO_4, 5.5mM D-glucose, 21mM HEPES, pH 7.05) and calcium chloride was added to a final concentration of 130mM. The mixture was allowed to stand at RT for 20 min until a fine precipitate developed. This DNA was added to 80% confluent monolayers of BHK-21 C13 cell in 50mm petri-dishes from which the medium had been drained. After 45 min of incubation cells were overlaid with ETC5. Four hour post infection cells were treated with 25% (v/v) DMSO in HEPES buffer for four min at RT. DMSO was removed gently by washing twice with ETC5 and cells were overlaid with ETC5 and incubated at 31°C for 3-4 days or until

cytopathic effect appeared. The cells were harvested, sonicated and stored at -70°C. Transfected plate stocks were titrated and single plaques were prepared for further restriction enzyme analysis of the genome. For marker rescue of the deletion in the variant 1704, the wild type fragment spanning the deletion was added to the transfection mix at a 5, 10 and 20 fold molar excess with the intact deletion variant genome.

2.19 Preparation and analysis of HSV infected cell polypeptide.

This method was essentially that described by Marsden *et al.*, (1976). Confluent BHK-21 C13 cells in Linbro wells were infected at a moi of 20 pfu/cell. After absorption for an hr at 37°C, the monolayers were washed twice with Eagles's medium containing 20% the normal concentration of methionine and 2% calf serum (Emet/5C2) and the same medium was used to overlay the monolayers. After 4 hr incubation at 37°C, 100 uCi/ plate of ^{35}S-methionine was added and incubation continued at 37°C for 24 hr. Samples were washed twice with PBS, harvested into 500ul sample buffer (150mM Tris-HCl pH 6.7, 6.28% (w/v) SDS, 0.15% (v/v) 2-mercaptoethanol, 0.3% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and after boiling for 5-10 min were analysed by SDS-PAGE (described in section 2.20)

2.20 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Two glass plates separated by 1.5mm thickness perspex were cast vertically and sealed with Scotch tape. Two types of resolving gels were used (i) single concentration gels containing the appropriate amount of acrylamide cross linked with 2.5% (w/w) N,N' methylene bisacrylamide in resolving gel buffer (375mM Tris-HCl

pH 8.9, 0.1% (w/v) SDS and (ii) gradient gels containing 5%-12.5% gradient acrylamide cross linked with 5% (w/w) N,N' methylene bis-acrylamide in resolving gel buffer . Ammonium persuphate (APS) and N, N, N, N' tetramethylethylene diamine (TEMED) were added to a final concentration of 0.006% (w/v) and 0.004% (v/v) respectively prior to pouring. After polymerization gels were overlaid with butan-2-ol to get a smooth top surface which was then washed off with deionized water. The stacking gels contained 5% acrylamide (cross linked with the same ratio of N, N' methylene bisacrylamide used in resolving gel) in gel buffer (0.11mM Tris-HCl pH 6.7, 0.1% (w/v) SDS). APS and TEMED were added to the stacking gel solution as above and a teflon coated comb was inserted prior to pouring. Samples were boiled for 5-10 min in sample buffer, loaded and electrophoresed in tank buffer (52mM Tris-HCl, 53mM glycine, 0.1% (w/v) SDS) at 60mA for 3-4 hr (Marsden *et al.*, 1976, 1978). Gels were fixed, stained for 1 hr by shaking in methanol:H_2O:acetic acid 50:43:7 in the presence of 0.2% (w/v) Coomassie Brilliant Blue R250. Gels were destained for 1-2 hr in methanol:H_2O:acetic acid 5:88:7, dried under vacuum and exposed for autoradiography at RT.

2.21 Glycerol stocks of bacteria.

Bacterial stocks were prepared from a 5ml standing culture grown overnight at 37°C in L-broth containing the appropriate antibiotic. The bacterial stocks were pelleted at 5000 rpm for 5 min at 4°C and pellets were resuspended in L- broth containing 50% (v/v) glycerol. The stocks were stored at -70°C.

2.22 Preparation of L-broth/agar plates.

L-broth/agar was melted by placing a 350 ml bottle in a

boiling water bath for about 1 hr or in a microwave oven for 8 min at medium range setting and gradually allowing to cool until comfortable to hold. For L-broth/agar plates alone, approximately 20 ml was poured onto 90mm bacterial petri dishes, allowed to set at RT for about 30 min, and the plates dried for 20 min at 37°C in an inverted position with the lids loose. Plates were stored at 4°C for up to 1 month prior to use. For L-broth/agar plates containing antibiotic, the antibiotic was added at the appropriate concentration just prior to pouring.

2.23 Construction of recombinant plasmids.

The linearised plasmid vector pUC19 (Yanisch-Perron *et al.*, 1985) was treated with CIP at a concentration of 5 units/ug of plasmid DNA. After incubation at 37°C for 4 hr, the DNA was extracted once with saturated phenol, once with phenol:choloroform (1:1), once with chloroform alone and precipitated with two volumes of ethanol in the presence of 1/10 volume of 3M Na acetate. The DNA pellet was washed with 70% ethanol, dried and resuspended in an appropriate amount of water to give 40ng/ul. A 3-4 times molar excess of the purified HSV DNA fragment relative to the phosphatase treated vector (40ng) was ligated overnight at 15°C in a 20ul ligation reaction containing 2 units of T4 DNA ligase and 1xligase buffer (10mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM DTT and 1mM ATP).

2.24 Transformation of bacterial cells with plasmids.

This procedure was based on the method described by Bankier *et al* (1987). Host bacterial cell *E.coli* strain *DH5* (Hanahan., 1985) were grown to an optical density at 600nm (OD_{600}) of 0.3 and 30 ml of bacteria pelleted by centrifuging at 2000 rpm for 10 min at

4°C. This pellet was resuspended gently in 2.5 ml of transformation buffer (TFB)(10mM MES, 100mM rubidium Cl (RbCl), 45mM manganese Cl($MnCl_2.4H_2O$), 10mM Calcium Cl($CaCl_2.H_2O$) and 3mM Hexaminecobaltic Cl) and incubated on ice . After 15 min the cells were treated with 0.1 ml of DMSO and 0.1 ml of DTT/KAC (2.25M DTT and 40mM KAc pH 6.0) and kept on ice for 5 min. The vector and HSV fragment ligation mixture was diluted 5 fold and 1, 3 and 5ul were incubated on ice for 30 min. with 20ul of competent cells . The cells were heat shocked at 42°C for 2 min and transferred to SOC medium (Bactotryptone 2% (w/v), Yeast extract 0.5% (w/v), NaCl 10mM, KCl 2.5mM, $MgCl_2.MgSO_4$ 20 mM (10mM each) and Glucose 20mM) and grown with shaking at 37°C in a orbital shaker for 2 hr to express the antibiotic resistant gene (Hanahan, 1985). 100ul of each sample was spread on L-broth agar plates containing 100ug/ml ampicillin and incubated overnight at 37°C. Single bacterial colonies were picked from the plates and analysed.

2.25 Small scale isolation of plasmid DNA.

Bacteria from single colonies were grown in 1.5 ml L-broth containing 100ug/ml ampicillin in an orbital shaker at 37°C. The cells were pelleted at 1300 rpm for two min at RT in a microfuge. The supernatant was carefully discarded and the pellet resuspended in 50ul freshly prepared solution I (50mM Glucose, 10mM EDTA, 25mM Tris-HCl pH 8.00 containing 4mg/ml lysozyme) and incubated on ice for 5 min. 100ul of freshly prepared solution II (0.2 M NaOH, 1% (w/v) SDS) was added and icubation continued for 5 min. Ice cold solution III (KAc ph 4.8) was added and incubation continued on ice for 5 min. The cell debris and chromosomal DNA were pelleted by centrifuging at 13000 rpm for 10 min. Plasmid DNA was extracted by treating the supernatant twice with an equal volume of phenol:choloroform (1:1 v/v) and precipitated by the

addition of 2 volume of ethanol at RT for 20 min. The DNA pellet was washed with 70% ethanol dried in a vacuum desiccator and resuspended in 50ul of H_2O. Usually 10ul of this was used for restriction enzyme digestion.

2.26 Large scale isolation of plasmid DNA.

This method is basically that described by Birnboin and Doly (1979) as modified by Maniatis *et al* (1982). Single bacterial colonies from L-broth agar plates or 10ul from bacterial glycerol stocks were inoculated into 10 ml of L-broth containing 100ug/ml ampicillin and incubated at 37°C overnight in an orbital shaker. 2ml of the overnight culture was inoculated in 350 ml L-broth containing 100ug/ml ampicillin and shaken overnight at 37°C. Bacteria were pelleted at 10,000 rpm for 8 min at 4°C in a Sorvall GSA rotor. The supernatant was discarded carefully and the pellet resuspended in 7 ml of freshly prepared solution I (see section above) containing 4mg/ml lysozyme incubated at RT for 10 min. 14 ml of freshly made solution II was added and incubation continued for 10 min on ice . Ice cold solution III was added and incubation continuted for 10 min on ice. The cell debris and chromosomal DNA were pelleted at 12000 rpm for 30 min in a Sorval SS34 rotor at 4°C. The supernatant was treatedmore than once with phenol:chloroform (1:1 v/v) until there was no interphase. DNA was precipitated by adding two volumes of absolute alcohol, washed with 70% ethanol, dried and redissolved in 20 ml of TE pH 7.4. Closed circular plasmid DNA was isolated by CsCl gradient centrifugation.

2.27 CsCl / Et. Br. gradient purification of recombinant plasmid.

This is the method described by Maniatis *et al* (1982).

CsCl was added to the plasmid so that the final density of the solution was 1.55g/ml and the final concentration of EtBr 600ug/ml. The solution was transferred to a Beckman Type-50 tube, sealed and centrifuged at 45,000 rpm for 36 hr at 16°C. The DNA was visualized under long wave UV light. Normally two bands should be seen, the upper one is chromosomal and nicked circular DNA and the lower is closed circular DNA. The lower band was removed through a # 21 hypodermic needle. The ethidium bromide was removed by extracting the DNA 3-4 times with an equal volume of isoamyl alcohol and the CsCl by dialysing the sample overnight in 5 litre of 0.1xSSC at 4°C. The DNA was recovered by precipitating with 2 volumes of 3M NaAc. The DNA was pelleted by centrifuging at 2000 rpm for 10 min at 4°C, washed with 70% ethanol, dried in a desiccator, redissolved in 1 ml of H_2O and quantitated at OD_{260}. The conversion factor for double stranded DNA is 1 O.D=50ug/ml.

2.28 Transfer of DNA fragments to nitrocellulose.

This method is basically as described by Southern (1975)and modified by Maniatis *et al* (1982). Well separated DNA fragments in agarose gels were denatured in Gel Soak I (200mM NaOH, 600mM NaCl) for 45 min. and neutralised in Gel Soak-II (1M Tris-HCl pH 8.00, 0.59M NaCl) for another 45 min . The gel was transferred onto two sheets of 3mm filter paper presoaked in 10xSSC (1XSSC is 15mM Trisodium citrate, 150mM NaCl). A sheet of Nytran blotting membrane cut to the exact size of the gel was placed on top of the gel, followed by several similar sized sheets of Whatman 3mm filter paper wet with blotting buffer. A stack of absorbent paper and finally a weight was placed on top to keep the surface of towels and filter papers in touch with each other . After 16-24 hr the Nytran blotting membrane was air dried and the DNA, UV crosslinked for

5 min at a 312mm wave length on a transilluminator.

2.29 Hybridisation procedure.

Nytran blotting membrane was hybridised in hybridisation buffer (0.5mM $NaHPO_4$ pH 7.4, 7% (w/v) SDS) with either a nick translated or randomly primed ^{32}P labelled probe of DNA. Hybridisation was allowed to proceed for 24 hr at 65°C. The Nytran blotting membrane was removed, washed 3x with 0.1xSSC and 1% SDS (w/v) each for 30, 15, and 15 min respectively at RT. The membrane was sealed in a plastic bag and placed in contact with Kodak XOMAT-S film at -70°C with an intensifying screen .

2.30 *In vitro* ^{32}P labelling of DNA by nick translation.

This method was as described by Rigby *et al* (1977). 0.5ug of the DNA to be used as a probe was labelled in a reaction mixture containing 2 units of DNA Polymerase I, 50mM Tris-HCl pH 7.8, 5mM $MgCl_2$, 10mM DTT, 10-4 mg/ml DNAse, 10ug/ml BSA, 2uCi [α-^{32}P] dCTP, 2uCi[α-^{32}P]dGTP, 0.2mM dATP and 0.2mM dTTP in a final volume of 30ul and incubated at 15°C for 2hr. The DNA was precipitated on dry ice for 15 min with 0.6 volume of isopropyl alcohol and 0.1 volume of 3M NaAcetate. The DNA was precipitated and the procedure repeated twice. The resultant pellet was redissolved in 10ul of water and 80% (v/v) deionised formamide and denatured by boiling at 100°C for 5 min.

2.31 *In vitro* ^{32}P labelling of double stranded DNA by random priming.

This method was as described by Sambrook *et al* (1989). 200ng of double stranded denatured λ DNA was mixed with 75ng of hexadeoxyribonucleotides in a mixture containing 1ul of 20mM

dithiothreitol, 1ul of 5mM solution of each dGTP, dTTP, dATP, 1ul of 10x RP buffer (900mM HEPES pH 6.6 adjusted with 4N NaOH and 100mM $MgCl_2$), 3ul of [α^{32}P] dCTP (specific avtivity >3000 Ci/mmol), 5 units of Klenow polymerase and H_2O up to 10ul. Incubation was carried out at room temperature overnight. 10ul of buffer A (50mM Tris.Cl pH 7.5, 50mM Nacl, 5mM EDTA pH 8.0) was mixed with the probe. The probe was then boiled for a minute and quickly added to the blot.

2.32 Oligonucleotide synthesis and purification.

Oligonucleotides were synthesized on the Biosearch 8000 DNA synthesizer. The DNA was eluted by resuspending in 1 ml of ammonia and incubating at 55°C for 5hr. The ammonia was removed in a vacuum desiccator overnight. The DNA was resuspended in 50ul of water, vortexed and centrifuged in a microfuge for three minutes. The supernatant was transferred to 50ul of sample buffer (28ul 10xTBE, 117ul H_2O, 800ul deionised formamide) boiled for 10 min, cooled on ice briefly and loaded immediately onto a 16% acrylamide gel containing 1 part in 30 N, N'methylene bis-acrylamide and 8.3M urea in 1xTBE. Two ul of formamide dye was loaded in a separate well to act as a mol. wt. marker. The gel was electrophoresed slowly, at 3.5mA overnight in TBE.

DNA was visualised by separating the plate, wrapping the gel in cling film and viewed against a white chromatoghraphic plate by angled long wave UV light. Successful synthesis of an oligonucleotide will show a strong, predominant band with a few minor lower mol. wt. bands. The top band was cut with a sharp scalpel, mashed with a glass rod and incubated at 42°C for 16 hr in 1ml of elution buffer (0.5M Ammonium accetate 1mM EDTA and

0.5% (w/v) SDS). This was filtered through glass wool, phenol chloroform (1:1) extracted, ethanol precipitated, washed in 70% ethanol, dried, redissolved in water and the DNA quantitated at OD_{260}. The conversion factor for synthetic oligonucleotides is 1 OD= 20ug/ml.

2.33 *In vitro* ^{32}P labelling of synthetic oligonucleotides by a forward reaction using T4 polynucleotide kinase.

This method was as described by Maniatis *et al* (1982) . DNA(0.1-0.2 ug)to be used as a probe was labelled in a reaction containing 2 units of T4 DNA polynucleotide kinase in 2ul of 5x linker kinase buffer (330mM Tris-Hcl pH 7.5, 50mM $MgCl_2$ and 50mM DTT) and 50uCi gamma ^{32}P dATP (specific activity >3000 Ci/mmol) and H_2O added to a total volume of 10ul. The reaction was carried out at 37°C for an hr and stopped by the addition of 0.25M EDTA.

2.34 Construction of recombinant M13.

The double stranded replication form (RE) of bacteriophage M13 mp18 and mp19 (Norrender *et al.*, 1983) was linearised with the appropriate enzyme or enzymes. The HSV DNA insert in pUC19 was cut out by digesting it with *Eco*RI and *Bam*HI in the case of 1704 and 1705 and with *Bam*HI for 1706. 40 ng of the DNA insert plus 2 units of T4 DNA ligase in ligase buffer was incubated at RT overnight (Sanger *et al.*, 1980).

2.35 Transfection of bacterial cells with M13.

E.coli strain *JM 101* (Messing., 1979) were grown in 2xYT broth to an OD_{630} of 0.3 and made competent as described (section 2.24) The ligation mix was added to 100ul aliquots of *JM101* cells and

incubated on ice for 30 min with occasional shaking. Cells were heat shocked at 42°C for 45 seconds. 3 ml of melted top agar containing 20ul of 2.5% (w/v) isopropyl-D-thiogalactoside (IPTG) and 25ul of 2% (w/v) 5-chloro-4-bromo-3-indolyl-β-D-galctoside (BCIG) in dimethyl formamide was added to the sample and the mixture was poured onto a 90mm L-broth agar plate allowed to stay at RT for 15 min and incubated at 37°C overnight . Colourless plaques inside the haze lawn of bacteria indicate tranfected recombinant clones. Non recombinant plaques will express β-glactosidase and appear blue.

2.36 Growth and extraction of recombinant M13 clones.

An overnight standing culture of *E.coli* strain *JM101* was used to inoculate 2xYT broth (1:100). This was dispensed in 1.5ml aliquots in 25ml universal bottles. The recombinant M13 clones were tooth picked from the plates into the broth and icubated at 37°C for 6-8h in an orbital shaker. The cells were transferred to an eppendorf tube and centrifuged at 13000 rpm for 5 min in a microfuge to pellet the cells. The supernatant was carefully transferred to another tube without carrying any cells and phage DNA was precipitated by the addition of 200ul of 20% (w/v) polyethylene glycol (PEG Mr 6000) in 2.5M NaCl for at least 30 min at RT and centrifuged at 13000 rpm for 5 min. The supernatant was completely removed by recentrifuging the pellet with a glass capillary tube. The phage pellet was resuspended in 100ul TE, the DNA extracted with phenol, precipitated by the addition of two volumes of ethanol and 0.1 volume 3M sodium acetate, washed with 70% ethanol, dried in a vacuum desiccator, redissolved in 30ul of TE and stored at -70°C (Sanger *et al.*, 1980).

2.37 Sequence analysis of recombinant M13 clones.

Sequencing was carried out by the di-deoxynucleotide chain termination reaction method. This method basically is as described by Sanger *et al* (1977) with modification in the ratio of de-oxy and di-deoxy nucleotides to facilitate HSV DNA sequencing. The single stranded DNA template was annealed with M13 forward sequence primer(Universal primer) or in the case of the 1704 *Hpa*I *o** fragment construct, the oligonucleotide number 2 (5' TGGAGCCCGGCAGAACA 3') was used as a primer in the presence of annealing buffer (10mM Tris-HCl pH 8.5 and 10mM $MgCl_2$). The volume was made up to 10ul with H_2O at 37°C for 30min. Samples were allowed to stand at RT for at least 10 min to complete the process of annealing. To the annealed DNA, 2 unit of klenow polymerase I was added and the mixture aliquoted in 2ul fractions into four wells of a U-bottom microtitre plate corresponding to the specific T, C, G and A reaction of each clone. An equal volume of the nucleotide mixture containing dNTP's and specific ddNTP's, 1ul of 11.8uM dATP, 0.3uCi[α-^{32}P] dATP was added to each well and the reaction allowed to proceed for 10 min at 37°C. The reaction was chased by addition of 2ul of chase solution (0.5mM uniform mixture of all four dNTP's) and incubation continued for 30 min at 37°C. The reaction was stopped by the addition of 2ul formyl dye mixture(0.1% (w/v) bromophenol blue and 0.1% (w/v) xylene cynol in de-ionised formamide). The samples were heated for 1 min at 100°C to denature DNA and electrophoresis was carried out on polyacrylamide gels.

2.38 Electrophoresis and autoradiography of sequencing gels.

Vertical plates 40x20x0.03 cm in size were used and spacer gel combs were cut from plastic card. The notched plate was treated with repelcote. The plain plate was treated with 0.5% (v/v) Wacker's

silicon in 0.3% (v/v) acetic acid and ethanol (Garoff and Ansorge., 1981), which bonds the acrylamide to the plate . Generally the electrophoresis was carried out through 6% acrylamide gels. In this system the top mix used was 0.5%xTBE, 6% acrylamide (Electron grade) and 9M urea (McGeoch *et al.*, 1986). ASP and TEMED were added to the top mix to a final concentration of 0.016% (w/v) and 0.16% (w/v) respectively . The contents were poured, the gel comb was inserted and gel rested in a nearly horizontal position until polimerization was complete. The tape was removed from the bottom of the gel and the plates set with 0.5xTBE in both the top and bottom tank of the gel kit. Before loading the DNA sample, gel wells were flushed with 0.5% TBE and the gel run at a constant power of 40W for 2 hr. After electrophoresis the plates were dismantled and the gel bonded to the plain plate immersed in a 10% acetic acid bath for 30 min to fix DNA and remove the urea from the gel. The gel was dried down on the plate in an oven at 120^{o}C for 1-2 hr and exposed against Kodak XS-I film at RT.

2.39 Accumulation and handling of the sequence data.

DNA sequence data was handled and interpreted using the Institute of Virology's VAX/VMS computer system running the GCG software (Devereux *et al.*, 1984). The gel readings were read and typed into an account using the EDIT program which stores data from gel reading under a chosen file name. The data was converted into GCG sequence using the FROMSTADEN program. The gel readings were compared against each other using the program BESTFIT to determine overlapping. The gel readings were aligned and compared against the known sequence currently held, using the BESTFIT and GAP programmes.

CHAPTER THREE

RESULTS

RESULTS

3.1 SEQUENCE ANALYSIS OF DELETION VARIANTS OF HSV-1 STRAIN 17 SYN$^+$.

3.1.1 Introduction.

Genomic analysis of single plaque isolates allows the identification of individual viruses with genomic rearrangements which would not normally be detected in a pooled virus genomic population. Variations in the genomic structure of HSV have frequently been reported (Brown and Harland, 1987; Harland and Brown, 1985; MacLean and Brown, 1987b). The frequency of variants with rearrangements in long repeat region of the genome within the wild type stock of HSV-2 strain HG52 was found to be 24% (Harland and Brown, 1985). However, in HSV-1 strain 17 syn$^+$ this value was found to be 0.02% (MacLean and Brown, 1987b). Although rearrangement of the genome within the repeat sequences of HSV could be due to the reiterated sequences found in these regions of the genome and which cause variation in the size of restriction fragments, (Rixon *et al.*, 1984; MacLean and Brown, 1987b) isolation of HSV-1 strain 17 syn$^+$ genomes displaying extensive variation, not prevously identified, raised the possibility of certain regions of the genome being particularly involved in the recombination/rearrangement process (MacLean and Brown, 1987b).

Three of the plaques isolated from a single transfection experiment, exhibited extensive variation in the IR_L/U_L region of the genome. These three deletion variants were designated 1704, 1705 and 1706. It appeared therefore that they may have been closely related to each other and could have arisen from a single progeny molecule, which thereafter underwent several rounds of rearrangement, to give rise to the deletion variants (MacLean and

Brown, 1987b). Several findings were in favour of this hypothesis; (i) similarity of the deletions at the right end of IR_L in 1704 and 1705 and in U_L in 1705 and 1706, (ii) all the variants exhibited similar growth properties *in vitro* and (iii) reduced levels of production of VmwIE63 in 1705 and 1706.

In order to study the genomic structure around the deletions precisely and to determine whether any specific sequences flanking the deletions, might play a part in the rearrangements, the variants 1704, 1705 and 1706 were sequenced across the end points of the deletions.

3.1.2 Sequence analysis across the U_L/IR_L and TR_L deletions in the variant 1704.

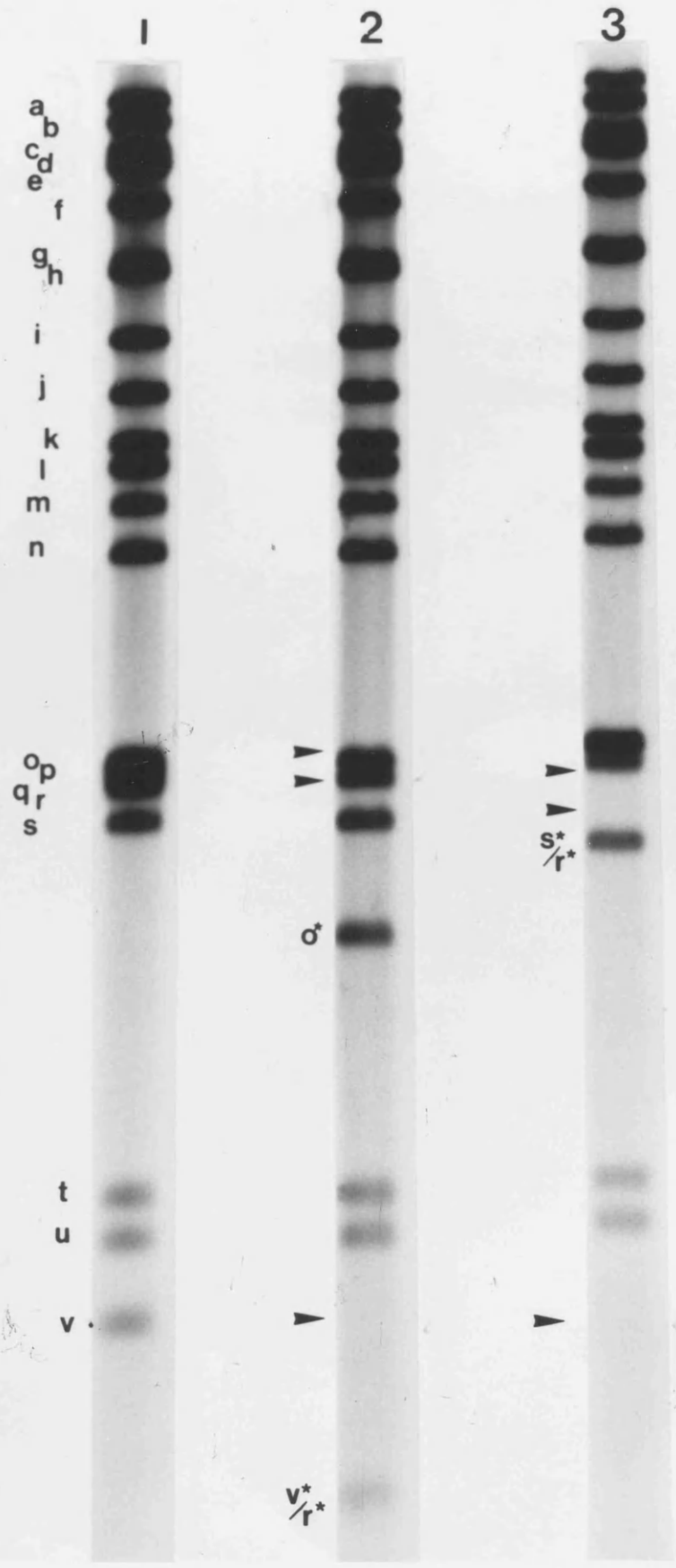
Restriction enzyme analysis of the 1704 genome revealed a deletion both in TR_L and U_L/IR_L. Digestion with *Hpa*I showed that the *o* fragment (np 6068-9618, $2.3x10^6$ Mr) was reduced by approximately $0.8x10^6$ Mr and migrated below the *s* band (np 112512-115763, $2.1x10^6$ Mr) making a novel fragment of approximately $1.5x10^6$ Mr designated as *o**. The s and m fragments (np 1-5900 and np 120300-126200, $3.9x10^6$ Mr) were unaltered. Fragments *v* (np 115763-117007, $0.8x10^6$ Mr) and r (np 117008-120298, $2.1x10^6$ Mr) affected by the $2.5x10^6$ Mr deletion within U_L/IR_L were absent. Remnants of the *v* and *r* fragments were fused to form a novel fragment of approximately 700bp designated as *v*/r** which runs at the bottom of the gel (Figures 3.1 and 3.2) (MacLean and Brown, 1987b).

3.1.2.a Oligonucleotide mapping of the U_L/IR_L deletion in 1704.

To estimate the end points of the deletions more precisely before sequencing, the sequence of the wild type genome in the

Figure 3.1

Autoradiograph of *Hpa*I digest of viral DNA ^{32}P labelled *in vivo* of 17 syn^+ (lane 1), 1704 (lane 2) and 1705 (lane 3). Letters refer to specific fragments; arrowheads indicate the position where fragments are missing and stars indicate novel fragments. The DNA products were separated on a 0.8% agarose gel.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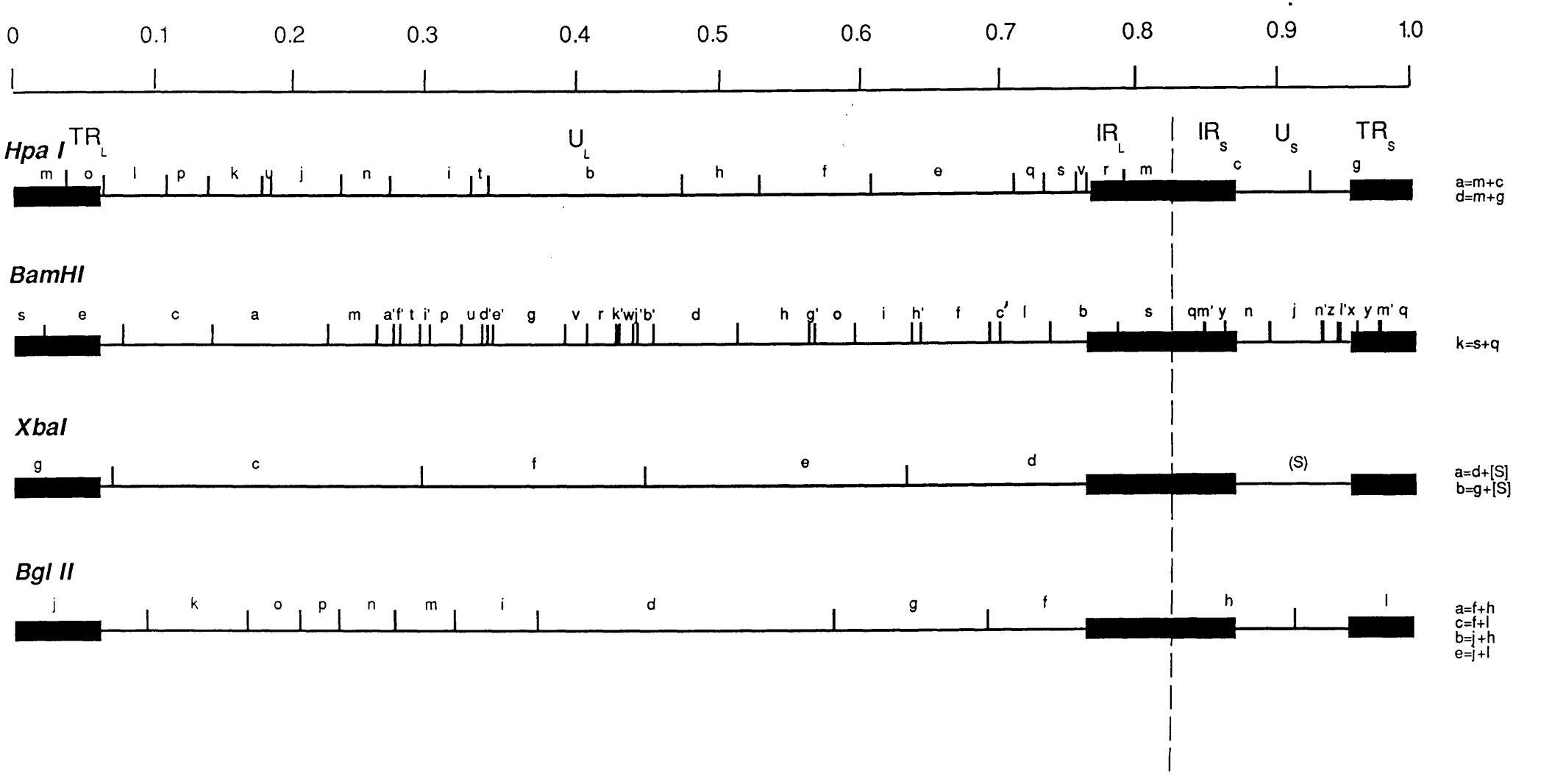
s*/r*

Figure 3.2

*Hpa*I, *Bam*HI, *Xba*I and *Bgl*II restriction maps of the DNA of HSV-1 strain 17 syn^{+}.(Wilkie, 1976; Davison, 1981)


0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Hpa I
TR L
U L
IR L
IR S
U S
TR S
m o l p k u j n i t b h f e q s v r m c g
a=m+c
d=m+g
BamHI
s e c a m a'f' t i' p u d'e' g v r k'w j'b' d h g' o i h' f c' l b s qm' y n j n'z l'x y m' q
k=s+q
XbaI
g c f e d (S)
a=d+[S]
b=g+[S]
Bgl II
j k o p n m i d g f h l
a=f+h
c=f+l
b=j+h
e=j+l

region of the deletions was examined and a series of oligonucleotides thought to span the deletions, were synthesised. These were used as probes in Southern blot hybridisation experiments. As we were particularly interested in the location of the deletions with respect to the LATs and the LAT promoter, the oligonucleotides were chosen to map only the end of the IR_L deletion affecting the LATs. The oligonucleotides from np 119561 to np 120377 are shown in Table 3.1. Oligonucleotides 6 to 9 within the *Hpa*I *r* fragment failed to hybridise and oligonucleotide No. 10 within *Hpa*I *m* showed positive hybridisation (gels not shown)(see Figure 3.2).

This analysis showed that one end of the deletion was between np 120177 and np 120300 (the *Hpa*I *r*/*m* junction) i.e within 123 bp.

3.1.2.b Cloning of the *Hpa*I *v**/*r** fragment of 1704 and nulceotide sequence analysis.

The DNA of variant 1704 was digested with a five fold excess of *Hpa*I and run on a 0.8% agarose gel. The novel fragment *v**/*r** was identified, cut out from the gel, electroeluted and cloned into pUC19. Since pUC19 has no *Hpa*I site, it was cloned into the compatible blunt ended *Sma*I site. Large scale preparation and $CsCl_2$ gradient purification of plasmid DNA was carried out. The cloned fragment was cut by double enzyme digestion with *Bam*HI and *Eco*RI. This fragment was subsequently cloned into M13 mp18 and mp19. Sequencing was carried out using the dideoxynucleotide chain termination reaction method by annealing recombinant M13 single stranded DNA with universal primer. Sequencing of the *v**/*r** fragment showed that the deletion is 3758 bp in length spanning the U_L/IR_L junction (Figures 3.3 and 3.4a); starting at nucleotide position 116502 and ending at np 120260. The deletion removes 655 bp of U_L

Table: 3.1 Southern blot analysis of *HpaI* v^*/r^* restriction fragment of the variant 1704 with synthetic oligonucleotides.

OLIGONUCLEOTIDE	HYBRIDISATION TO
No: Coordinates (np)	*HpaI* v^*/r^* fragment (1704)
6. 119561-119577	–
7. 119771-119587	–
8. 119961-119977	–
9. 120161-120177	–
10. 120361-120377!	+

! Oligonuleotide No. 10 is from *HpaI m* (see Figure 3.2)

Figure 3.3

A portion of an autoradiograph showing the start (np 116502) and end (np 120261) of the deletion in the variant 1704 in U_L/IR_L. Sequencing products were separated on a 6% denatured polyacrylamide gel (Sanger *et al.*, 1980) containing 9M urea (McGeoch *et al.*, 1986).

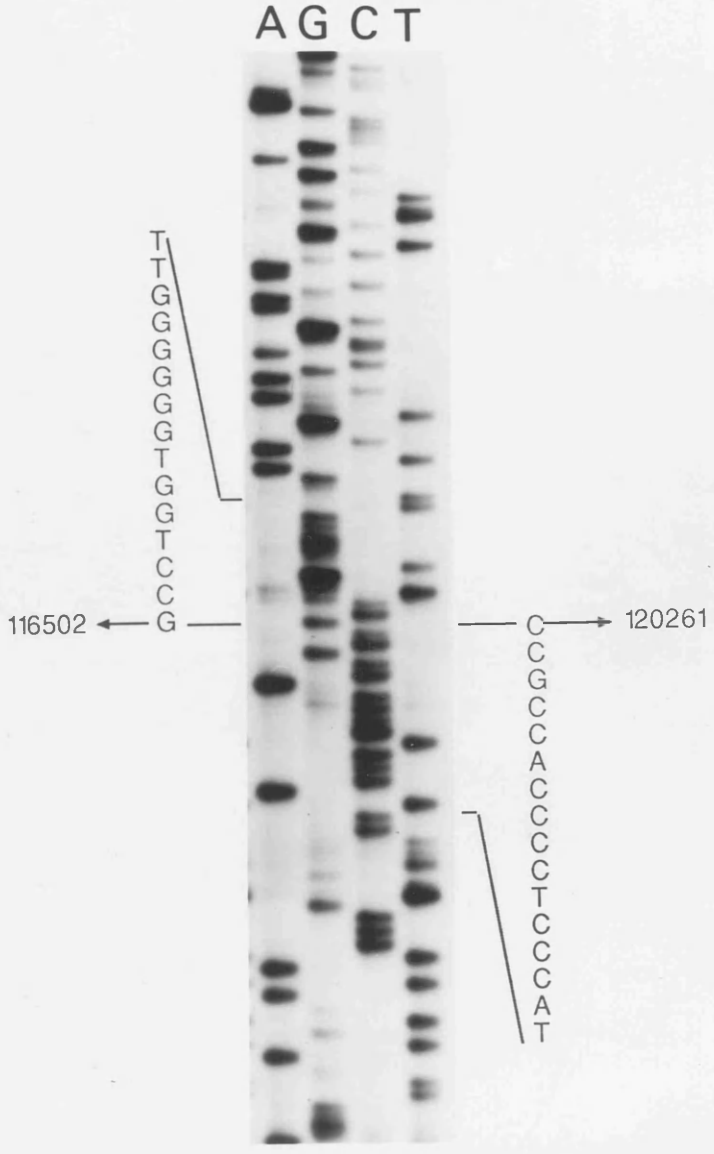
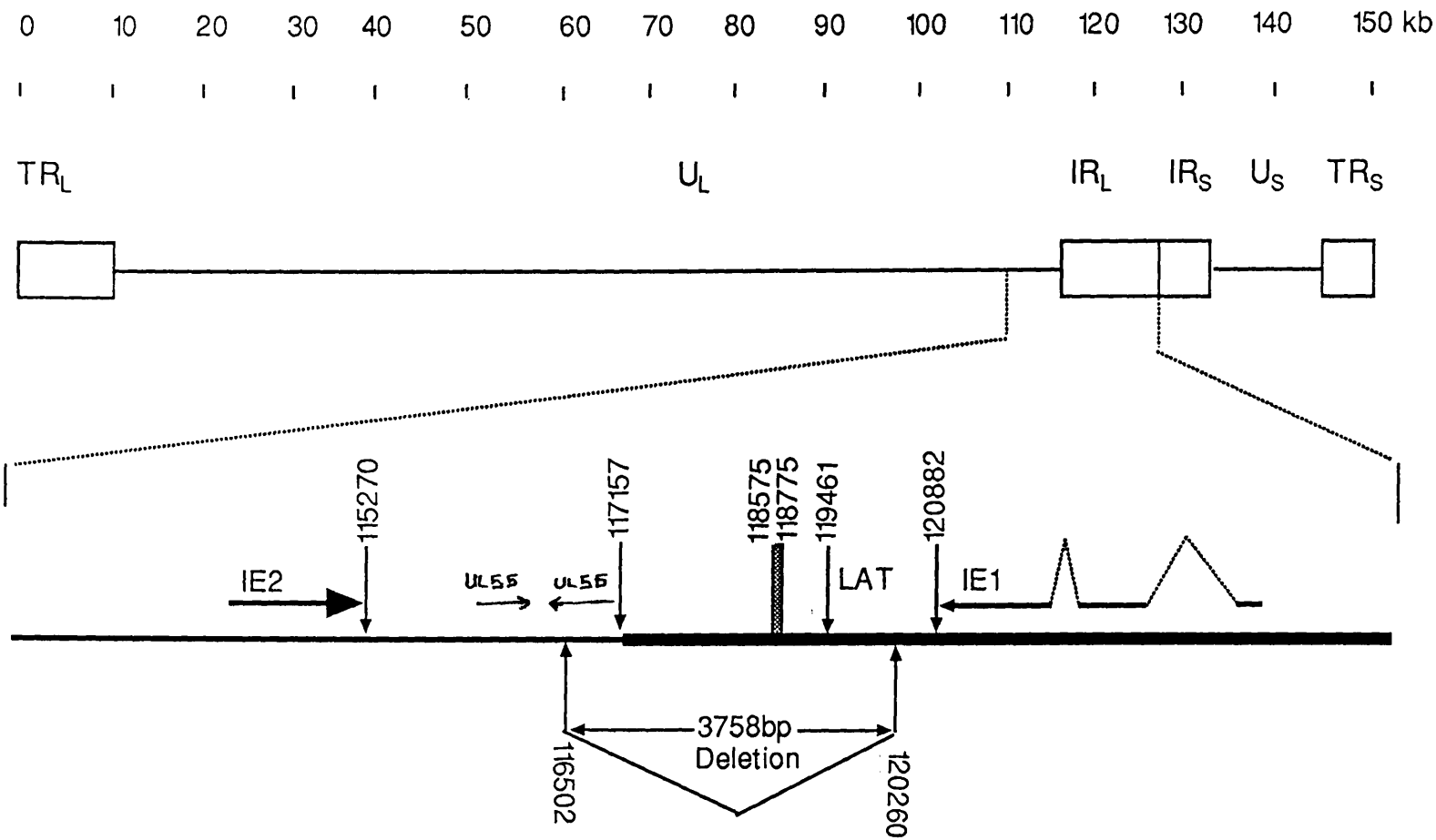

A G C T
TTGGGGGGTGGTCCCG
116502
C
120261
CCGCCACCCCCTCCCCAT

Figure 3.4.a

Structure of the HSV-1 genome (top line) showing U_L and U_S flanked by TR_L/IR_L and IR_S/TR_S respectively. The second line shows expansion between 110kb map position and the IR_L/IR_S junction. Thin and thick lines indicate unique and repeat portions of the long region of the genome. Above the line, the position of 3' end of IE1, 3' end of IE2, 5' end of the LAT and U_L/IR_L junction are indicated by the arrows. The LAT promoter region is indicated by a hatched bar. The extent of the deletion in the variant 1704 in U_L/IR_L is marked below the line. Coordinates are given as nucleotide positions (McGeoch *et al.*, 1988).


0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150 kb
TRL
UL
IRL
IRS
US
TRS
115270
117157
118575
118775
119461
120882
IE2
UL55
UL56
LAT
IE1
3758bp
Deletion
116502
120260

and 3103 bp of IR_L starting 622 bp downstream of the 3' end of IE1. Only 170 bp of the 3' end of UL56 (np 116930-116332) are retained and 799bp from the 5' end of the LATs (np 119461) are deleted as well as the LAT promoter region (np 118775-118575) (TATAA & CAAT boxes and SpI binding sites) (Weschler *et al.*, 1988; Zwaagstra *et al.*, 1990)

3.1.2.c Oligonucleotide mapping of the TR_L deletion in 1704.

An initial estimate of the extent of the deletion in TR_L in 1704 was obtained by Southern blot hybridisation experiments using a range of five 17-mer synthetic oligonucleotides. These oligonucleotides were selected from np 6291 to np 9507 and the hybridisation results revealed that out of the five only one oligonucleotide failed to hybridise (Figure 3.4b .) indicating that the deletion was between np 7057 and np 8720 (Table 3.2).

3.1.2.d Cloning of *Hpa*I o* fragment of 1704 and nucleotide sequencing analysis.

The 1704 *Hpa*I *o** fragment was cloned into the SmaI site of pUC19. Cloning was confirmed by:

(a) *Hpa*I digested Southern blots of 1704 and 17 syn^+. Figure 3.5(a) shows that the *o** fragment hybridised to the 1704 *o** fragment and the *o* fragment in 17 syn^+. As a control randomly primed 17 syn^+ DNA was hybridised to the same digest of 17 syn^+ and 1704 in order to identify the position of the relevant fragments on the gel (Figure 3.5b).

(b) Restriction enzyme analysis of the cloned *Hpa*I *o** fragment was carried out with *Sph*I, blotted to nitrocellulose and hybridised to *Hpa* I *r* (*Hpa*I *r* and *o* share the same R_L sequences) The sequence of

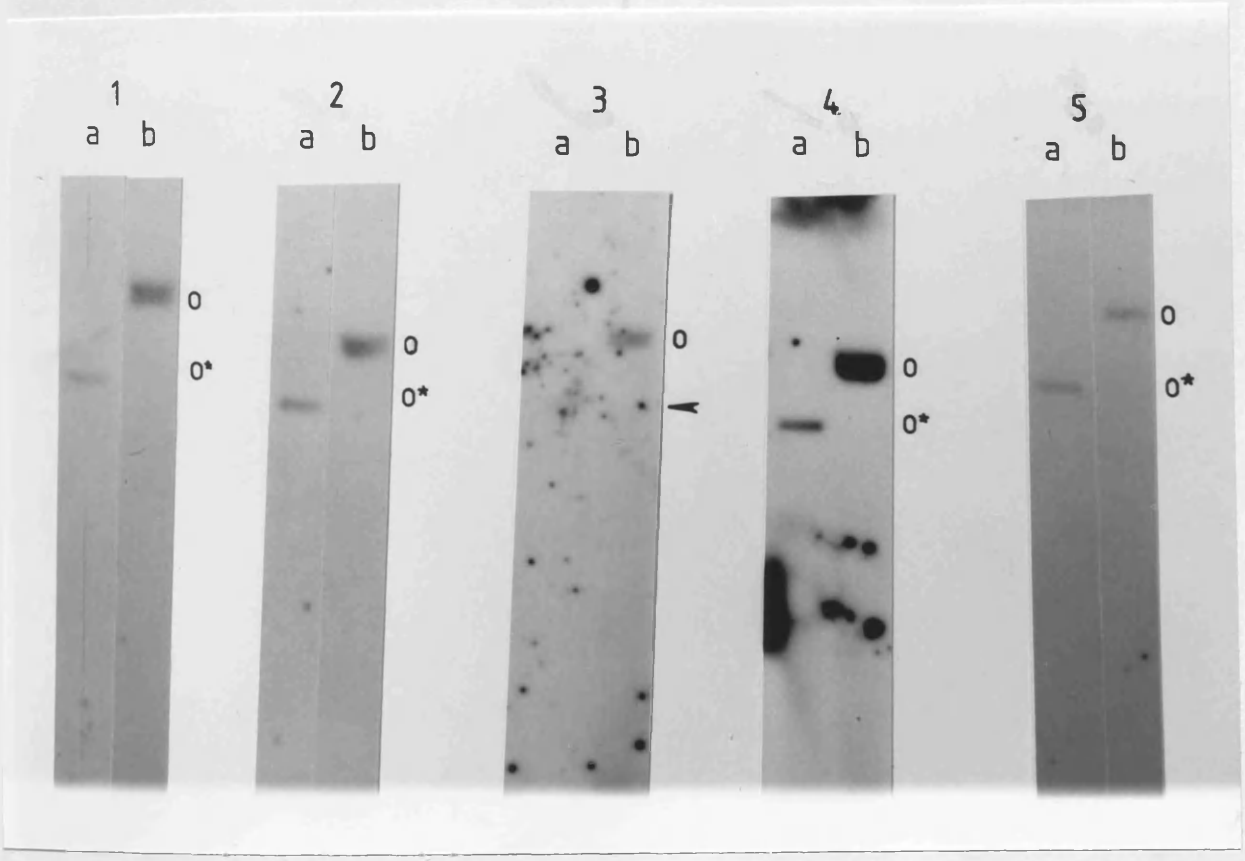


Figure 3.4b

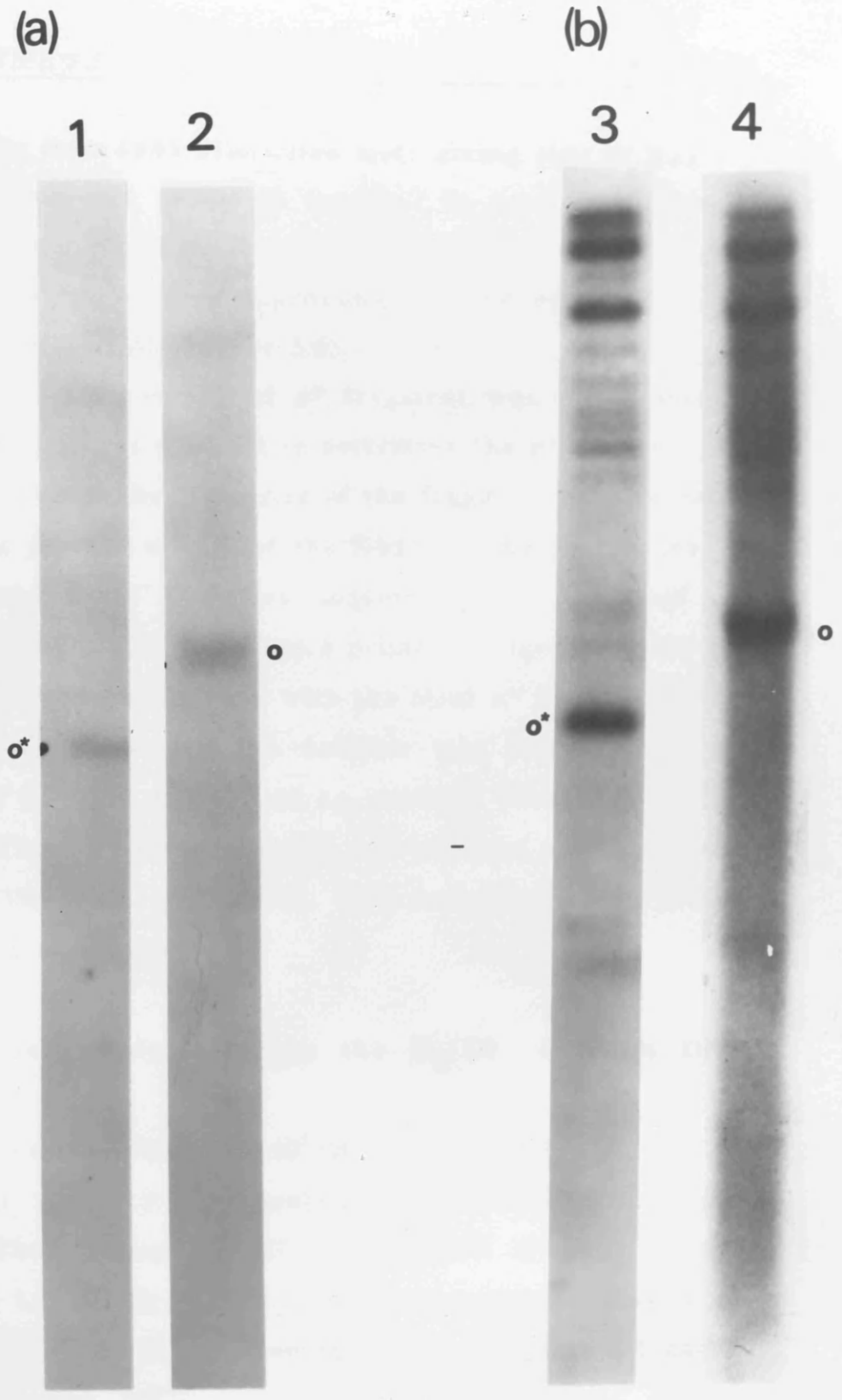
Autoradiograph of Southern blot in which ^{32}P labelled *in vitro*, oligonucleotides (1-5) were hybridised to DNA of strain 17 syn^{+} (lane b) and 1704 (lane a), which had been digested with *Hpa*I. Letters indicate specific fragments, stars indicate the novel fragments and arrowhead indicates where the specifc oligonucleotide failed to hybridise.

Table: 3.2 Southern blot analysis of *HpaI o** restriction fragment of the variant 1704 with synthetic oligonucleotides.

OLIGONUCLEOTIDE	HYBRIDISATION TO
No: Coordinates (np)	*HpaI o** fragment (1704)
1. 6291-6370	+
2. 7041-7057	+
3. 8054-8070	–
4. 8721-8737	+
5. 9491-9507	+

Figure 3.5

Autoradiograph of Southern blots in which randomly primed ^{32}P labelled *in vitro* probes of (a) *Hpa*I *o** and (b) 17 syn^{+} were hybridised to DNA of 1704 (lane 1 and 3) and 17 syn^{+} (lane 2 and 4), which had been digested with *Hpa*I. Letters refer to specific fragments and stars indicate novel fragments.


(a)
(b)
1
2
3
4
o*
o
o*
o

*Hpa*I *o* contains three *Sph*I restriction sites giving rise to fragments of 231, 786, 2516 and 18 bp in length. In agreement with the oligonucleotide mapping data the 2516 bp SphI restriction fragment was reduced to a fragment of approximately 1300 bp containing the end points of the deletion (Figure 3.6).

The 2.8 kb novel *Hpa*I *o** fragment was cloned into M13 mp18 and mp19. It was difficult to determine the precise end points of the deletion due to the large size of the fragment and the location of the deletion in the middle of the fragment, by sequencing from one end or the other therefore sequencing was initiated using oligonucleotide No. 2 (Table 3.2) as a primer. Oligonucleotide No. 2 had shown positive hybridisation with the *Hpa*I *o** fragment of 1704.

It was found that the deletion was 942 bp in length exending from np 7202 to np 8144 i.e. entirely within TR_L (Figures 3.7 and 3.8). The LAT transcripts are not affected and the promoter region at np 7596-7796 is removed. This includes TATAA & CAAT boxes and SpI binding sites.

3.1.3 Sequencing analysis across the U_L/IR_L deletion in the variant 1705.

This variant has a 3.5×10^6 Mr deletion in U_L/IR_L. On *Hpa*I digestion the *s, v* and *r* fragments are absent and the *m* band is unaltered. The deletion in U_L/IR_L created a novel band of approximately 1.8×10^6 Mr consisting of the remnants of *Hpa*I *s* and *r* designated as *s*/r** which is running below (Figures 3.1 and 3.2) (MacLean and Brown, 1987b).

3.1.3.a Oligonucleotide mapping of the U_L/IR_L deletion in the variant 1705.

In order to narrow down the end points of the deletion a

Figure 3.6

Autoradiograph of Southern blot in which randomly primed ^{32}P labelled *in vitro HpaI r* was hybridised to *SphI* digested DNA of the *HpaI o** fragment of the variant 1704. Numbers indicate size of the specific fragments in base pairs.

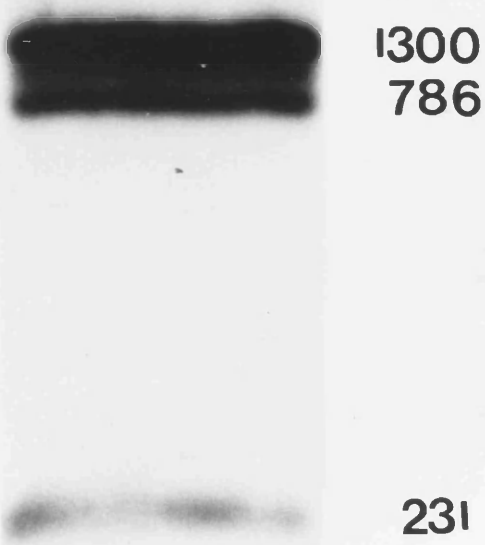

1300
786
231

Figure 3.7

A portion of an autoradiograph showing the start (np 7202) and end (np 8145) of the deletion in the variant 1704 in TR_L. Sequencing products were separated on a 6% denatured polyacrylamide gel (Sanger *et al.*, 1980) containing 9M urea (McGeoch *et al.*, 1986).

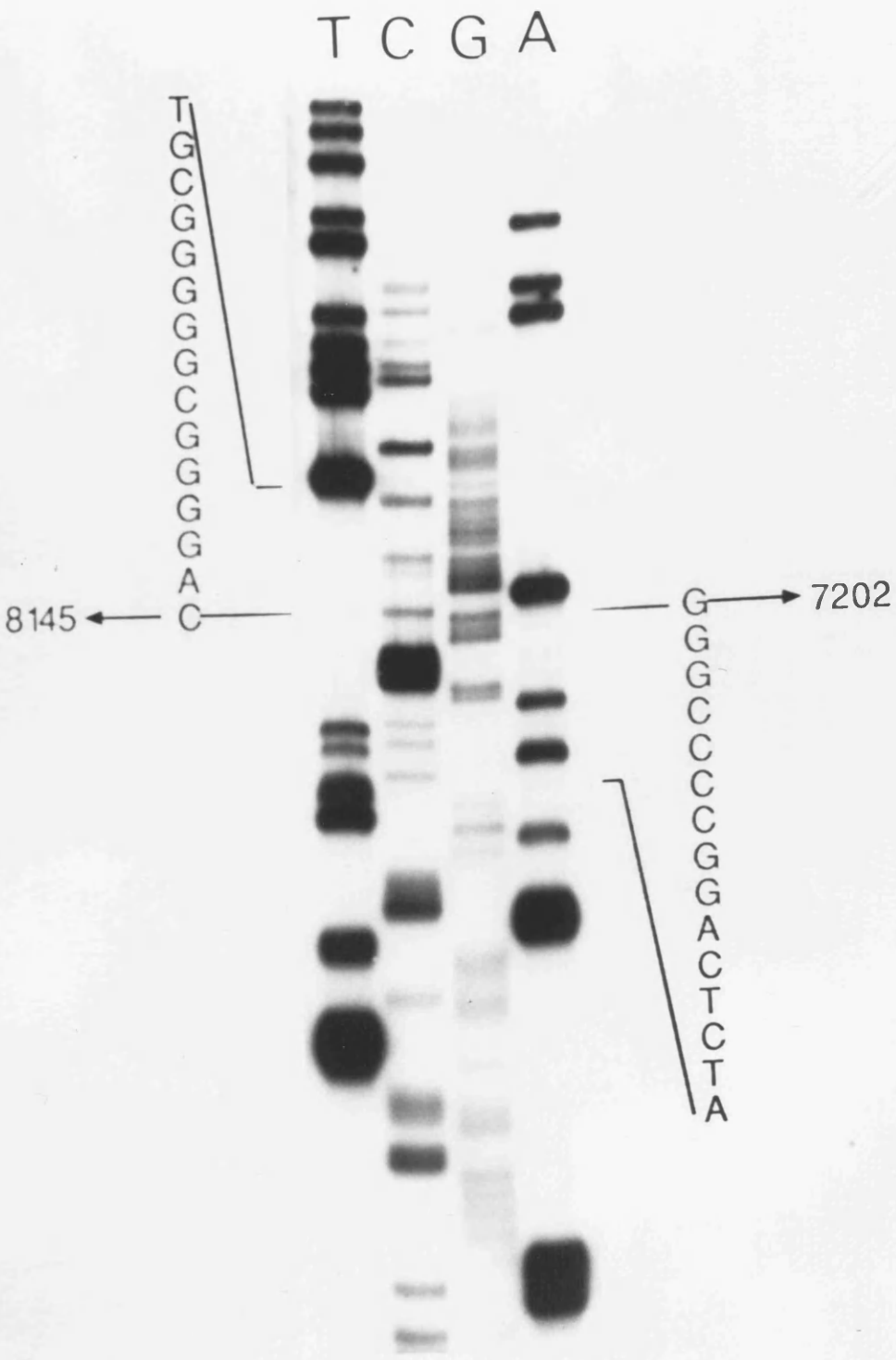
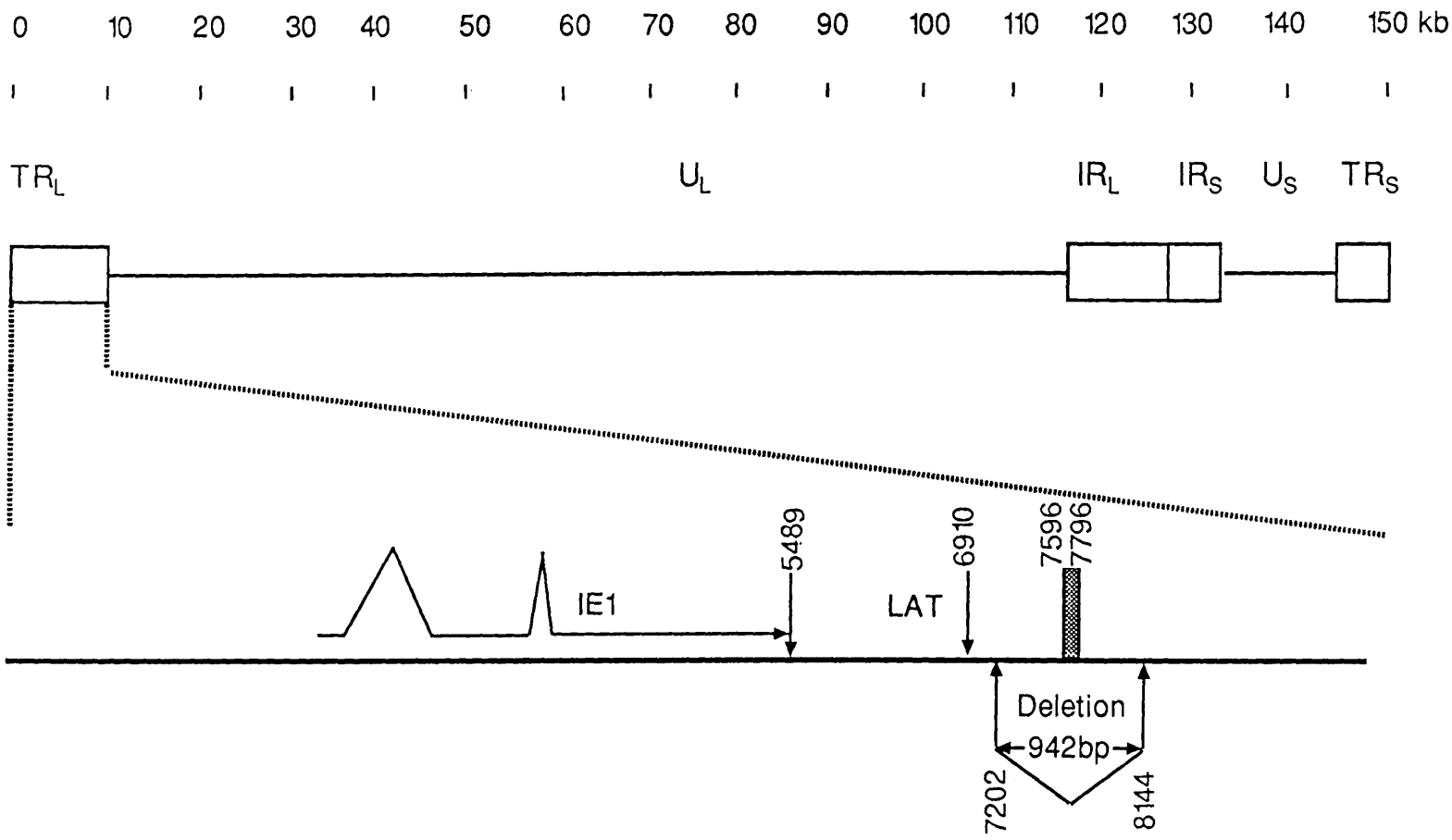

T C G A
TGCGGGGGGCCGGGGGA
C
8145
G
7202
GGGCCCCCGGACTCTA

Figure 3.8

Structure of the HSV-1 genome (top line) showing U_L and U_S flanked by TR_L/IR_L and IR_S/TR_S respectively. The second line shows expansion of the TR_L region of the genome. Above the line, the position of 3' end of IE1, 5' end of the LAT are indicated by arrows. The LAT promoter region is indicated by a hatched bar. The extent of the deletion in the variant 1704 in TR_L is marked below the line. Coordinates are given as nucleotide positions (McGeoch *et al.*, 1988).


0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150 kb
TRL
UL
IRL
IRS
US
TRS
5489
6910
7596
7796
IE1
LAT
Deletion
942bp
7202
8144

number of relevant oligonucleotides were used in Southern blot hybridisation experiments with 1705. None of the oligonucleotides hybridised to *Hpa*I *s**/*r** of 1705 (Table 3.3, gels not shown). This variant does synthesise VmwIE63 from IE2 (MacLean, 1988) indicating that one end of the deletion must be between np 115270 (the 3' end of IE2) and np 115460 (the first nucleotide of oligonucleotide No. 11) i.e within 190 bp.

3.1.3.b Cloning of the *Hpa*I *s**/*r** fragment of 1705 and nucleotide sequencing analysis.

The novel *Hpa*I *s**/*r** fragment of 1705 was cloned into the blunt ended SmaI site of pUC19. Cloning was confirmed by a Southern blot (Figure 3.9). Large scale preparation and $CsCl_2$ gradient purification of the cloned plasmid was carried out . As the cloned 1705 fragment was almost the same size as the vector a double digest was carried out with *Eco*RI and *Dra*I using the same restriction endonuclease enzyme buffer and additionally with *Xba*I. The 1705 *Hpa*I *s**/*r** fragment does not have a *Dra*I site but the vector contains two sites giving fragments of 1975, 692 and 19 bp. The *Hpa*I *s**/*r** insert is therefore easily separated (Figure. 3.10) The fragment *s**/*r** was cloned into the *Eco*RI and *Xba*I sites of M13 mp18 and mp19. Recombinants were annealed with a universal primer and sequenced by the dideoxy chain termination reaction method. The deletion was found to be 4735 bp in length, extending from np 115453 to np 120188 (Figure 3.11 and 3.12). The deletion removes 3031 bp of IR_L and 1704 bp of U_L containing UL55 and UL56 and stops 183 bp and 694 bp downstream of the 3' ends of IE2 and IE1 respectively. 727bp from the 5' end of the LATs and the LAT promoter region have also been deleted. The variant 1705 is not deleted in TR_L.

Table: 3.3 Southern blot analysis of *Hpal s, s***/r** and *l* restriction fragments of 17^{+}, 1705 and 1706 respectively with synthetic oligonucleotides.

OLIGONUCLEOTIDE	HYBRIDISATION TO		
No: Coordinates (np)	*Hpal s* (17^{+})	*Hpal s***/r** (1705)	*Hpal l** (1706)
11. 115461-115477	+	–	–
12. 115561-115577	+	–	–
13. 115661-115677	+	–	–

Figure 3.9

Autoradiograph of Southern blot in which the randomly primed ^{32}P labelled *in vitro* *s**/*r** cloned fragment of the variant 1705 was hybridised to HpaI digested DNA of 1705 (lane 1) and 17 syn^+ (lane 2).

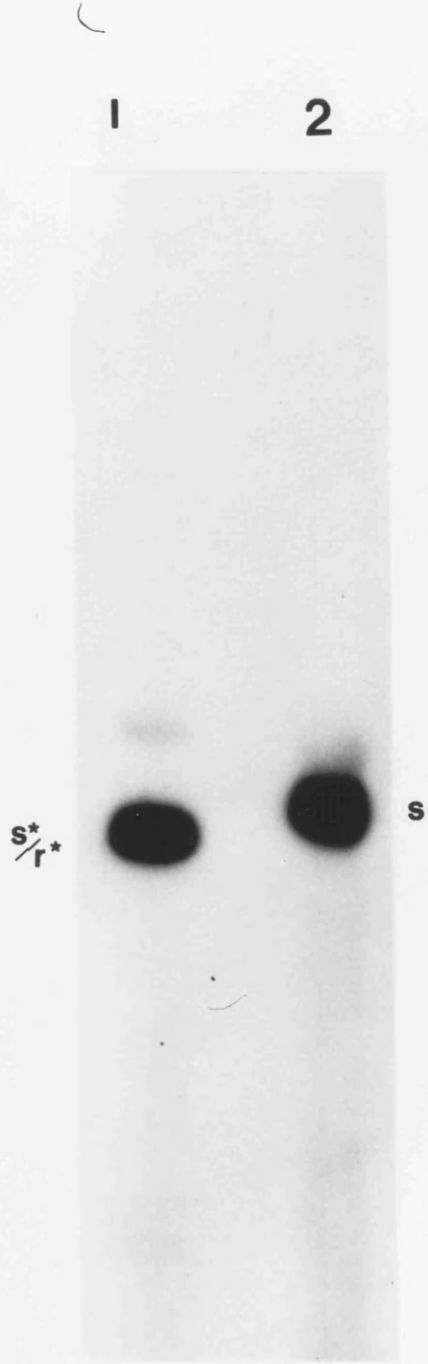

1
2
s*/r*
s

Figure 3.10

The map of a construct of *Hpa*I *s*/r** fragment (shaded area) of the variant 1705 in the plasmid pUC19. Various restriction sites and size of the fragments in base pairs is also indicated.

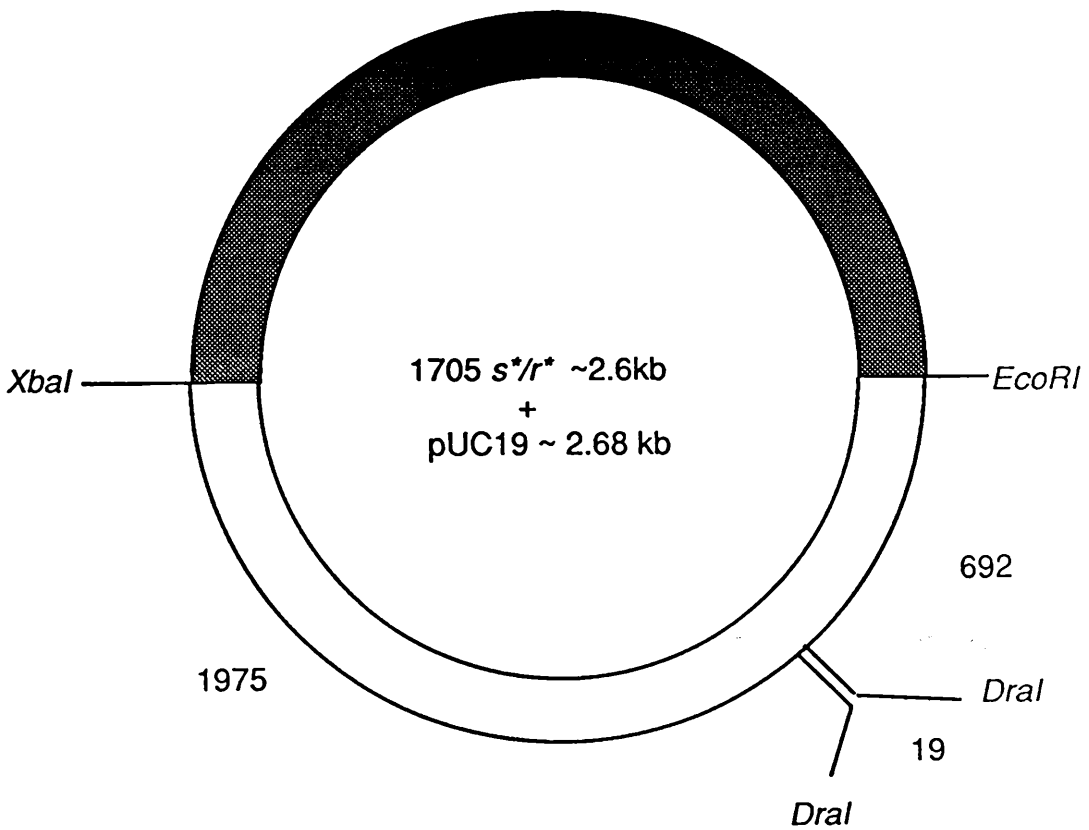

1705 s*/r* ~2.6kb
+
pUC19 ~ 2.68 kb
XbaI
EcoRI
692
1975
DraI
19
DraI

Figure 3.11

A portion of an autoradiograph showing the start (np 115453) and end (np 120189) of the deletion in the variant 1705 in U_L/IR_L. Sequencing products were separated on a 6% denatured polyacrylamide gel (Sanger *et al.*, 1980) containing 9M urea (McGeoch *et al.*, 1986).

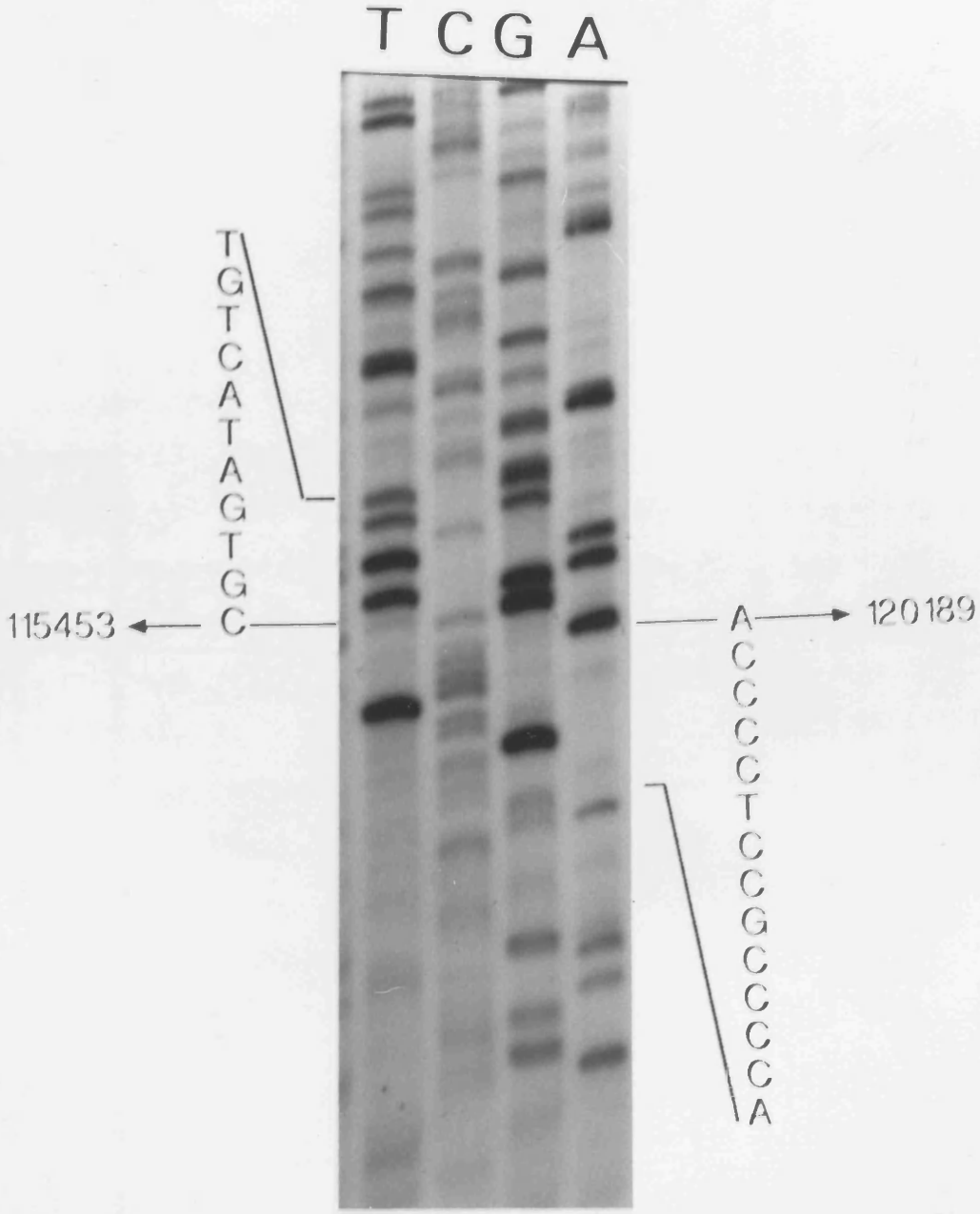
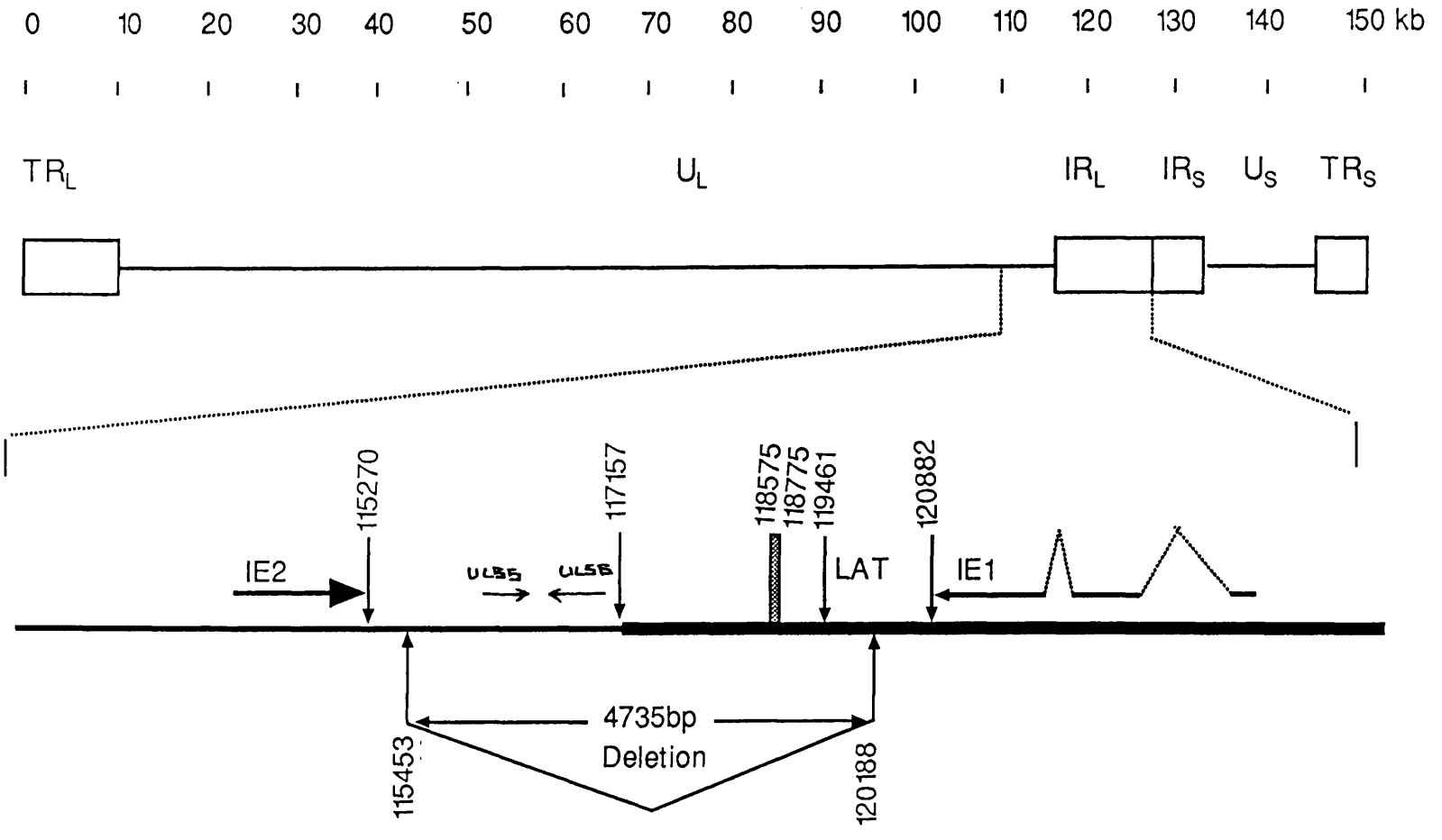

T C G A
TGTCATAGTGC
115453
A
120189
ACCCCCTCCGCCCCCA

Figure 3.12

Structure of the HSV-1 genome (top line) showing U_L and U_S flanked by TR_L/IR_L and IR_S/TR_S respectively. The second line shows expansion between map position 110kb and the IR_L/IR_S junction. Thin and thick lines indicate unique and repeat portions of the long region of the genome. Above the line, the position of 3' end of IE1, 3' end of IE2, 5' end of the LAT and U_L/IR_L junction are indicated by the arrows. The LAT promoter region is indicated by a hatched bar. The extent of the deletion in the variant 1705 in U_L/IR_L is marked below the line. Coordinates are given as nucleotide positions (McGeoch *et al.*, 1988).


0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150 kb
TRL
UL
IRL
IRS
US
TRS
115270
117157
118575
118775
119461
120882
IE2
UL55
UL56
LAT
IE1
4735bp
Deletion
115453
120188

3.1.4 Sequencing analysis across the deletion/insertion in the variant 1706.

The variant 1706 has a deletion of $1x10^6$ Mr involving the right end of U_L adjacent to IR_L. The deleted sequences have been replaced by sequences of approximately $3x10^6$ Mr from the left side of U_L, so that the total size of the 1706 genome has been increased by $2x10^6$ Mr (MacLean and Brown, 1987b). On *Bam*HI digestion the *b* fragment was absent and the *e* fragment appeared to be 2M. A novel band of approximately $2.7x10^6$ Mr running above *o* was the fusion product of part of *Bam*HI *b* and part of *Bam*HI *c* designated as *b*/c** (Figures 3.13 and 3.2).

On *Hpa*I digestion the *o* fragment was 2M and the *s*, *r* and *v* fragments were absent. A novel band of approximately $4.7x10^6$ Mr above *k*, the fusion product of *s* and *l*, designated as *l**, was present (Figures 3.14 and 3.2). (MacLean and Brown, 1987b).

3.1.4.a Oligonucleotide mapping of the U_L deletion/insertion in the variant 1706.

In order to map both the deletion and the insertion, a series of 17-mer synthetic oligonucleotides were made and used as probes in Southern blot hybridisation experiments with 1706 DNA. To delimit the span of the deletion three oligonucleotides No. 11, 12 and 13 from np 115461 to np 115667 were selected 83 bp equidistant from each other. Radiolabelled oligonucleotides were allowed to hybridise to *Hpa*I digested Southern blots of 1706 and 17 syn^+ DNA. All the oligonucleotides failed to hybridise to 1706 (gels not shown) demonstrating that the sequences between np 115461 and np 115667 are missing (Table 3.3). The variant 1706 expresses VmwIE63 from the IE2 gene (MacLean,. 1988), indicating that the termination of the

Figure 3.13

Autoradiograph of a *Bam*HI digest of viral DNA ^{32}P labelled *in vivo* of 17 syn^+ (lane 1) and 1706 (lane 2). Letters refer to specific fragments, arrowheads indicate the position where fragments are missing and stars indicate novel fragments. The DNA products are separated on a 1.2% agarose gel.

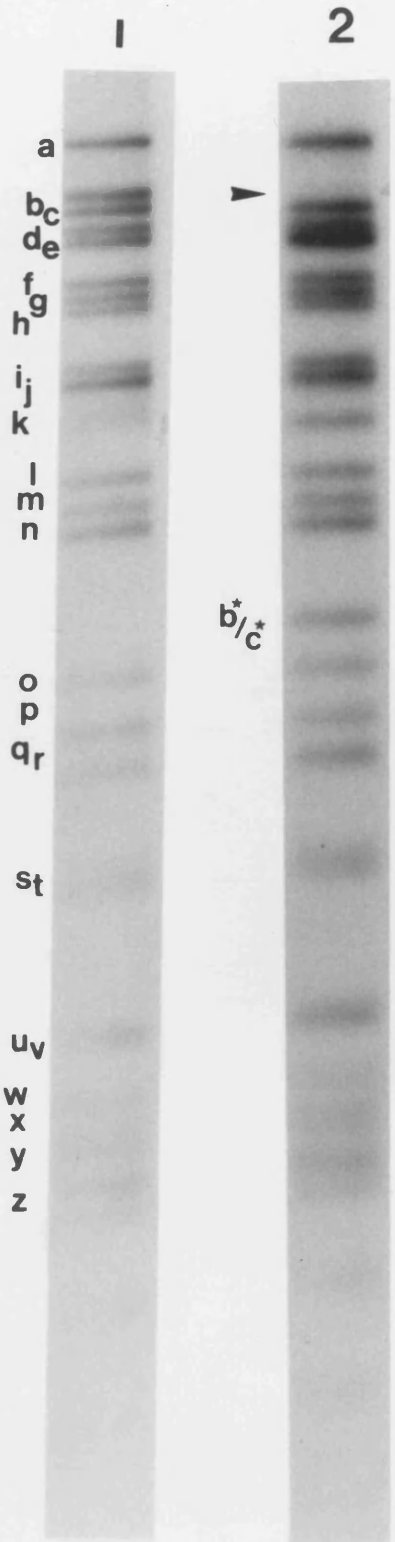
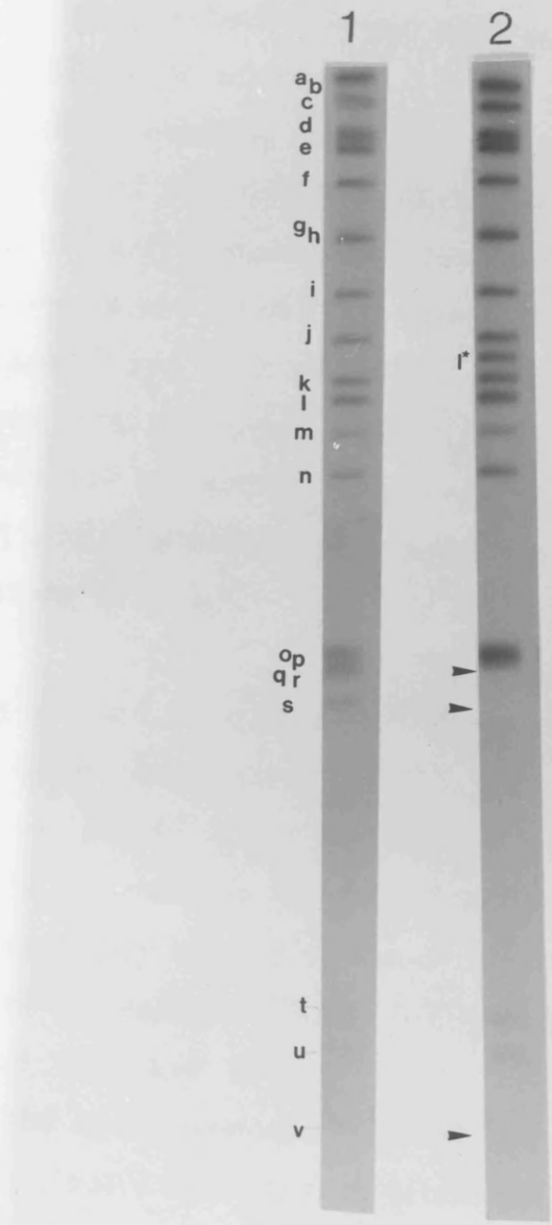

1
2
a
b c
d e
f g
h
i j
k
l
m
n
o
p
q r
s t
u v
w
x
y
z
b*/c*

Figure 3.14

Autoradiograph of a *Hpa*I digest of viral DNA ^{32}P labelled *in vivo* of 17 syn^{+} (lane 1) and 1706 (lane 2). Letter refer to specific fragments, arrowheads indicate the position where fragments are missing and stars indicate novel fragments. The DNA products are separated on a 0.8% agarose gel.


1
2
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
r*

deletion is between np 115270 (the 3' end of IE2) and np 115460 i.e within 190 bp.

To determine the extent of the insertion from the left end of U_L a series of six synthetic 17-mer oligonucleotides between np 10611 and np 15717 were selected and synthesised. *Hpa*I digested Southern blot hybridisation of 1706 and 17 syn^+ DNA with the oligonucleotides showed that numbers 14, 15, 16 and 17 hybridised to both *Hpa*I *l* and the *l** fragment of 1706 and *Hpa*I *l* of 17 syn^+. Oligonucleotide no. 18 and 19 showed only positive hybridisation to *Hpa*I *l* of 1706 and 17 syn^+ but not to the *Hpa*I *l** fragment of 1706 (Table 3.4, Figure 3.14, gels not shown). These results indicate that the fragment from the left end of U_L inserted in the right end starts before np 10601 and terminates between np 13657 and np 14691 i.e. it is at least 3055 bp in length.

3.1.4.b Cloning of the *BamH*I *b*/c** fragment of the variant 1706 and nucleotide sequencing.

The novel *BamH*I *b*/c** fragment of the variant 1706 was cloned into pUC19. Cloning in the pUC19 vector was confirmed by hybridising the randomly primed probe of the cloned novel *Bam*HI *b*/c** fragment of the variant 1706 with *Hpa*I and *Bam*HI digested Southern blots of 1706 and 17 syn^+ DNA. The *Hpa*I digestion showed that in 1706 hybridisation was specific to the *l* and *l** bands and in 17 syn^+ to *Hpa*I *l* and *Hpa*I *o* (Figure 3.15a). The *Bam*HI digestion confirmed the correct fragment as there was positive hybridisation to *Bam*HI *b* and c in 17 syn^+ and to *Bam*HI c and the novel fragment *b*/c** in 1706 (Figure 3.15b). This was further confirmed by hybridising randomly primed probe of the *Bam*HI *c* fragment with a *Bam*HI digested Southern blot of 17 syn^+ and the variant 1706. Figure 3.15(c) shows positive hybridisation with

Table: 3.4 Southern blot analysis of *Hpal I* restriction fragment of 17^{+} and *Hpal I* and I^{*} restriction fragments of 1706 with synthetic oligonucleotides.

OLIGONUCLEOTIDE	HYBRIDISATION TO		
No: Coordinates (np)	*Hpal I* (17^{+})	*Hpal I* (1706)	*Hpal* I^{*} (1706)
14. 10600-10617	+	+	+
15. 11631-11647	+	+	+
16. 12660-12677	+	+	+
17. 13641-13657	+	+	+
18. 14691-14707	+	+	–
19. 15701-15717	+	+	–

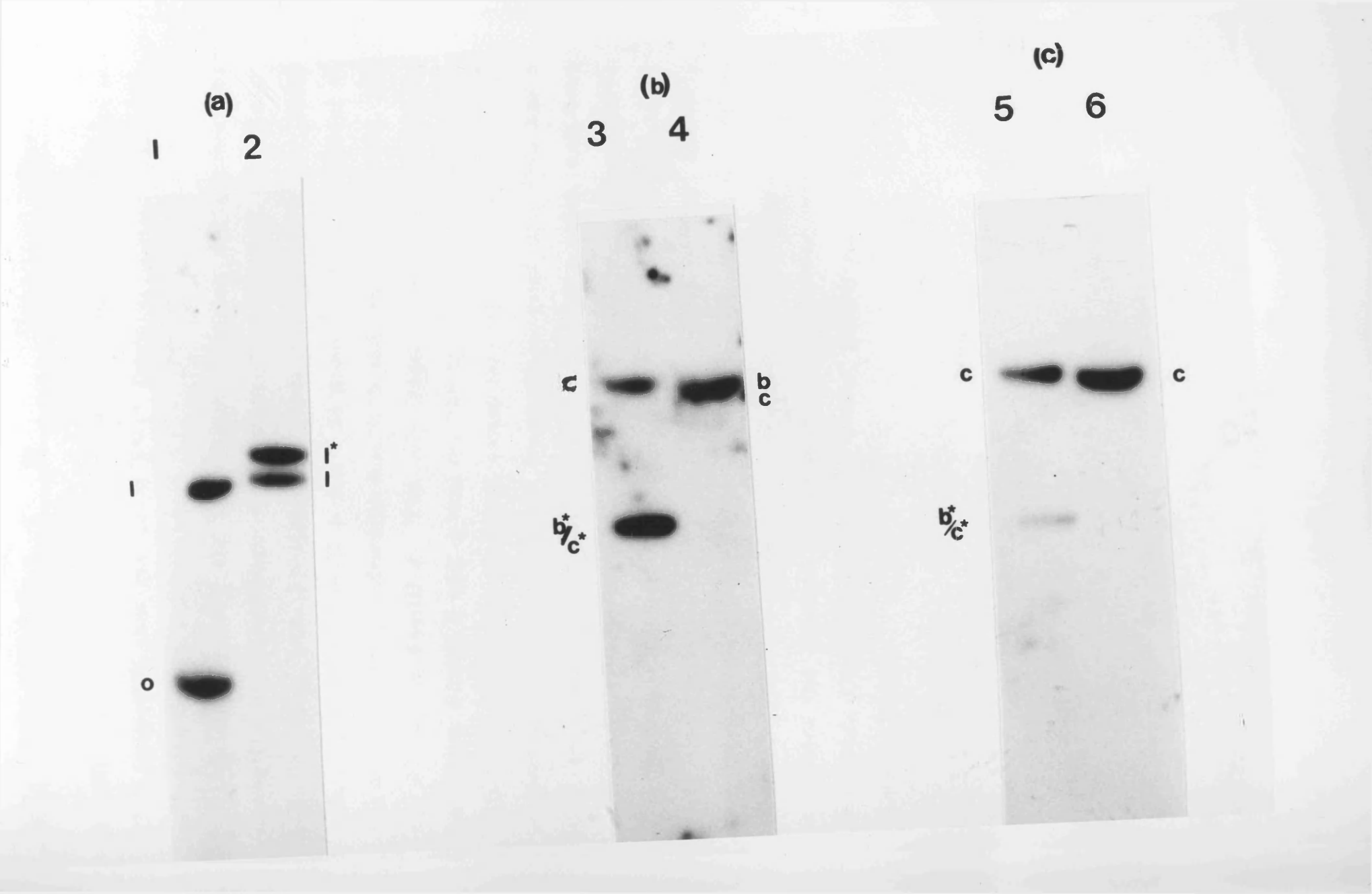
Figure 3.15

Autoradiograph of a Southern blot of the randomly primed ^{32}P *in vitro* labelled novel fragment *Hpa*I *l** of the variant 1706 was hybridised to:

(a) *Hpa*I digested 17 syn$^+$ (lane 1) and 1706 (lane 2) DNAs and

(b) *Bam*HI digested 1706 (lane 3) and 17 syn$^+$ (lane 4) DNAs.

(c) *Bam*HI digested of 17 syn$^+$ and 1706 DNAs: lane 5; 1706 probed with *Bam*HI *c*, lane 6; 17 syn$^+$ probed with *Bam*HI *c*. Letters refer to specific fragments and stars indicate the novel fragments.


(a)
1
2
I*
I
I
o
(b)
3
4
c
b
c
b*/c*
(c)
5
6
c
c
b*/c*

*Bam*HI *c* of 17 syn$^+$ and *Bam*HI *c* and the novel *b*/c** fragment of 1706 indicating that the novel *b*/c** fragment contains sequences both from *Bam*HI *b* and *c*.

To identify the subfragment spanning the deletion, the cloned *Bam*HI *b*/c** fragment was digested with *Bam*HI/*Hpa*I/*Sma*I and Southern blot hybridisation experiments carried out using randomnly primed *Bam*HI *b* and *c* as probes. The subfragment of 861 and 726 bp hybridised to *Bam*HI b and those of 726, 675, 516 and 271 bp to *Bam*HI *c*. The only fragment containing sequences both from *Bam*HI *b* and c was the 726 bp fragment (Figure 3.16). This fragment was electroeluted, cloned into M13 mp18 and mp19 and sequenced. Sequencing of the fragment revealed that 1706 had a 1807 bp deletion at the right hand end of U_L which had been replaced by 4754 bp from the left hand end (Figures 3.17 and 3.18). The deletion started 80 bp downstream of the 3' end of IE2 at np 11535 and terminated at np 117157. The deletion therefore completely removed UL55 and 56. The sequences from np 9215 to np 13969 have been repeated and inserted in an inverted orientation to replace the deletion. Consequently 1706 contains two copies of UL1, UL2, UL3 and UL4 and a second partial copy of UL5 (np 13969-np 12487).

Figure 3.16

Autoradiograph of a Southern blot in which randomly primed ^{32}P labelled *in vitro* *Bam*HI *b* (1) and c (2) were hybridised to the *Bam*HI *b*/c** fragment of 1706 DNA which was digested with *Bam*HI/*Hpa*I/*Sma*I.

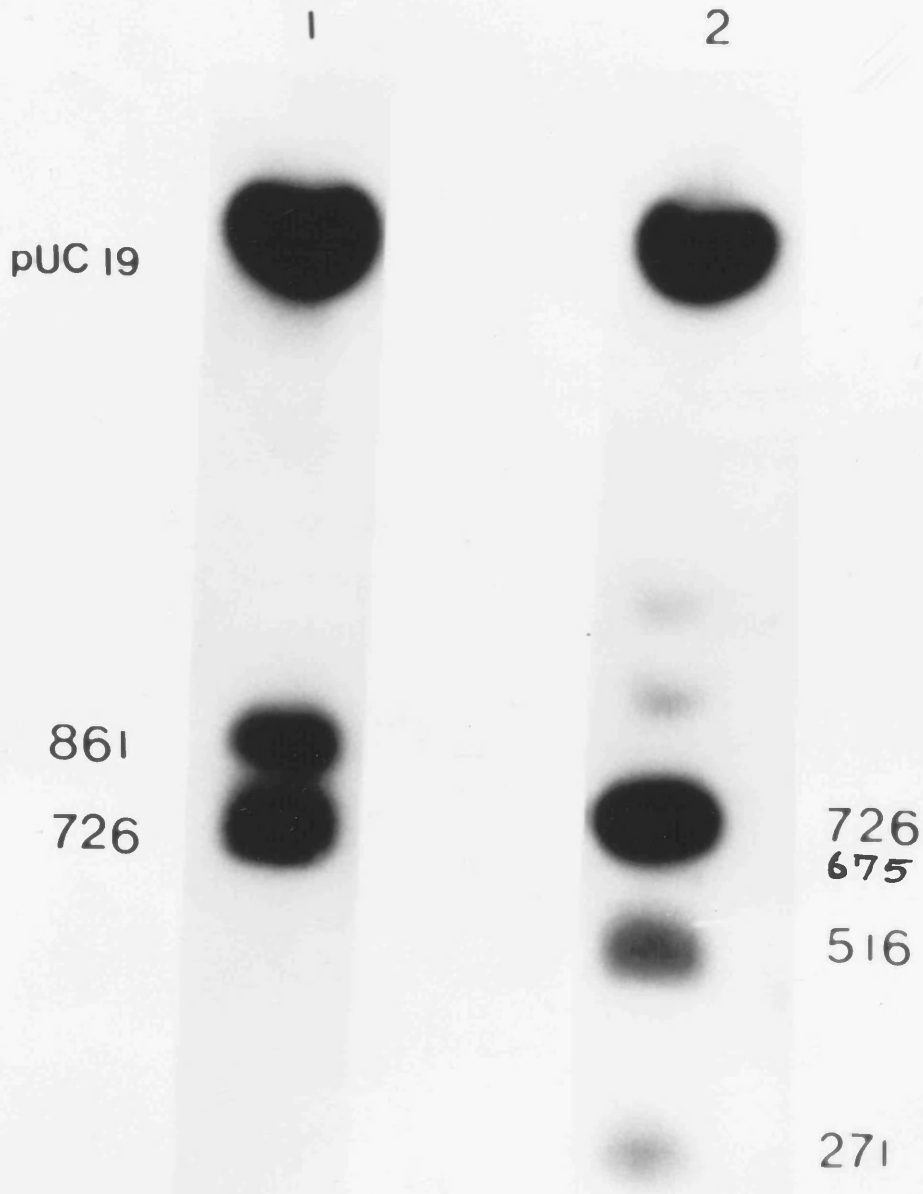

1
2
pUC 19
861
726
726
675
516
271

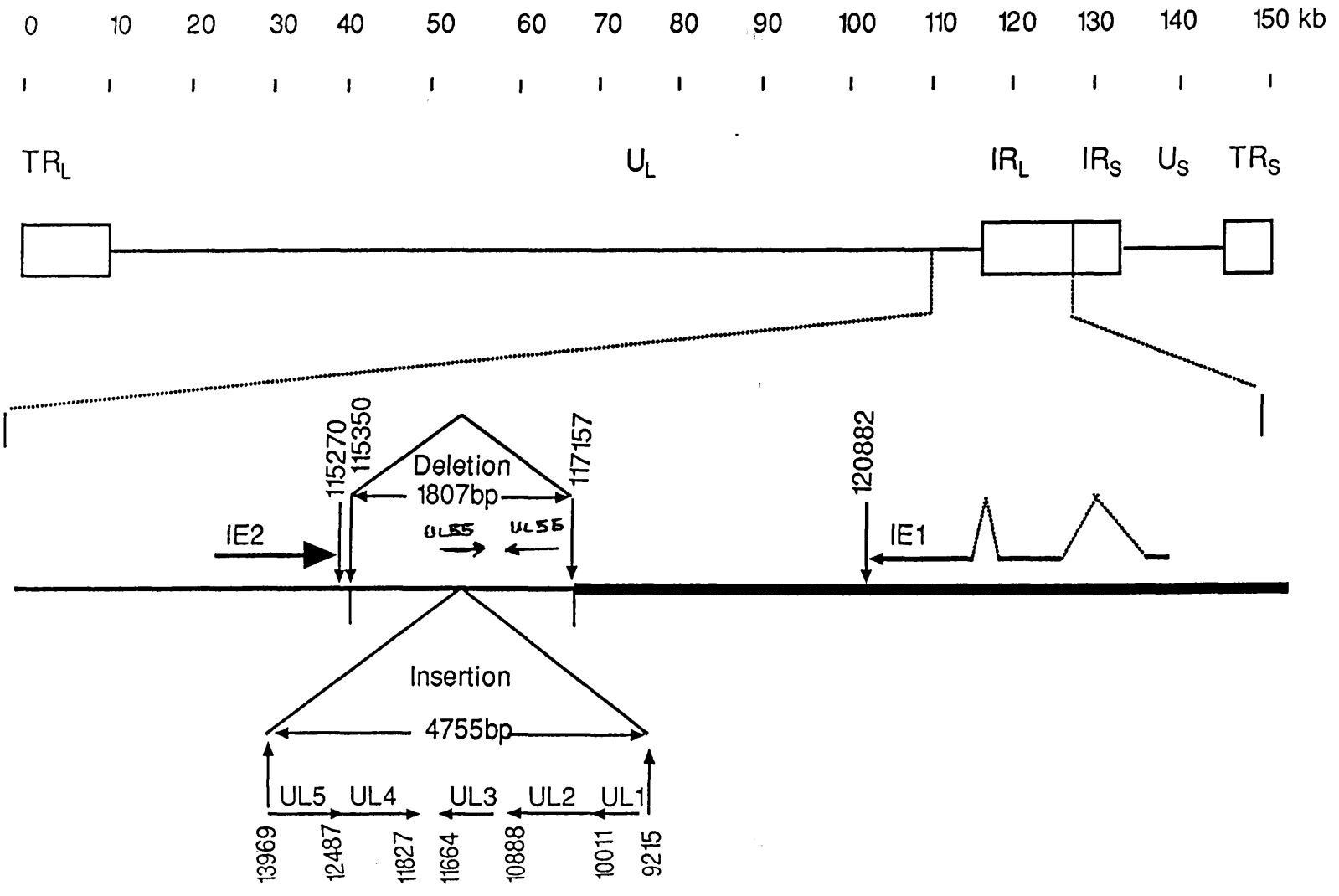
Figure 3.17

A portion of an autoradiograph of a sequencing gel showing deletion and insertion at the right end of U_L in the variant 1706 . The deletion starts at np 115350 and ends at np 117157. The insertion is between np 13969 and np 9215. Please note the inversion of the sequence represented by AGCT at the top and TCGA at the bottom of the gel.


A G C T
CGTCCAGCCGGG
13969
G
T
115350
GGGTGCGGGGC
T C G A

Figure 3.18

Structure of the HSV-1 genome (top line) showing U_L and U_S flanked by TR_L/IR_L and IR_S/TR_S respectively. The second line shows expansion between map position 110kb and IR_L/IR_S junction. Thin and thick lines indicate unique and repeat portions of the long region of the genome. Above the line, the position of 3' end of IE1, 3' end of IE2, the U_L/IR_L junction and the extent of the deletion in the variant 1706 are indicated by the arrows. The extent of the insertion in the variant 1706 is marked below the line showing inversion of the genes UL1,UL2, UL3, UL4 and a partial copy of the gene UL5. Coordinates are given as nucleotide positions (McGeoch *et al.*, 1988).


0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150 kb
TRL
UL
IRL
IRS
US
TRS
115270
115350
Deletion
1807bp
117157
120882
IE2
UL55
UL56
IE1
Insertion
4755bp
UL5
UL4
UL3
UL2
UL1
13969
12487
11827
11664
10888
10011
9215

3.2. VIRULENCE ANALYSIS OF THE DELETION VARIANTS OF HSV-I STRAIN 17 SYN$^+$.

3.2.1. Introduction.

HSV is a neurotropic virus. In humans, invasion of the nervous system frequently leads to a latent infection in sensory ganglia and in rare circumstances fatal encephalitis (Fenner *et al.*, 1974; Johnson, 1982). The virus host relationship is highly complex and influenced by the genotype of both virus and host. Many host factors have been shown to influence virulence including route of inoculation (Caspary *et al.*, 1980), age (Kohl and Loo, 1980) and strain of experimental animal (Lopez, 1975). Virus strain (Dix *et al.*, 1983) and serial passage of virus *in vivo* (Kaerner *et al.*, 1983) and *in vitro* (Goodman and Stevens, 1986) may also effect the virulence. Individual strains of HSV differ in their level of neurovirulence (Dix *et al.*, 1983; Richards *et al.*, 1981). Specific viral factors required for the replication of virus at the periphery and for spread to the central nervous system may be the underlying cause of heterogeneity of individual virus strains. Viral factors playing a part in neuroinvasiveness have been reported (Javier *et al.*, 1986; Kaerner *et al.*, 1983).

Heterogeneity in the neurovirulence of plaque purified stocks of the HSV-2 strain HG52 has been reported (Taha *et al.*, 1988). Although detectable deletions in the genome of the elite stock of HSV-2 strain HG52 were found at a frequency of 24% (Harland and Brown, 1985), DNA profiles of the individual stocks inoculated intracranially had no obvious differences compared to wild type stock (Taha *et al.*, 1988). The variation affecting the phenotype of the virus is perhaps due to minor sequence alterations undectable by conventional restriction enzyme methods.

3.2.2 Neurovirulence of individual stocks of the elite stock of HSV-1 strain 17 syn $^{+}$.

3.2.1.b Isolation of single plaque stocks.

To determine the basis for evaluating neurovirulence, intracranial inoculation of single plaque stocks picked from the HSV-I 17 syn^{+} elite stock were performed.

This stock was plated on BHK-21 C13 cells and overlaid with methyl cellulose. Nine well separated plaques were picked after washing thoroughly with PBS. High titre virus was propagated with one further passage in BHK-21 C13 cell. All the plaque DNAs were *in vivo* radiolabelled with ^{32}P and subjected to restriction endonuclease digestion with *Xba*I (Figure 3.19) and *Hpa*I (Figure 3.20) (see method section 2.14). As a representative sample, the profiles of six of them are shown in Figures 3.19 and 3.20. The results show no apparent difference in the size of the fragments and distribution of sites in the DNA of individual plaque stocks.

3.2.2.b Neurovirulence of single plaque stocks of HSV-I strain 17 syn^{+}.

The nine plaques were separately inoculated intracranially into three week old BALB/c mice with doses ranging from 1-10^{2} pfu/mouse. During this series of experiments no mice died before day three post inoculation. The LD_{50} values were calculated by the method of Reed and Muench (1938). Table 3.5 shows LD_{50} values of the nine plaques stocks . The resuls show that each plaque stock has an LD_{50} similar to the elite parental 17 syn^{+} stock. There is therefore no apparent neurovirulence heterogeneity within the elite

Figure 3.19

Autoradiograph of *Xba*I digest of viral DNA ^{32}P labelled *in vivo* of individual plaque stocks of 17 syn^+ (from left to right 17 syn^+, Plaque Nos. 1, 3, 4, 5 and 6). Letters refer to specific fragments. The DNA products were separated on a 0.5% agarose gel.

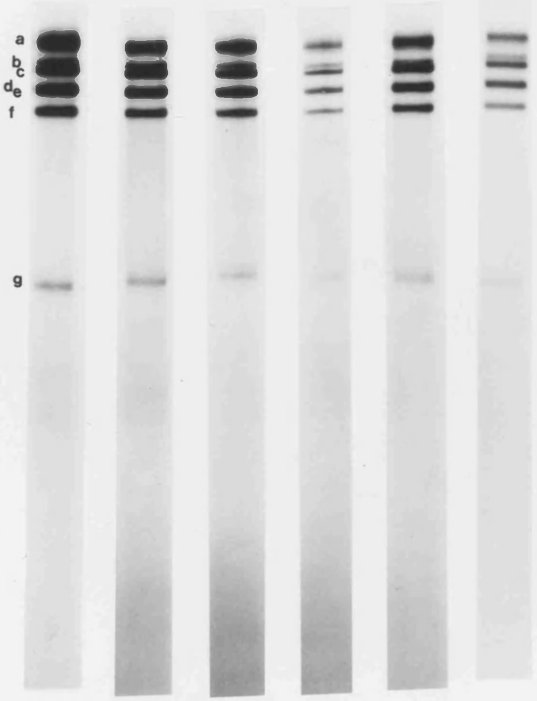

a
b
c
d
e
f
g

Figure 3.20

Autoradiograph of *Hpa*I digest of viral DNA ^{32}P labelled *in vivo* of individual plaque stocks of 17 syn^+ (from left to right, 17 syn^+, plaque Nos. 1, 3, 4, 5, and 6). Letters refer to specific fragments. The DNA products were separated on a 0.8% agarose gel.

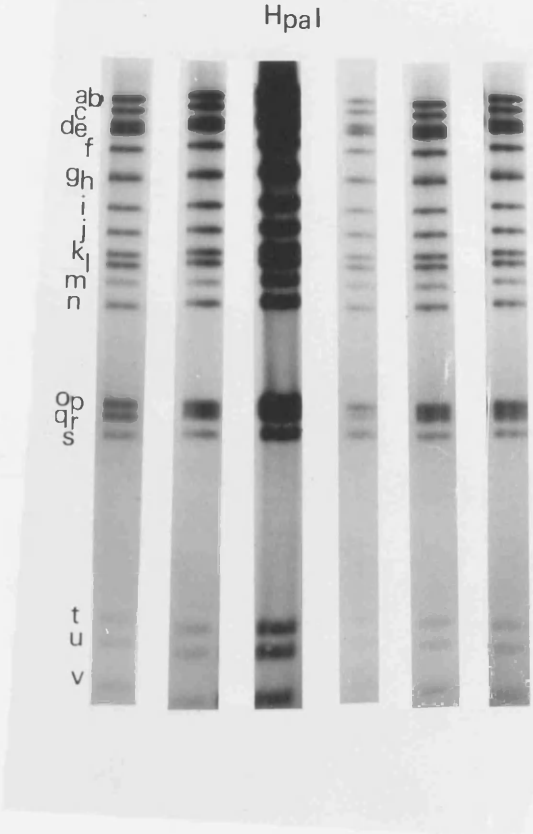

HpaI
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v

Table:3.5 LD_{50} values after intracranial inoculation of individual plaque stocks of 17 syn^{+}.

plaque No.	1pfu*	$1x10^{1}$	$1x10^{2}$	LD_{50} pfu/mouse
1.	0/5**	1/5	4/5	30.2
2.	0/5	5/5	5/5	3.2
3.	1/5	2/5	5/5	15
4.	0/5	2/5	5/5	14
5.	5/5	5/5	5/5	<1
6.	5/5	5/5	5/5	<1
7.	0/5	2/5	3/5	30.2
8.	1/5	4/5	5/5	3.2
9.	3/5	4/5	5/5	<1

* Dose pfu/animal
**Number of deaths/number of animals inoculated
3 week old BALB/c mice were used
0.025ml of virus inoculated

stock of HSV-I strain 17 syn^+.

3.2.3 Virulence of the variants 1704, 1705 and 1706.

To determine the pathogenic potential of the deletion variants, intracranial and foot pad inoculation of the viruses was carried out in three week old BALB/c mice.

3.2.3.a Intracranial inoculation.

Groups of 5 to 10, three week old BALB/c mice were inoculated with 1, 10, 10^2, 10^3, 10^4 and 10^5 pfu/ mouse of each variant. 0.025ml of each dilution was inoculated intracranially into the left cerebral hemisphere after anaesthesia (see method section 2.11). Death due to virus replication does not normally occur before day 3 post inoculation. Any death occurring before day three is assumed to be due to mechanical injury to the vascular or the neuronal system of the animal. The 50% lethal dose (LD_{50}) is calculated by the formula of Reed and Muench (1938) on the basis of death occurring between day three and day twenty one post inoculation and is the dose calculated to kill 50% of the infected animals. Clinical signs produced in mice following intracranial inoculation are closely observed. Some mice become hunched with ruffled fur and cease to be active with death following rapidly. The mice also show a wide range of neurological signs e.g fits and limb paralysis of varying severity. Mice showing severe neurological signs are killed immediately. The LD_{50} values of the variants compared to the wild type virus are shown in Table 3.6. It can be seen that 17 syn^+ had an LD_{50} of 10 pfu/mouse. 1704 an LD_{50} of 2×10^2 pfu/mouse, 1705 an LD_{50} of 11 pfu/mouse and 1706 an LD_{50} of 4.6×10^3 pfu/mouse. The variant 1705 was therefore not different from 17 syn^+ but 1704 and 1706 were 20 fold and 460 fold less neurovirulent

Table: 3.6 LD50 values after intracranial inoculation of 17 syn^+, 1704, 1705 and 1706.

Virus	1*	$1x10^1$	$1x10^2$	$1x10^3$	$1x10^4$	$1x10^5$	LD_{50} pfu/mouse
17^+	4/10**	5/10	9/10	5/5	ND	ND	10
1704	2/10	5/15	6/15	11/15	9/10	10/10	$2x10^2$
1705	2/15	7/15	15/15	5/5	5/5	5/5	11
1706	0/5	0/5	2/10	3/10	6/10	10/10	$4.6x10^3$

*Dose pfu/animal

**Number of deaths/number of animals inoculated.

N.D: Not done.

3 week old BALB/c mice were used.

0.025ml of virus inoculated.

respectively.

To determine whether the differences in the LD_{50} values of the virus stocks were related to the number of virus particles within the stocks, particle counts were performed (see method section 2.7) and the particle:pfu ratios are shown in Table 3.7. All the virus stocks had particle:pfu ratios within the acceptable range for HSV-I.

3.2.3.b Foot pad inoculation.

To determine the general pathogenicity of the variants, peripheral inoculation via the foot pads of BALB/c mice was carried out. In this system 0.025 ml of serially diluted virus from 10^3 to 10^7 pfu/mouse was inoculated in the left rear foot pad of three week old BALB/c mice. Following inoculation at the peripheral site , the virus replicates and travels via the sciatic nerve and dorsal root ganglia (DRG) to the spinal cord. It can become latent in the DRG or replicate and spread to the central nervous system thereby causing death. Clinial signs are similar to those seen following intracranial inoculation but limb paralysis is a common sequela following high dose inoculation in the foot pad. Table 3.8 shows that 17 syn^+ and 1705 are comparable with LD_{50} values of approximately 10^5 pfu/mouse. Doses of 10^7 pfu/mouse of 1704 and 1706 were not able to kill any of the mice. It was not possible to infect with a dose higher than 10^7 pfu/mouse.

3.2.3.c Intraperitoneal inoculation.

The HSV-I strain HFEM has a 4.1 kb deletion in the *Bam*HI *b* (Ben-Hur *et al.*, 1989). This virus has been shown to be avirulent on intraperitoneal inoculation for tree shrews and mice (Rosen and Darai., 1985, Becker *et al.*, 1986) and its avirulence was attributed to the deletion. The strain 17 syn^+ variant 1705 has a 4.7

Table: 3.7 Particle:pfu ratio of 17 syn^+, 1704, 1705 and 1706.

Virus	Particle: pfu
17^+	72:1
1704	64:1
1705	46:1
1706	125:1

Table: 3.8 LD_{50} values after footpad inoculation of 17 syn^+, 1704, 1705 and 1706.

Virus	$1x10^{3*}$	$1x10^4$	$1x10^5$	$1x10^6$	$1x10^7$	LD_{50} pfu/mouse
17^+	ND	3/10**	4/10	9/10	ND	$2x10^5$
1704	0/5	0/15	0/15	0/15	0/15	$>1x10^7$
1705	0/5	0/15	8/15	15/15	15/15	$9x10^4$
1706	0/5	0/5	0/5	0/10	0/5	$>1x10^7$

*Dose pfu/animal

**Number of deaths/number of animals inoculated.

N.D: Not done.

3 week old BALB/c mice were used.

0.025ml of virus inoculated.

kb deletion in a similar region and hence its intraperitoneal virulence has been tested.

Three week old BALB/c mice were inoculated IP with doses between 10^2-10^5 pfu/mouse. The results show (Table 3.9) that the LD_{50} for 1705 is $2.4x10^3$ pfu/mouse and for 17 syn^+ $1.4x10^3$ pfu/mouse, demonstrating that in HSV-I strain 17 syn^+ intraperitoneal virulence in mice is not associated with the deletion in the *Bam*HI *b* fragment.

3.2.4 Replication efficiences of 1704 and 1706 *in vivo*.

The possibility that the observed differences in the LD_{50} values of 17 syn^+, 1704 and 1706 following foot-pad inoculation were due to differential abilities to replicate in the mouse peripheral nervous system was tested. Three week old BALB/ c mice were inoculated separately in the left rear foot pad with 17 syn^+ and 1706 at an input dose of 10^5 pfu/mouse and 1704 at an input doses of 10^5 and 10^7 pfu/mouse. At daily intervals animals were sacrificed and nine DRG (one thoracic, six lumbar and two sacral) from the left side of the spinal cord were explanted, homogenised and assayed for virus by titration on BHK-21 C13 cells at 37°C. The results in Figure 3.21 show that the replication efficiency of 1704 and 1706 is poor compared to wild type. At an input dose of 10^5 pfu/mouse 1704 and 1706 titres reached their peak on day three and on the day five no virus was detectable. At an input dose of 10^7 pfu/mouse the variant 1704 was detected by day one post inoculation and continued to increase in titre to the third day, but no virus was detectable by day five. In contrast wild type growth reached its peak on the third day post inoculation, maintained its replication to day four and was detectable until day six. These results indicate that, the growth of 1704 and 1706 is considerably impaired in the peripheral nervous

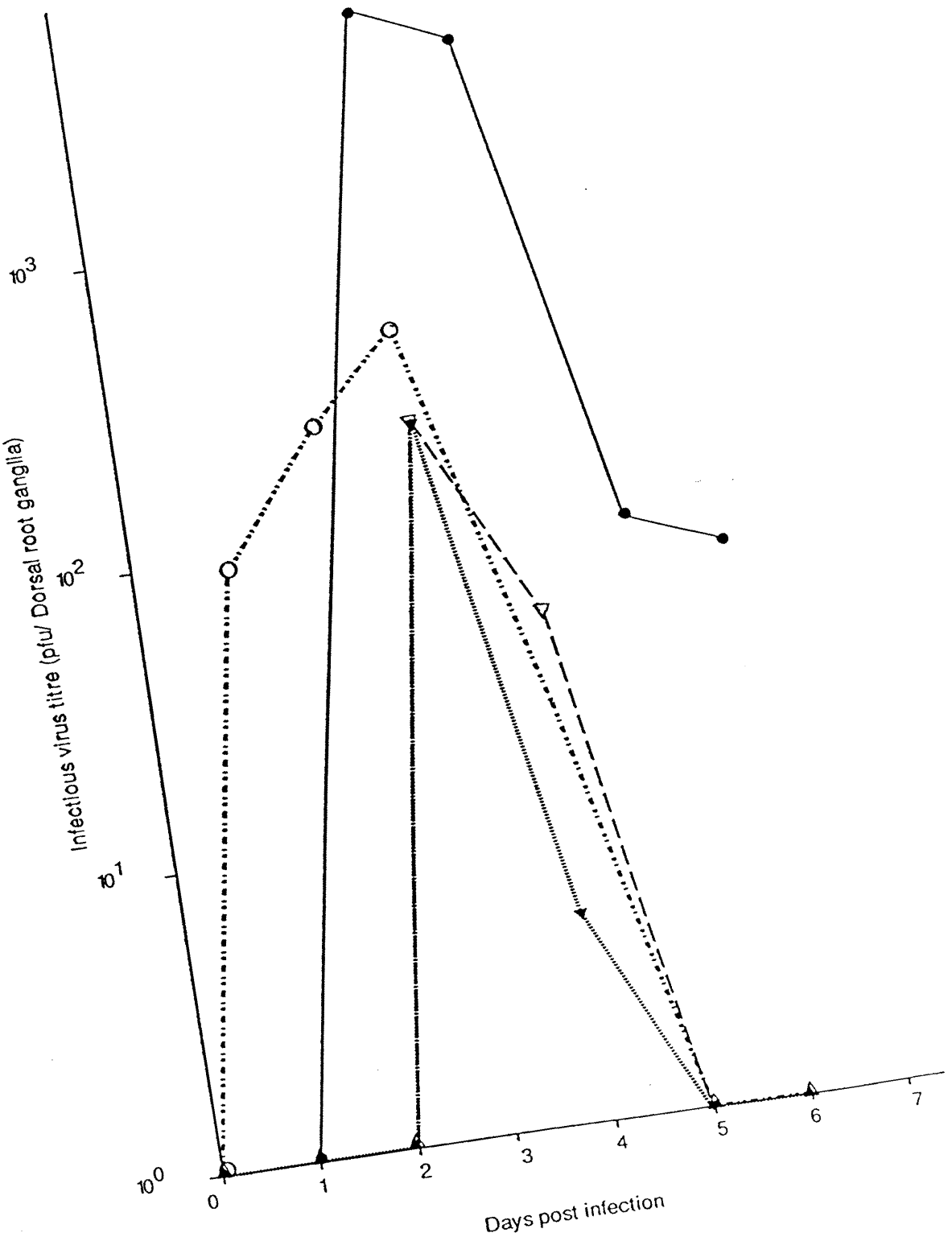
Table: 3.9 LD_{50} values after intraperitoneal inoculation of 17^{+} and 1705.

Virus	$1x10^{2*}$	$1x10^{3}$	$1x10^{4}$	$1x10^{5}$	LD_{50} pfu/mouse
17^{+}	0/5**	2/5	5/5	5/5	$1.4x10^{3}$
1705	0/5	1/5	5/5	5/5	$2.4x10^{3}$

*Dose pfu/animal
**Number of deaths/number of animals inoculated
3 week old BALB/c mice were used.
0.025ml of virus inoculated.

Figure 3.21

In vivo growth kinetics of HSV-1 strain 17 syn^+, 1704 and 1706. Three week old BALB/c mice were inoculated in the left rear footpad with 25ul of each virus with 17^+ (●), 1704 (▼) and 1706 (▽) at the dose 10^5 pfu/mouse and 1704 (○) at the dose 10^7 pfu/mouse. At indicated times post infection, two mice from each time point were killed. The DRGs from the left side of the spinal cord were removed and homogenised; the resulting cell suspension was sonicated and released infectious virus titrated on BHK-21 C13 cells at 37^oC.


Infectious virus titre (pfu/ Dorsal root ganglia)
10^3
10^2
10^1
10^0
0
1
2
3
4
5
6
7
Days post infection

system of the mouse, which could explain the avirulence of 1704 and 1706 following foot pad inoculation.

3.3. LATENCY ANALYSIS OF DELETION VARIANTS OF HSV-1 STRAIN 17 SYN$^+$.

3.3.1. Introduction.

Like other members of the α-herpesviridae family HSV-1 frequently establishes latent infections in sensory neurons from which it reactivates periodically (Cook *et al.*, 1974, MacLennan and Darby., 1980). In humans, invasion of the nervous system leads to latent infection in sensory ganglia and in rare circumstances to fatal encephalitis (Fenner *et al.*, 1974; Javier *et al.*, 1986). Several studies have been carried out to define the role of specific viral genes in latency. Specific transcripts (LATs) are abundantly expressed during latent infection of the virus and the molecular basis of this phenomenon has been studied in several animal models and sero-positive human cadavers (Croen *et al.*, 1987, Stevens *et al.*, 1987; Steiner *et al.*, 1988). At least three transcripts, 2.0, 1.5 and 1.45kb have been detected by Northern blot hybridisation and have been finely mapped. These transcripts are diploid and transcribed complementary to the IE1 gene transcripts (Spivak and Fraser, 1987, Wagner *et al.*, 1988; Wechsler *et al.*, 1988). The proposed promoter region which unusually is 686bp upstream from the 5' end of the LATs has proved to be a strong promoter both *in vivo* and *in vitro* and is neurospecific (Dobson *et al.*, 1989; Zwaagstra *et al*,. 1989, 1990). The presence of immediate early or early mRNA is not a prerequisite of LAT promoter expression in vitro (Zwaagstra *et al.*, 1989). The role of LATs in the establishment and maintenance of latency is obscure, but there is evidence that it may affect viral

reactivation (Dobson *et al.*, 1989; Leib *et al.*, 1989; Steiner *et al.*, 1989).

The behaviour of HSV-1 strain 17 syn^+ and the variant 1704 has been studied by Steiner *et al* (1989) in trigeminal ganglia of mice during acute infection, latent infection and explant reactivation following primary infection of the eye. The variant 1704 replicated in the trigeminal ganglia of infected mice to the same extent as 17 syn^+ and established latency in almost all of the infected animals. However, following trigeminal ganglia explant the reactivation kinetics were significantlly delayed for 1704 relative to 17 syn^+. HSV-1 LATs could not be detected with 1704 either by Nothern blot or by *in situ* hybridisation, suggesting a role for LATs in reactivation from latency.

From the sequencing analysis described in section 3.1, it can be seen that in the variant 1704, both copies of the LATs promoter region and one copy of the LAT coding region in IR_L is deleted. In 1705 only the IR_L copy of the LATs promoter region and the LAT coding region is deleted. 1706 has no deletion in the LAT gene. As a comparative analysis of latency of the variants 1704, 1705 and 1706 we have used the well established mouse foot pad model system, to investigate latency in the DRG of the spinal cord in 3 week old BALB/c mice.

3.3..2. Latency analysis of the deletion variants, 1704, 1705 and 1706.

Three week old BALB/c mice were inoculated separately in the left rear foot pad with 17 syn^+, 1704, 1705 and 1706. Six weeks post inoculation nine DRG (one thoracic, six lumbar and two sacral) from the left side of the spinal cord were explanted and transferred to individual microtitre plates containing ECS50. Screening for

release of reactivated virus was carried out by transferring the culture medium from individual microtitre wells to flat bottom microtitre plate wells containing semiconfluent BHK-21 C13 cells and incubated at 37°C. Released virus was detected by the appearence of cpe in the indicator BHK-21 C13 cells.

The results for 17 syn^+, 1705 and 1706 in Figure 3.22 show that at input doses of 10^5 pfu/mouse, 17 syn^+ and 1705 infected ganglia started reactivating at 5 and 6 days post-explantation and by day 10, 50% of the ganglia from each group of animals had reactivated i.e 28/54 ganglia for 17 syn^+ and 27/54 for 1705. The ganglia from 1706 infected animals also started to reactivate at about the same time but by the 10th day post inoculation only 16% i.e 6/36 ganglia were positive for virus release.

With 1704 infected animals at an input dose of 10^5 pfu/mouse there was no reactivation until day 12 and even then only 1/36 ganglia (3%) reactivated with no increase in number up to 22 days post explantation (Figure 3.23). The control group of ganglia from 17 syn^+ infected animals started reactivating on day 5 and began to plateau at 33% on day 7.

To determine whether this delay and low reactivation frequency with 1704 was dose dependent, a group of animals were infected with an input dose of 10^7 pfu/mouse. The input dose of 17 syn^+ could not be increased as all of the animals would have died. It can be seen in Figure 3.24 that at a 10^7 pfu input dose 1704 started reactivating on day 7 (4/36 ganglia), plateaued by day 12 and reached a final value of 30% (11/36 ganglia) by day 18. The 17 syn^+ infected ganglia started reactivating on day 5 i.e 5/18 positive and reached a final value of 72% of ganglia releasing virus (13/18) by day 18.

Figure 3.22

Explant reactivation time course of HSV-1 strain 17 syn^+ (□), 1705 (◆) and 1706 (■). Three week old BALB/c mice were inoculated into the left rear footpad with 25ul of each virus at a dose of 10^5 pfu/mouse. Six weeks later, mice were killed and the DRGs from the left side of the spinal cord were removed and cultured in ECS50. The supernatant was overlaid on indicator BHK-21 C13 cells. A ganglion was scored positive for reactivation when cytopathic effect was detected on the BHK21-C13 cell monolayers. Data given as percentage of reactivation at each time point.

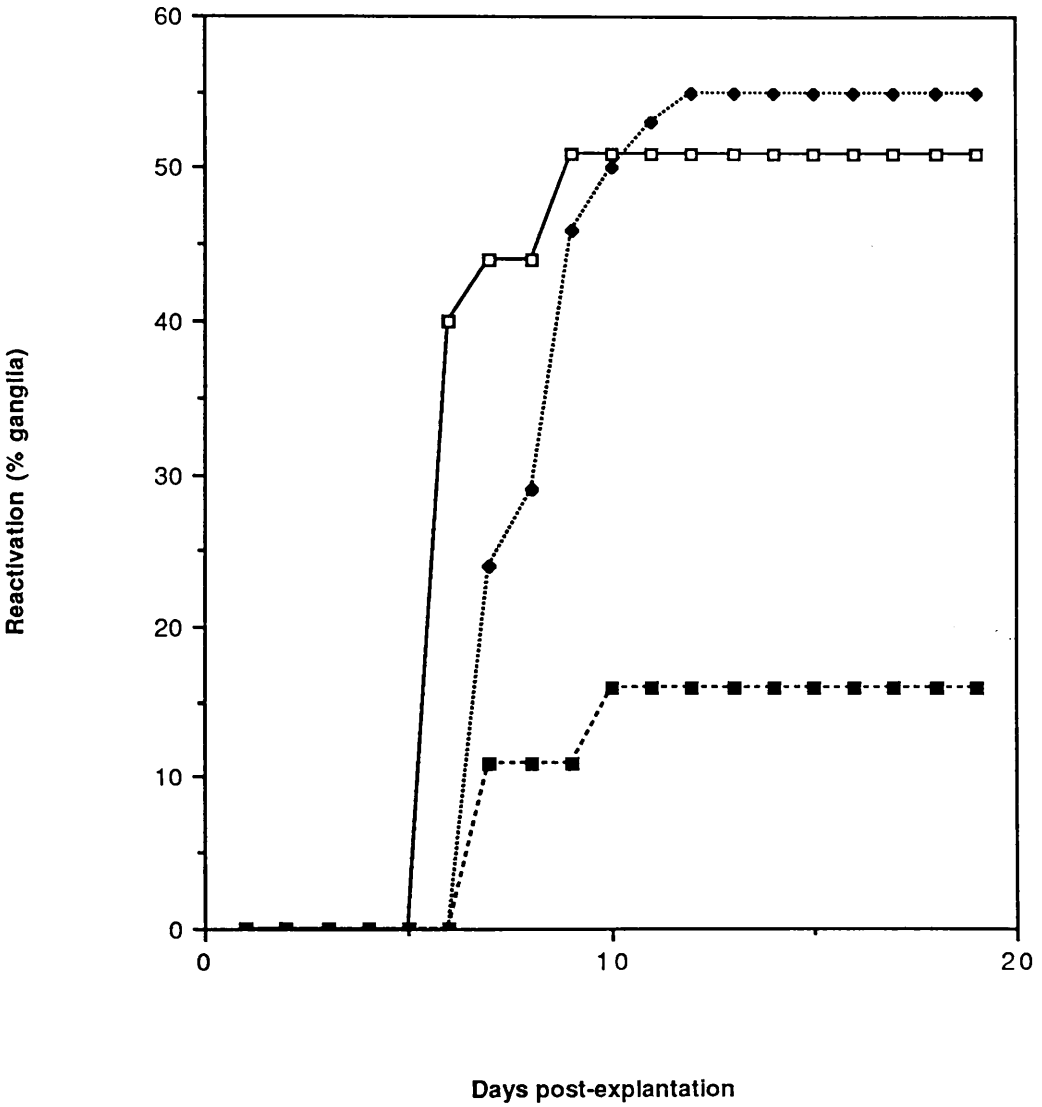

60
50
40
30
20
10
0
0
10
20
Reactivation (% ganglia)
Days post-explantation

Figure 3.23

Explant reactivation time course of HSV-1 strain 17 syn^+ (□) and the variant 1704 (■). Three week old BALB/c mice were inoculated into the left rear footpad with 25ul of each virus at a dose of 10^5 pfu/mouse. Six weeks later mice were killed and the DRGs from the left side of the spinal cord were removed and cultured in ECS50. The supernatant was overlaid on indicator BHK-21 C13 cells. A ganglion was scored positive for reactivation when cytopathic effect was detected on the BHK21-C13 cell monolayers. Data given as percentage of reactivation at each time point.

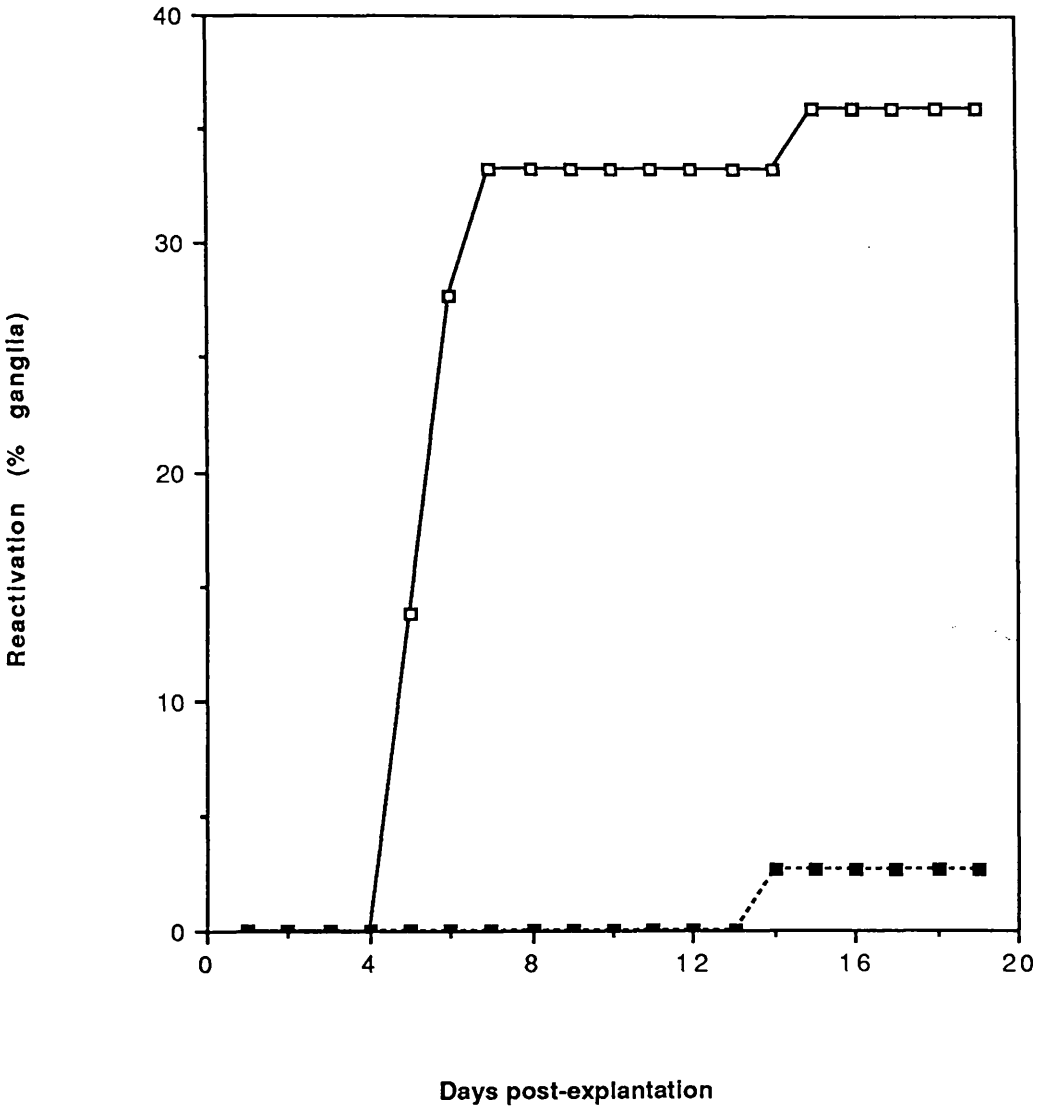
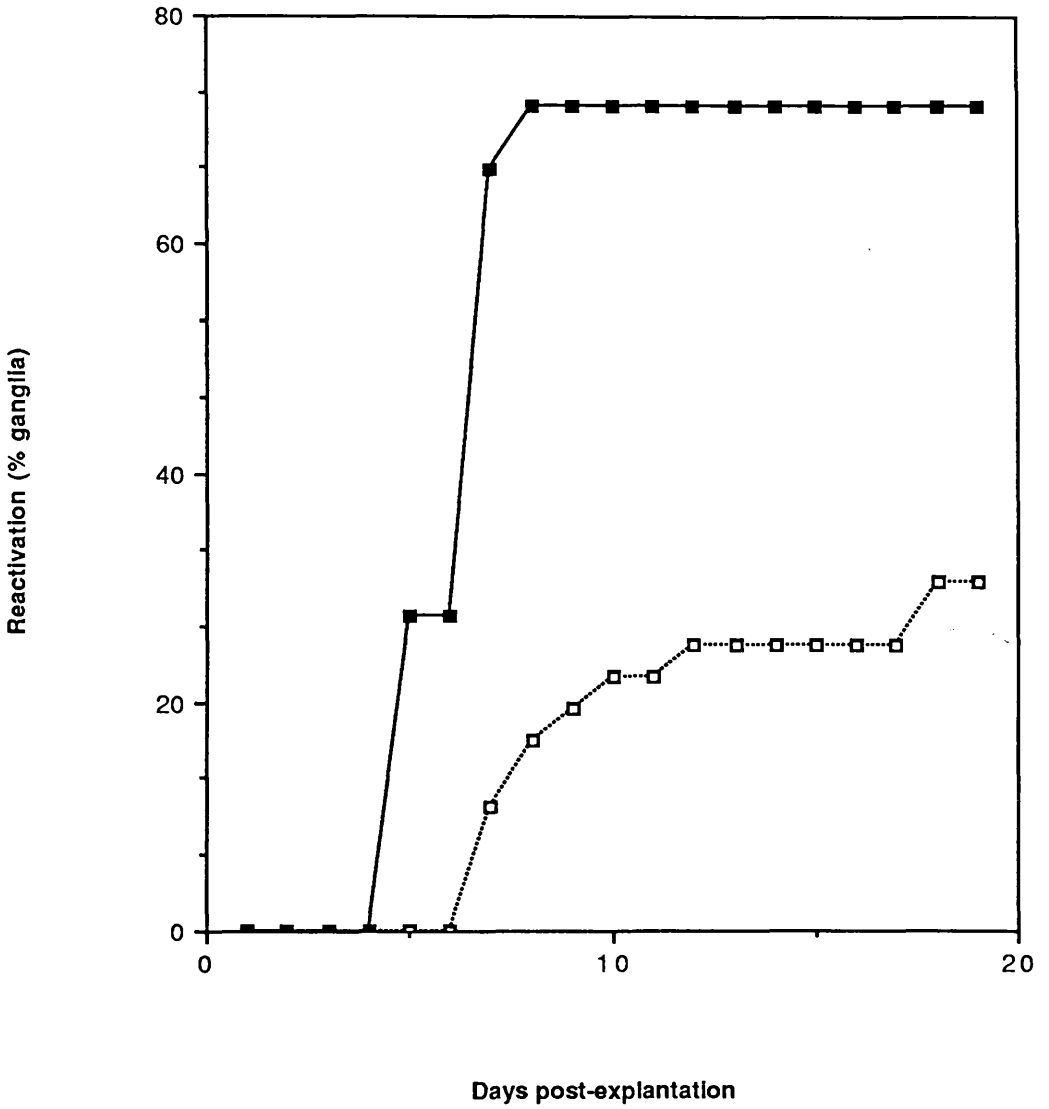

40
30
20
10
0
0
4
8
12
16
20
Reactivation (% ganglia)
Days post-explantation

Figure 3.24

Explant reactivation time course of HSV-1 strain 17 syn^+ (■) and the variant 1704 (□). Three week old BALB/c mice were inoculated into the left rear footpad with 25ul of virus at a dose of 10^5 pfu/mouse of 17^+ and 10^7 pfu/mouse of 1704. Six weeks later mice were sacrificed and the DRGs from the left side of the spinal cord were removed and cultured in ECS50. The supernatant was overlaid on indicator BHK-21 C13 cells. A ganglion was scored positive for reactivation when cytopathic effect was detected on the BHK21-C13 cell monolayers. Data given as percentage of reactivation at each time point.


80
60
40
20
0
0
10
20
Reactivation (% ganglia)
Days post-explantation

3.3.3. Genome analysis of reactivated 1704,1705 and 1706.

To determine if there was any change in the genomic structures of the variant 1704, 1705 and 1705 following inoculation in the mouse foot pad and reactivation from latency, a plate stock was grown from a representative plaque of each variant, its DNA extracted, radiolabelled with ^{32}P and subjected to restriction endonuclease digestion with HpaI and *Bam*HI. The results are shown in Figure 3.25. The DNA profiles of the reactivated variants compared with the input viruses show no apparent differences.

3.4. CORRECTION OF THE DELETION IN THE VARIANT 1704.

To determine whether the phenotype of 1704 was entirely due to the deleted sequences it was necessary to correct the deletion in TR_L and IR_L and determine whether the phenotype of the resultant recombinant was that of the parental virus. To do this, unit length 1704 genomes and the *Bam*HI *b* restriction endonuclease fragment spanning the deletion from the 17 syn^+ genome were cotransfected onto BHK-21 C13 cells. The progeny virus was titrated and individual plaques were picked. Their DNAs were analysed by restriction endonucleases using the method of Lonsdale (1979). The strategy is outlined in Figure 3.26. Recombinant viruses were identified, plaque purified and their DNA profiles, pathogenicity and latency potential determined.

The deletion in 1704 was shown to be within *Hpa*I *o* in TR_L and within *Hpa*I *v* and *r* in U_L/IR_L (Figures 3.1 and 3.2). The *Bam*HI *b* fragment covers the *Hpa*I *s*, *v*, *r* and *m* fragments (Figure 3.2). The selection of this particular fragment for marker rescue experiments was based on the assumption that if recombination occurs in IR_L the deletion will also be corrected in TR_L. Also the larger

Figure 3.25

Autoradiograph of restriction enzyme digests of viral DNA ^{32}P labelled *in vivo* of reactivated virus recovered from DRG of BALB/c mice. *Hpa*I (lanes 1 to 5) and *Bam*HI (lanes 6 to 10) of 17 syn^+ (lanes 1 and 6) reactivated 17 syn^+ (lanes 2 and 7), reactivated 1704 (lanes 3 and 8), reactivated 1705 (lanes 4 and 9) and reactivated 1706 (lanes 5 and 10). Letters refer to specific fragments, arrowheads indicate the position where the fragments are missing and stars indicate the novel fragments.

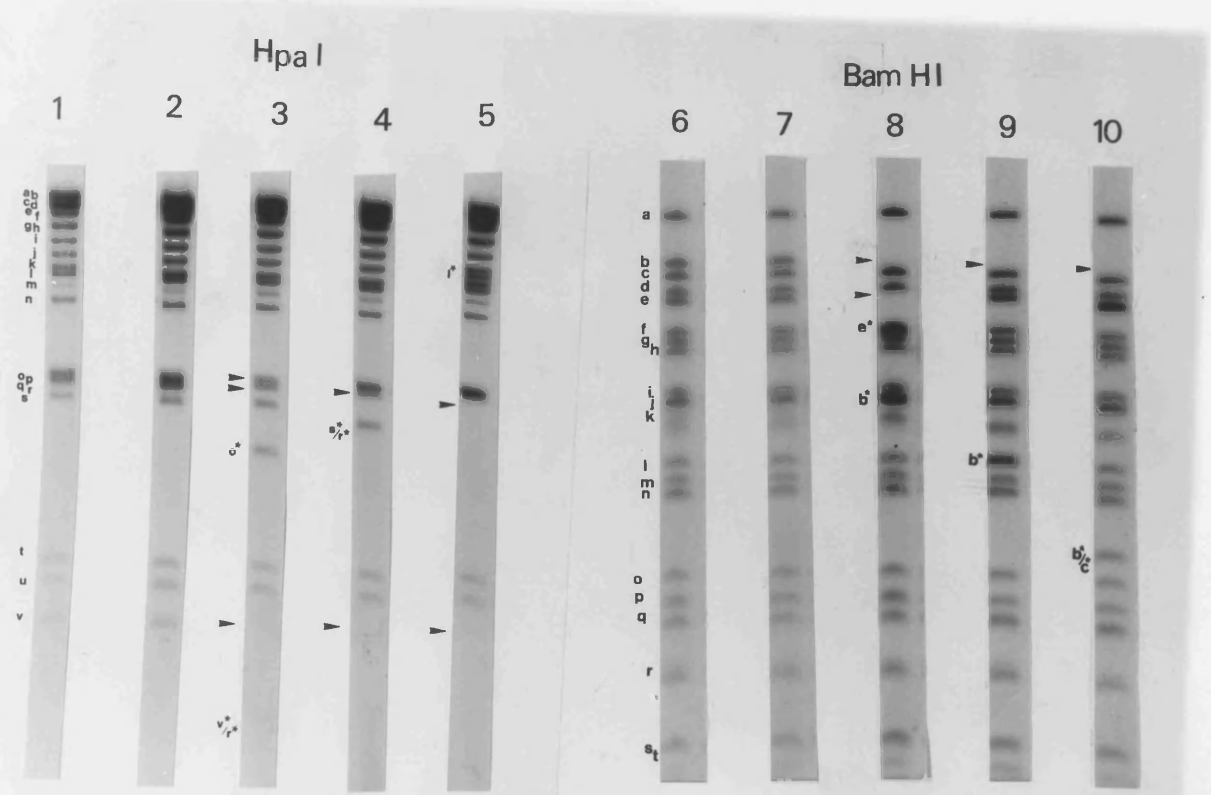
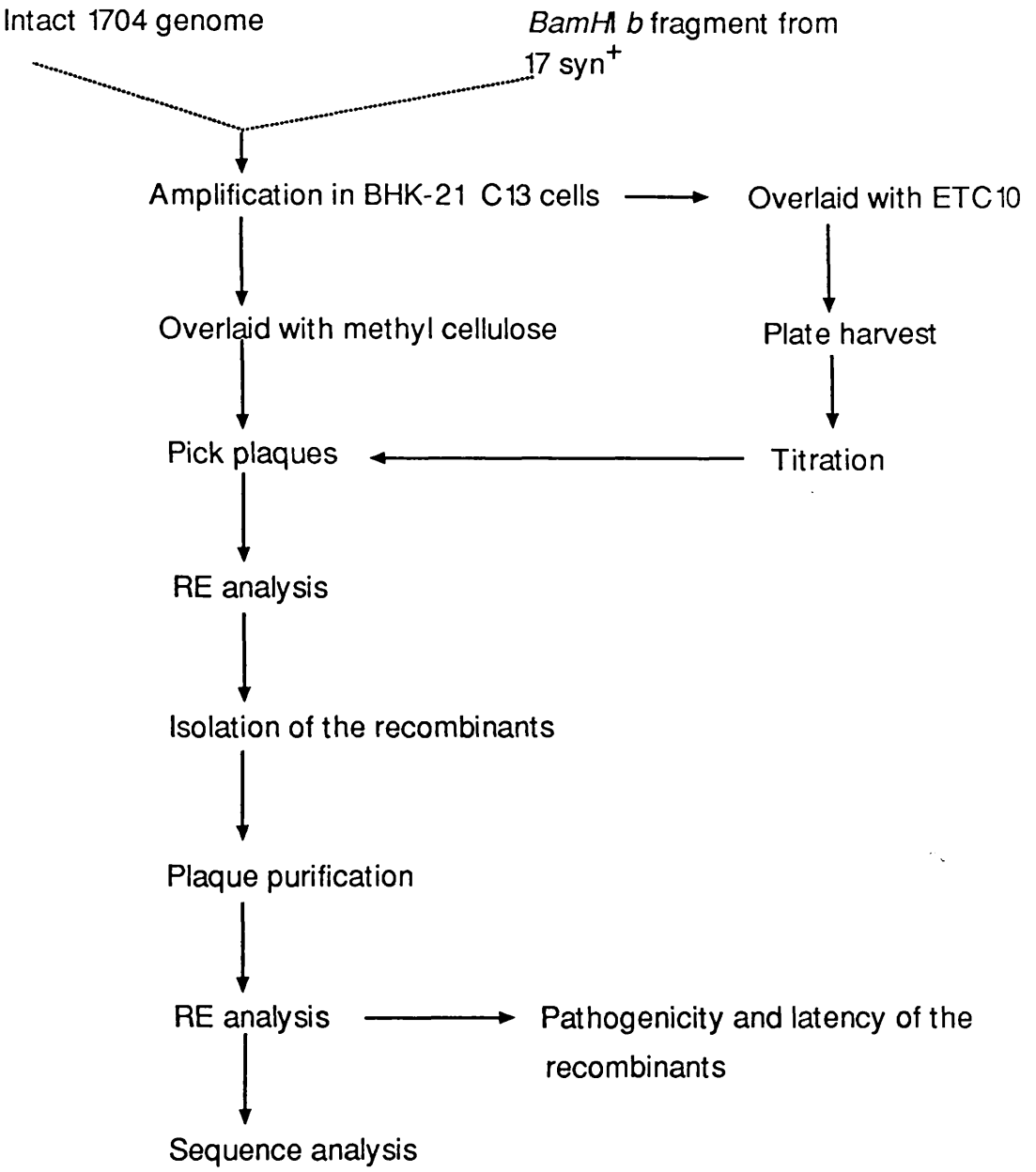

Hpa I
Bam HI
1
2
3
4
5
6
7
8
9
10

Figure 3.26

Strategy employed for the correction of the deletion in the variant 1704.


Intact 1704 genome
*BamH*I *b* fragment from 17 syn+
Amplification in BHK-21 C13 cells
Overlaid with ETC10
Overlaid with methyl cellulose
Plate harvest
Pick plaques
Titration
RE analysis
Isolation of the recombinants
Plaque purification
RE analysis
Pathogenicity and latency of the recombinants
Sequence analysis

*Bam*HI *b* fragment was used to facilitate recombination between the fragment and the intact genome (the larger the fragment the greater the chances of recombination occurring) as no selection system was available . In cotransfection experiments the fragment was used at a 5, 10 and 20 fold molar excess to the intact 1704 DNA. After three days incubation only the 20 fold molar excess plates had plaques. Seventy plaques were picked and the DNAs of 66 of them were analysed with *Bgl*II. Recombination between *Bam*HI *b* and the intact 1704 genome will be demonstrated on *Bgl*II digestion by the appearance of the *f* band in the wild type position, the return of the *j* band to its normal position and also the reappearance of the joint fragment *a (f+j)* (Figure 3.2).

Four isolates showed *Bgl*II profiles identical to that of wild type 17 syn^+ hence indicating that recombination correcting the deletion in 1704 had taken place. The recombinant was designated as 1704R. Figures 3.27 and 3.2 show the *Bgl*II profile of a recombinant compared to wild type and 1704. The DNA of the plaques digested with *Bam*HI and *Hpa*I confirm their structure. Digestion of 1704 DNA with *Bam*HI (Figures 3.28 and 3.2) shows that the *b* fragment is reduced by 2.5×10^6 Mr and the *e* fragment by 0.7×10^6 Mr. In 1704R the *b* fragment was running at the wild type position as was the *e* fragment.

On *Hpa*I digestion 1704R *Hpa*I *v*, *r* and *o* were all running in the wild type position indicating that the deletion both in U_L/IR_L and TR_L has been corrected (Figure 3.29 and 3.2).

3.5. ISOLATION OF THE VARIANT 1704 LP⁻

Of the 66 plaques analysed from the transfection of 1704 DNA+*Bam*HI *b* of 17 syn^+, one showed a DNA profile which indicated that there was partial correction of the deletion in U_L/IR_L

Figure 3.27

Autoradiograph of *Bgl*II digest of viral DNA ^{32}P labelled *in vivo* of 17 syn^+ (lane 1), 1704 (lane 2) and 1704R (lane 3). Letters refer to specific fragments, arrowheads indicate the position where fragments are missing and stars indicate novel fragments. The DNA products were separated on a 0.6% agarose gel.

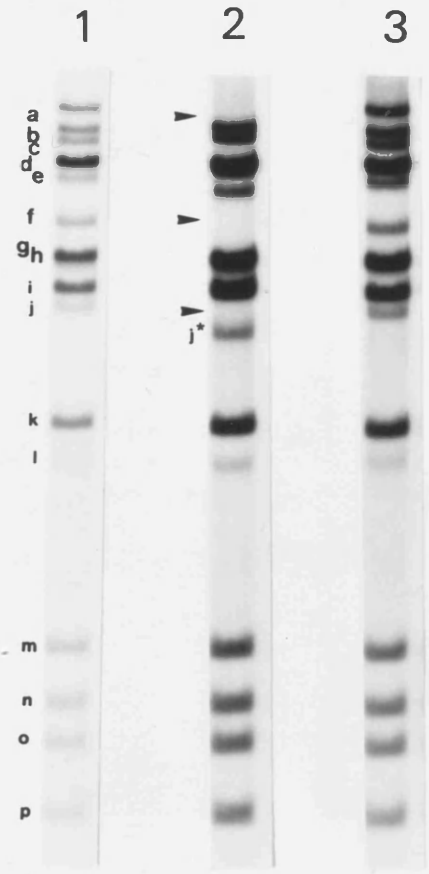

1
2
3
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

Figure 3.28

Autoradiograph of *Bam*HI digest of viral DNA ^{32}P labelled *in vivo* of 17 syn^+ (lane 1), 1704 (lane 2),1704R (lane 3) and 1704LP$^-$(lane 4). Letters refer to specific fragments, arrowheads indicate the position where fragments are missing and stars indicate novel fragments. The DNA products were separated on a 0.8% agarose gel.

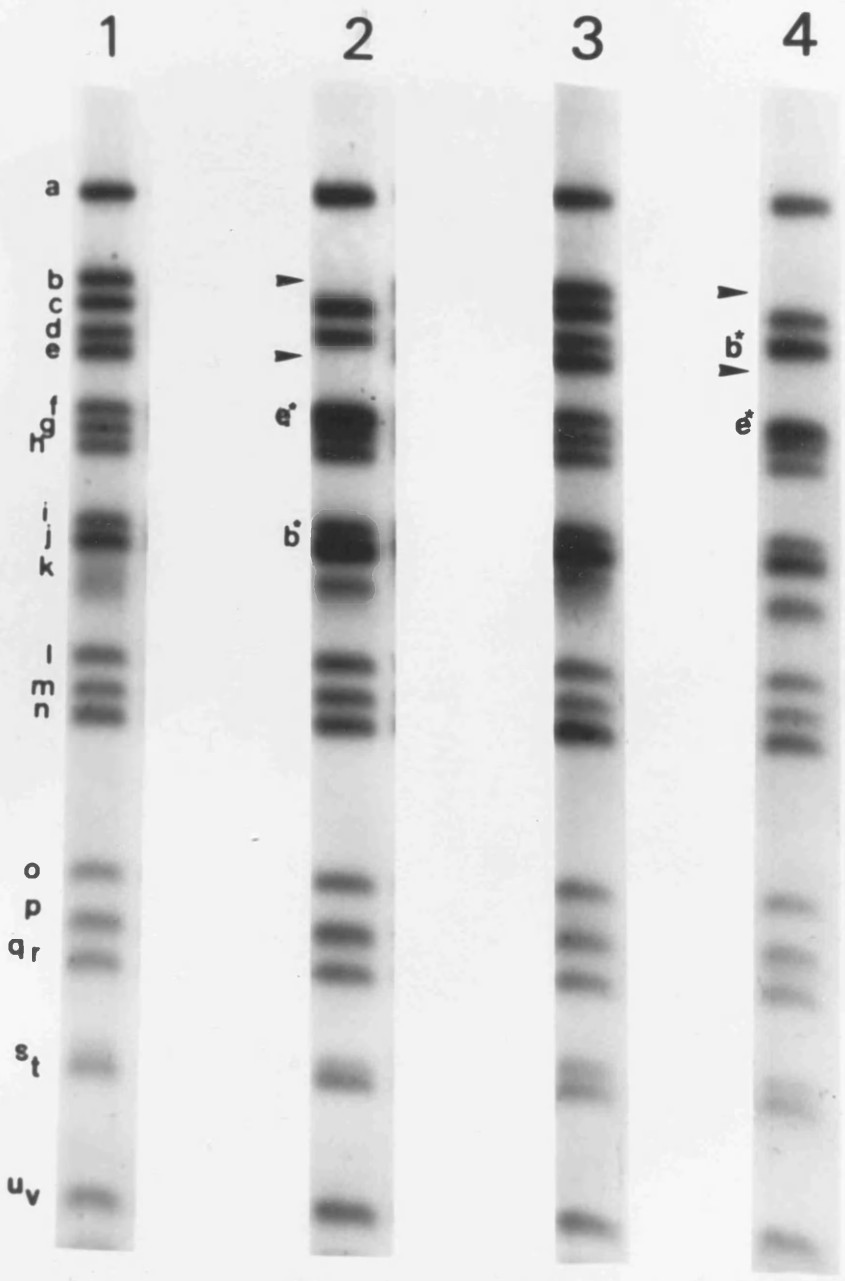

1
2
3
4
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v

Figure 3.29

Autoradiograph of *Hpa*I digest of viral DNA ^{32}P labelled *in vivo* of 17 syn+ (lane 1), 1704 (lane 2),1704R (lane 3) and 1704LP$^-$ (lane 4). Letters refer to specific fragments, arrowheads indicate the position where fragments are missing and stars indicate novel fragments. The DNA products were separated on a 0.8% agarose gel.


1
2
3
4
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
o*
o*
r*

and in TR_L it had not been corrected at all. This was shown by the appearance of the *f* and *a* bands on *Bgl*II digestion running lower than the equivalent wild type bands (Figure 3.30). The *Bgl*II *j* fragment which had been reduced by $0.7x10^6$ Mr in 1704 was running at the same position as in 1704 DNA showing that the deletion has not been corrected in TR_L. Overall the restriction profile indicated an approximately $0.7x10^6$ Mr deletion both in TR_L and IR_L. After three rounds of plaque purification this was further confirmed by *Hpa*I and *Bam*HI digestion.

*Hpa*I digestion (Figures 3.29 and 3.2) showed that the *Hpa*I *o* fragment was deleted by $0.7x10^6$ Mr and was running below *s*. The *s* and *v* fragments were unaltered indicating that the deletion involving the U_L part of 1704 had been corrected, *m* was unaltered and *r* was also reduced by $0.7x10^6$ Mr and was running below *o*. The *v* fragment was running in the wild type position showing that the deletion is confined to *r* only.

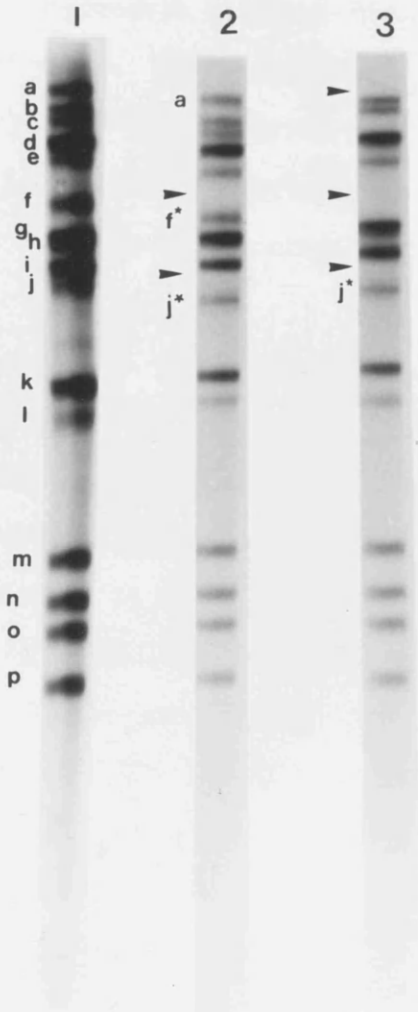
A *Bam*HI digest showed that the *e* band was reduced by $0.7x10^6$ Mr and was comigrating with *f*, while the *b* band was reduced by the same amount and was comigrating with *c*, suggesting that the deletion in IR_L and TR_L is probably identical (Figures 3.28 and 3.2).

3.5.1. Fine mapping of the deletion in the variant 1704 LP$^-$

Fine mapping of the deletion in TR_L and IR_L has been carried out by Southern blotting using an 18-mer synthetic oligonucleotide as the probe. As the 1704 *Hpa*I *o** fragment has been sequenced across the deletion (see section 3.1.2.d), the oligonucleotide was selected from the *Hpa*I *o** fragment of 1704 from np 7194-7202 together with np 8145-8154 i.e nine base pairs from each end point of the deletion. The sequence of this oligonucleotide

Figure 3.30

Autoradiograph of *Bgl*II digest of viral DNA ^{32}P labelled *in vivo* of 17 syn^+ (lane 1), $1704LP^-$ (lane 2) and 1704 (lane 3). Letters refer to specific fragments, arrowheads indicate the position where fragments are missing and stars indicate novel fragments. The DNA products were separated on a 0.6% agarose gel.


1
2
3
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
a
f*
j*
j*

is shown in Figure 3.31. The oligonucleotide was designated as 'E'. The logic behind choosing the sequence from 1704 *Hpa*I o* is that if 1704LP$^-$ shares identical deleted sequences as those within the 1704 TR_L deletion, the novel *Hpa*I *o** and *r** fragments should show positive hybridisation with oligonucleotide 'E'. The oligonucleotide 'E' probe was allowed to hybridise to *Hpa*I digested Southern blots of 1704, 1704LP$^-$ and 17 syn$^+$. Oligonucleotide 'E' showed positive hybridisation with the *Hpa*I *o** and *r** bands of 1704LP$^-$ and *Hpa*I *o** of 1704 (Figure 3.32). Since these sequences were not present in 17 syn$^+$ the oligonucleotide 'E' failed to hybridise to the wild type *Hpa*I *o* band.

In order to confirm that the deletion in the variant 1704LP$^-$ is exactly as in 1704 TR_L, another 18-mer oligonucleotide was selected from the wild type sequences which are deleted in 1704 TR_L. This was designated as oligonucleotide 'F'. The oligonucleotide showed positive hybridisation to the wild type *Hpa*I *o* and *r* bands but failed to hybridise to 1704 and 1704LP$^-$ DNA (Figure 3.32).

These results suggests that 1704LP$^-$ has a deletion identical to that in 1704 TR_L from np 7202 to 8144 in TR_L and np 118228 to np 119168 in IR_L i.e. 942 bp in length (Figure 3.33). This deletion involves both copies of the LAT promoter region i.e TATAA & CAAT boxes and SpI binding sites.

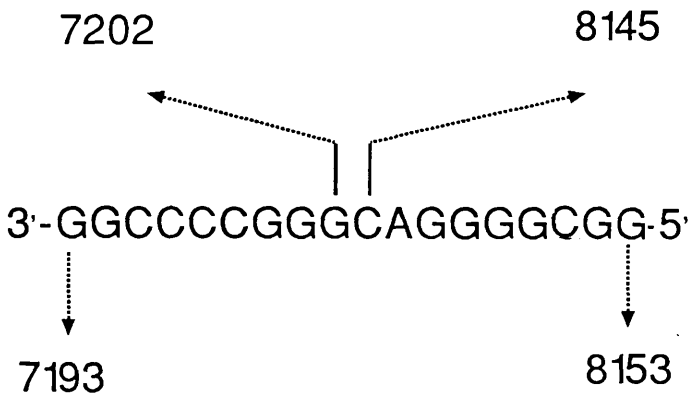
3.6 NEUROVIRULENCE OF 1704R AND 1704LP$^-$.

Groups of four, three week old BALB/c mice were inoculated with 1, 10, 10^2 and 10^3 pfu/mouse of 1704R and 1704LP$^-$; wild type and 1704 were used as controls in this experiment. The results in the Table 3.10, show that 1704R and 1704LP$^-$ vary in their pathogenic phenotype. The LD_{50} of 1704LP$^-$ is that of wild type but

Figure 3.31

Sequence of the oligonucleotides 'E' and 'F'. The oligonucleotide 'E' was designed to the corresponding sequences in the *Hpa*I *o** fragment of the variant 1704. The oligonucleotide 'F' was taken from the *Hpa*I *o* fragment of the 17 syn^+ sequences, which are deleted in the variant 1704. Coordinates are given as nucleotide positions (McGeoch *et al.*, 1988).

Sequence of the oligonucleotide E .



Sequence of the oligonucleotide F

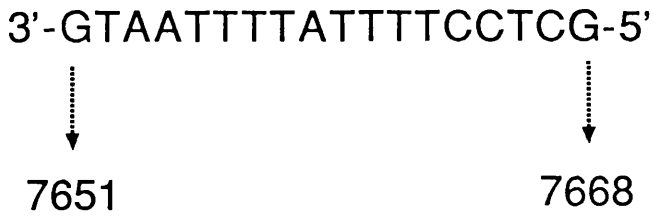


Figure 3.32

Autoradiograph of Southern blots in which ^{32}P labelled *in vitro:* oligonucleotide 'E': panel (a) 17 syn^+ (lane 1), 1704 (lane 2) and 1704LP^- (lane 3) and oligonucleotide 'F': panel (b) 1704 (lane 1), 1704LP^- (lane 2) and 17 syn^+ (lane 3) were hybridised to *Hpa*I digested DNA of 17 syn^+, 1704 and 1704LP^-. Letters refer to specific fragments.

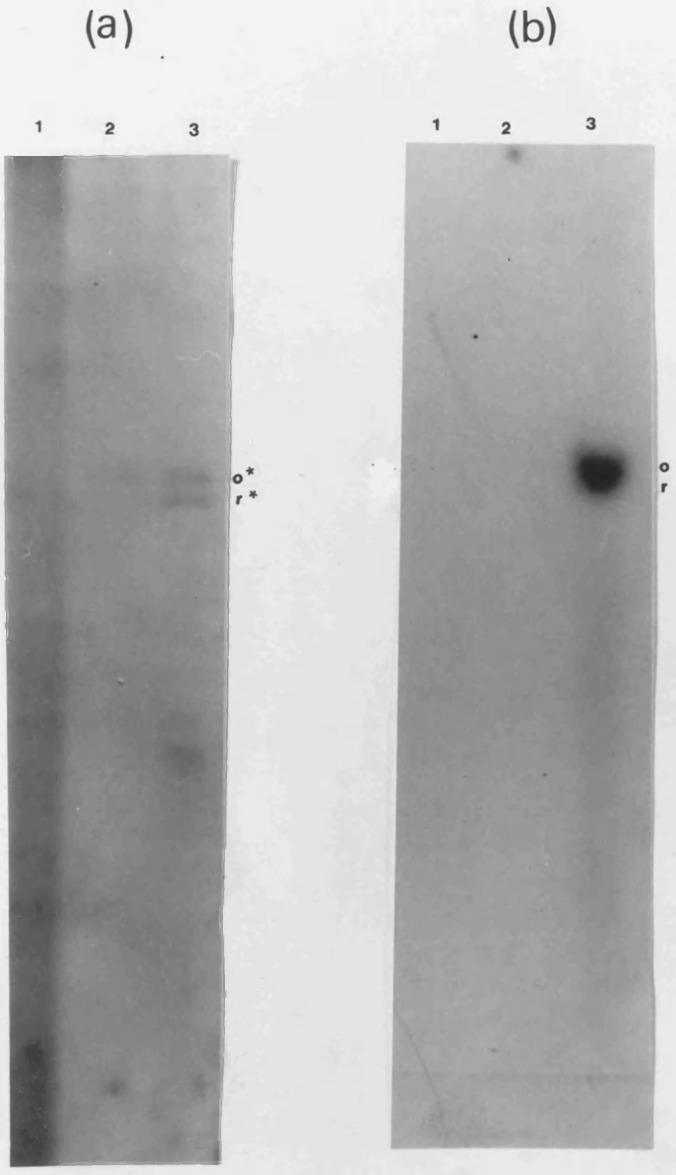

(a)
(b)
1
2
3
1
2
3
o*
r*
o
r

Figure 3.33

Structure of the HSV-1 genome (top line) showing U_L and U_S flanked by TR_L/IR_L and IR_S/TR_S respectively. The second line shows expansion of the TR_L and IR_L regions of the genome. Above the line, the position of the 3' end of IE1 and the 5' end of the LAT are indicated by arrows. The LAT promoter region is indicated by a hatched bar. The extent of the deletion in 1704LP$^-$ in R_L is marked below the line. Coordinates are given as nucleotide positions in the TR_L region of the genome (McGeoch et al., 1988).

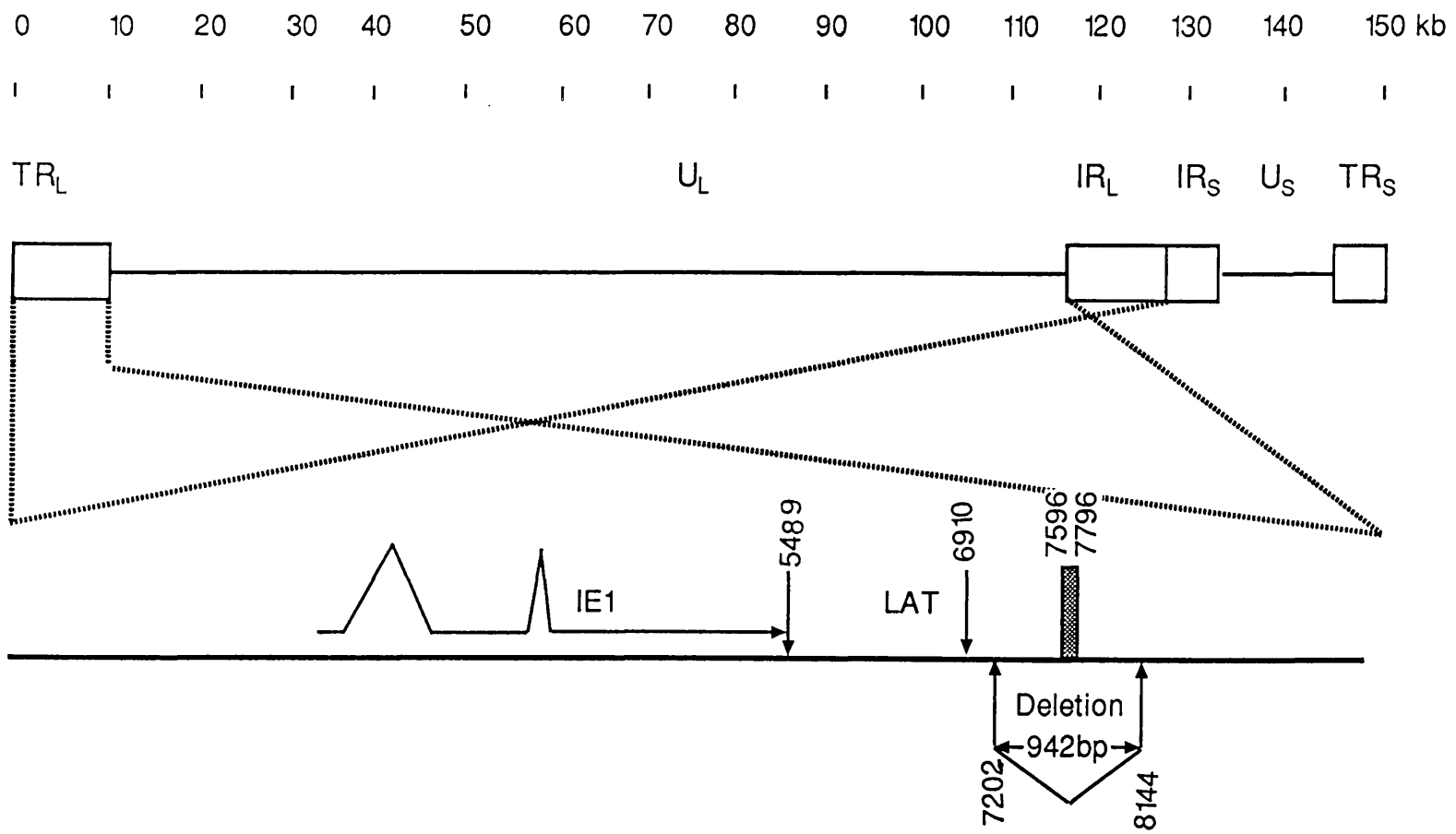

0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150 kb
TRL
UL
IRL
IRS
US
TRS
5489
6910
7596
7796
IE1
LAT
Deletion
942bp
7202
8144

Table: 3.10. LD50 values after intracranial inoculation of 17 syn^+, 1704, 1704R and $1704LP^-$

Virus	1*	$1x10^1$	$1x10^2$	$1x10^3$	LD_{50} pfu/mouse
17^+	0/4**	1/4	4/4	ND	22
1704	ND	1/4	2/4	4/4	$1x10^2$
1704R	ND	0/4	0/4	1/4	$>1x10^3$
$1704LP^-$	ND	1/4	3/4	3/4	32

* Dose pfu/animal

**Number of deaths/number of animals inoculated.

N.D: Not done.

3 week old BALB/c mice were used.

0.025ml of virus inoculated.

1704R shows an LD_{50} of $>10^3$ pfu/mouse.

3.7 LATENCY ANALYSIS OF 1704R AND 1704LP⁻

Three week old BALB/c mice were inoculated separately via the left rear foot pad with 10^5 pfu of 17 syn^+, 1704, 1704R and $1704LP^-$. Six weeks post inoculation mice were sacrificed. Nine DRG (last thoracic, six lumbar and two sacral) from the left side of the spinal cord were explanted, and transferred to individual microtitre plates containing ECS50 and screened for release of infectious virus (see method section 2.13). The results are shown in Figure 3.34. Detection of 1704R on the sixth day post explantation demonstrates that the kinetics of reactivation of 1704R have returned to that of 17 syn^+ suggesting that the sequences deleted in 1704 confer the slow phenotype. However the frequency of reactivation in 1704R remained poor indicating that this virus had not reverted fully to wild type behaviour. The kinetics of reactivation of $1704LP^-$ on the other hand mimic those of 1704. The variant $1704LP^-$ fails to make detectable LATs by Nothern blotting (N.W. Fraser, personal communication).

3.8 GROWTH PROPERTIES OF 1704R AND 1704LP⁻ IN VITRO.

One step growth properties of 1704R, $1704LP^-$, 1704 and 17 syn^+ were carried out over a 24h period in BHK-21 C13 cell at 37^oC and the results are shown in Figure 3.35. It has been previously shown that 1704 grows at a slightly slower rate than the wild type virus and produces a lower 24h yield (MacLean and Brown, 1987b). Although $1704LP^-$ grows similarly to the parent, 1704R was slightly impaired in its rate of growth and gave a 24h yield similar to 1704. This could explain the difference in the LD_{50}'s of 1704R and 1704,

Figure 3.34

Explant reactivation time course of HSV-1 strain 17 syn^+ (□), 1704 (■), 1704R (◇) and 1704LP (◆). Three week old BALB/c mice were inoculated into the left rear footpad with 25ul of each virus at a dose of 10^5 pfu/mouse . Six weeks later mice were killed the DRGs from the left side of the spinal cord were removed and cultured in ECS50. The supernatant was overlaid on indicator BHK-21 C13 cells. A ganglion was scored positive for reactivation when cytopathic effect was detected on the BHK21-C13 cell monolayers. Data given as percentage of reactivation at each time point.

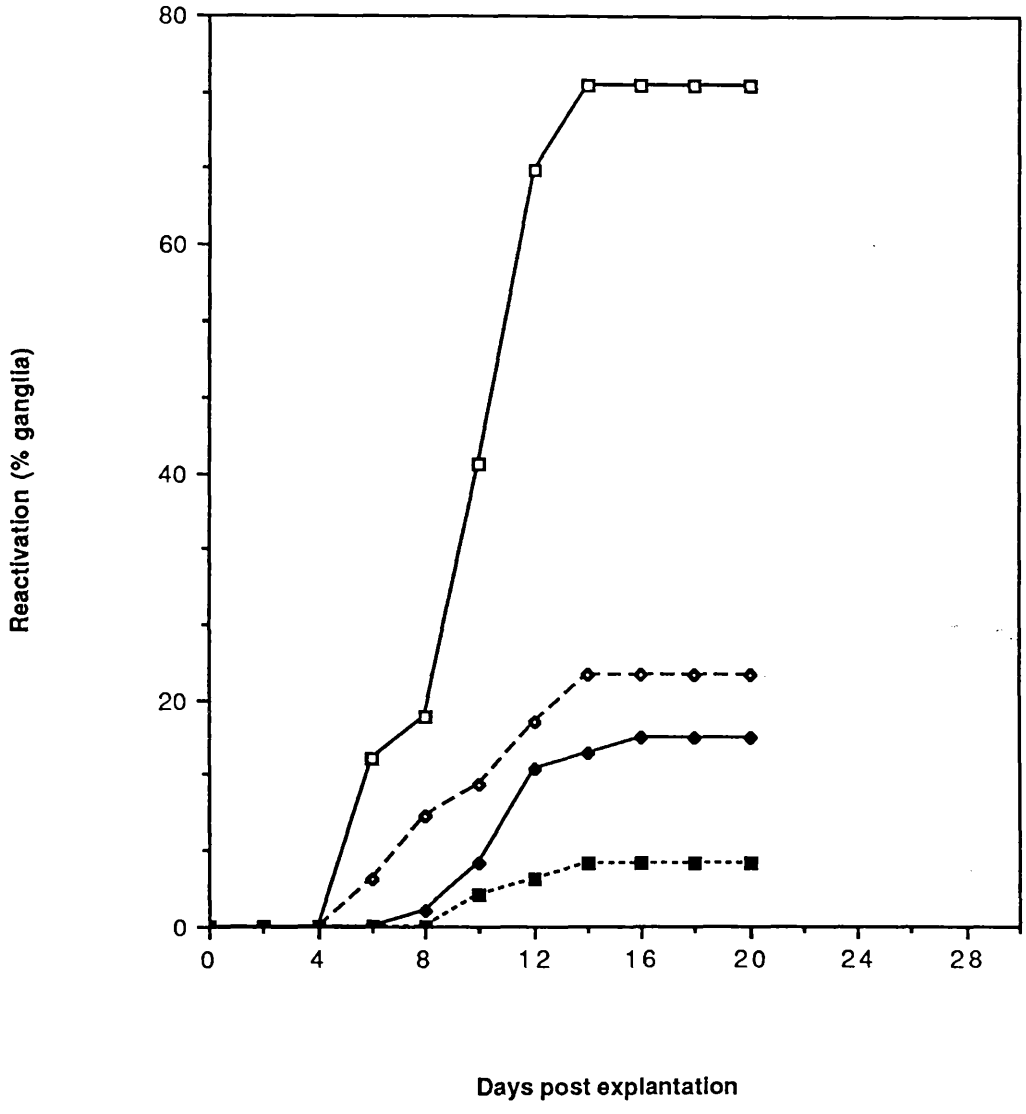
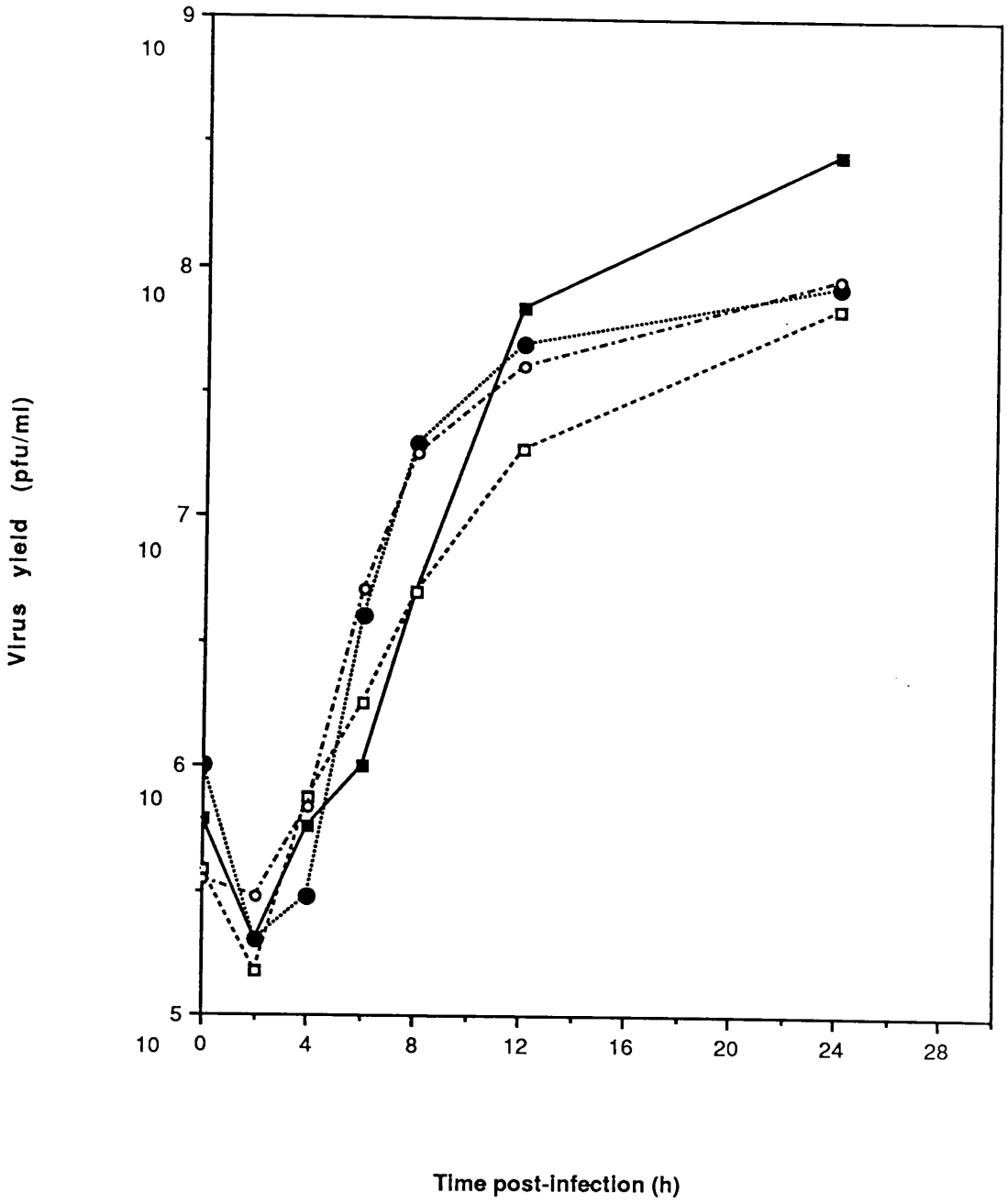

80
60
40
20
0
0
4
8
12
16
20
24
28
Reactivation (% ganglia)
Days post explantation

Figure 3.35

One step growth curves of HSV-1 strain 17 syn^+ (■), 1704 (●), 1704R (□) and 1704LP$^-$ (○) in BHK-21 C13 cells. Cells were infected at a multiplicity of 5 pfu/cell, the monolayers washed twice with PBS/calf serum, overlaid with ETC10 and incubated at 37^{o}C. Plates were harvested at 0, 2, 6, 8, 12 and 24 h post infection and titrated as normal.


Virus yield (pfu/ml)
9
10
8
10
7
10
6
10
5
10
0
4
8
12
16
20
24
28
Time post-infection (h)

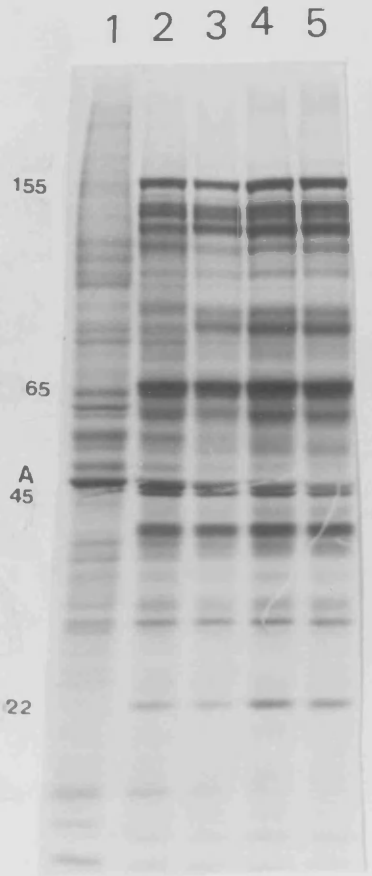
where 1704R has a LD_{50} of $>10^3$ pfu/mouse (see section 3.4.4).

3.9. INFECTED CELL POLYPEPTIDE SYNTHESIS BY 1704R AND 1704LP⁻.

It has been shown previously that 1704 synthesises normal amounts of HSV-1 induced immediate early polypeptides under immediate early conditions (MacLean and Brown, 1987b). The infected cell polypeptides of 17 syn^+, 1704, 1704R and $1704LP^-$ were labelled with ^{35}S methionine and analysed by SDS-PAGE (see method section 2.39). The results are shown in Figure 3.36. There were no detectable differences between the infected cell polypeptides synthesized by 17 syn^+ (lane 2), 1704 (lane 3), 1704R (lane 4) and $1704LP^-$ (lane 5), compared with mock infected cells (lane 1), indicating that although, apparently there is no effect on detectable general polypeptide synthesis of 1704R and $1704LP^-$, detailed analysis might be necessary for further investigation .

Figure 3.36

Autoradiograph of infected cell polypeptides induced in BHK-21 C13 cells, labelled with ^{35}S-methionine from 4-24 h post infection and separated on SDS-PAGE using a 5-12.5% polyacrylamide gradient gel. Molecular weights (x 10^{-3}) of predominant HSV-1 polypeptides are given on the left hand side. A is actin. Lane 1, mock infected; lane 2, 17 syn^{+}; lane 3, 1704; lane 4, 1704R and lane 5, 1704LP^{-}.


1 2 3 4 5
155
65
A
45
22

CHAPTER FOUR

DISCUSSION

DISCUSSION

The aim of the work presented in this thesis was to further characterise three HSV-1 strain 17 syn^+ deletion variants isolated from a single recombination experiment. The end points of the deletions of the variants were precisely sequenced by the dideoxy chain termination reaction method and the biological properties of the variants were studied *in vivo* using BALB/c mice. The variants 1704, 1705 and 1706 with $U_L/IR_L/TR_L$ rearrangements were isolated following restriction enzyme analysis of 80 progeny plaques from a single recombination infection. In addition, within the same progeny, 11 isolates showed extensive variation up to several hundred base pairs long within the R region of the genome excluding the 'a' sequence (MacLean and Brown, 1987b).

It was apparent therefore that these progeny molecules contained a higher than expected proportion of genomes with alterations and rearrangements involving the long repeat region of the genome. This was concordant with findings on HSV-2 strain HG52 in which several genomes with rearrangements of the short region of the genome had been isolated following a single infection whereas variants with long region alterations had arisen from a separate experiment (Harland and Brown, 1985; Brown and Harland, 1987). This "clustering" of rearrangements to particular regions of the genome in any one experiment pointed to them having arisen from a single or related events.

The favoured explanation at the time of isolation of 1704, 1705 and 1706 was that one variant genome had arisen which due to instability went through several rounds of rearrangement prior to the formation of stable genomes; thus an initial event could potentially lead to the isolation of several variants in a population.

It was further postulated that since the deletion occurred within or adjacent to the long repeat regions of the genome it could be due to illegitimate recombination involving the sets of reiterated sequences found within these regions of the HSV-1 genome (MacLean and Brown, 1987b).

To test this hypothesis the altered regions of the genomes in 1704, 1705 and 1706 were subjected to dideoxysequence analysis. The variant 1704 is deleted between np 116502 in U_L and 120260 in IR_L. Therefore 6112bp of IR_L has been retained between the 'a' sequence and the start of the deletion, 3103bp has been lost in addition to 655bp of U_L. In TR_L, the deletion is between np 7202 and np 8144, therefore 7202bp has been retained between the 'a' sequence and the start of the deletion plus 1071bp between the deletion and the start of U_L.

The 4735bp deletion in 1705 started at np 115453 in U_L and terminated at 120188 np in IR_L. There was therefore 1049bp between the start of the U_L deletion in 1704 and 1705. Within IR_L there was only 172bp difference between the end points of the 1704 and 1705 deletions. . The variants, 1704 and 1705 may therefore have arisen from a common process involving a nicked DNA strand and recombination event traversing to different points in the genome. It seems likely that further extension of the deletion at the right end of IR_L in 1704 resulted in the variant 1705 and the variants arose from the same progenitor molecule.

In 1706 there is no R_L deletion and the deletion in U_L extends to np 115350 which is 103bp to the left of the 1705 deletion point. The deleted sequences are replaced by sequences from the left end of U_L. A model for the origin of 1706 involving recombination between 1705 in the prototype orientation and either 17^+ or 1705 in the I_L orientation was proposed by MacLean and Brown (1987b).

Recombination could occcur homologously in the repeat regions and illegitimately through short homologous sequences possib ly having partial homology. As a result one end of U_L would give an extended repeat while the other end became deleted. It was assumed that if the end points of the deletions in 1705 and 1706 were the same, the recombination between IR_L of 1705 and TR_L of wild type virus in the opposite orientation could have resulted in 1706. This depends on the U_L/IR_L novel juction being unstable and therefore prone to disruption thus facilitating such an event. The closeness of the two end points in 1705 and 1706 would not therefore refute this model. Sequence comparison has demonstrated no gross homology between the sequences around np 115350 and those around 13969 the insert point of the DNA from the left hand side of U_L.

There are six sets of contiguous tandem reiterations between the 'a' sequence and the U_L/R_L junction (Perry and McGeoch, 1988). The first set of 11 reiterations each containing 17bp of T and C residues is located between np117158 and np117341. The second set of 7 reiterations each containing 3bps, two G's and one T residue, is located between np117544 and np117565. The third one of 6 reiterations, of three C's and two G's is located between np117785 and np117815. The fourth set of reiterations is located in close proximity to the 3' end of the IE1 gene. This set consists of 9 reiterated sequences of 16bp each mainly consisting of C's located between np120491 and np120636. The two other reiterated sets are upstream of the 5' end of IE1 and are therefore not relevant to the position of the 1704, 1705 and 1706 deletions. The IR_L end points of the deletions in the variants 1704 and 1705 are 656bp and 1705bp respectively away from the first set of reiterations, so that the first, second and third sets are deleted in both the variants. The left ends of the deletions in 1704 and 1705 are 232bp and 304bp respectively

away from the fourth set of reiterations. The end points of the deletion in 1704 in TR_L are 661bp and 1324bp respectively away from the third and fourth sets of reiterations. The TR_L and IR_L deletions had not arisen from the same point and are not within or immediately adjacent to any of the 4 sets of tandem reiterations located in the long repeat region of HSV-1 (Perry and McGeoch, 1988). In the variant 1706 in which the deletion is entirely confined to the right end of U_L, the right end of the deletion is immediately adjacent to the first reiteration set. The sequence analysis therefore determined that the initiation of deletions in R_L is probably independent of the reiterated sets.

The model proposed for the expansion or contraction of the repeats (McGeoch, 1984; Whitton and Clements, 1984) depends on the essential nature of the genes in U_L adjacent to R_L. The genes UL55 and 56 are non-essential and UL54 is essential (Sacks *et al.*, 1985). The termination of the deletions within U_L is interesting. All the variants have been deleted in UL55 and UL56 but the extent of the end point of the deletions from the 3' end of IE2 (UL54) gene in 1704, 1705 and 1706 is 1232bp, 183bp and 80bp respectively. A YGTGTTYY (Y= pyrimidine either C or T) motif located downstream from the poly A signal is required for efficient formation of mRNA 3' end termini (McLauchlan *et al.*, 1985). This motif is present 40bp downstream from the 3' end of the IE2 gene in HSV-1. Although according to our sequence analysis (Figure 4.1) these sequences are not deleted in any of the variants, synthesis of VmwIE63 is reduced to approximately half the wild type level in 1705 and 1706 but normal levels in 1704 in which the end point of the deletion is 1232bp downstream from the 3' end of IE2 gene (MacLean, 1988). Surprisingly in another variant 1703 in which the deletion terminated approximately 500bp downstream from the 3' end of IE2,

Figure 4.1

The sequence of HSV-1 17 syn^+ between 115101-116600bp (Perry and McGeoch, 1988). The end points of the deletions in U_L/IR_L in 1704, 1705 and 1706, the 3' of UL54 (IE2), UL55 and UL56 and the 5' of UL55 are indicated. The poly A site downstream of the 3' of UL54 is underlined and YGTGTTY element (McLauchlan *et al.*, 1985) is indicated in the dotted box.

115101 CGGAGAGAAG ATGCATTTCT ACCTCCCCGG GGCCTGCATG GCGGGCCTGA

115151 TCGAAATCCT AGACACGCAC CGCCAGGAGT GTTCGAGTCG TGTCTGCGAG

115201 TTGACGGCCA GTCACATCGT CGCCCCCCCG TACGTGCACG GCAAATATTT

3' end of IE2 (UL54)

115251 TTATTGCAAC TCCCTGTTTT AGGTACAATA AAAACAAAAC ATTTCAAACA

Poly A

115301 AATCGCCCCT CGTGTTGTCC TTCTTTGCTC ATGGCCGGCG GGGCGTGGGT

1706 U_L Deletion

115351 CACGGCAGAT GGCGGGGGTG GGCCCGGCGT ACGGCCTGGG TGGGCGGAGG

115401 GAACTAACCC AACGTATAAA TCCGTCCCCG TTCCAAGGCC GGTGTCATAG

1705 U_L/IR_L Deletion

5' end of UL55

115451 TGCCCTTAGG AGCTTCCCGC CCGGGCGCAT CCCCCCTTTT GCACTATGAC

115501 AGCGACCCCC CTCACCAACC TGTTCTTACG GGCCCCGGAC ATAACCCACG

115551 TGGCCCCCCC TTACTGCCTC AACGCCACCT GGCAGGCCGA AACGGCCATG

115601 CACACCAGCA AAACGGACTC CGCTTGCGTG GCCGTGCGGA GTTACCTGGT

115651 CCGCGCCTCC TGTGAGACCA GCGGCACAAT CCACTGCTTT TTCTTTGCGG

115701 TATACAAGGA CACCCACCAC ACCCCTCCGC TGATTACCGA GCTCCGCAAC

115751 TTTGCGGACC TGGTTAACCA CCCGCCGGTC CTACGCGAAC TGGAGGATAA

115801 GCGCGGGGTG CGGCTGCGGT GTGCGCGGCC GTTTAGCGTC GGGACGATTA

115851 AGGACGTCTC TGGGTCCGGC GCGTCCTCGG CGGGAGAGTA CACGATAAAC

115901 GGGATCGTGT ACCACTGCCA CTGTCGGTAT CCGTTCTCAA AAACATGCTG

115951 GATGGGGGCC TCCGCGGCCC TACAGCACCT GCGCTCCATC AGCTCCAGCG

116001 GCATGGCCGC CCGCGCGGCA GAGCATCGAC GCGTCAAGAT TAAAATTAAG

3' end of UL55

116051 GCGTGATCTC CAACCCCCCC ATGAATGTGT GTAACCCCCC CCAAAAAAAT

116101 AAAGAGCCGT AACCCAACCA AACCAGGCGT GGTGTGAGTT TGTGGACCCA

116151 AAGCCCTCAG AGACAACGCG ACAGGCCAGT ATGGACCGTG ATACTTTTAT

116201 TTATTAACTC ACAGGGGCGC TTACCGCCAC AGGAATACCA GAATAATGAC

116251 CACCACAATC GCGACCACCC CAAATACAGC ATGGCGCCAC ACCACGCCAC

3' end of UL56

116301 AACAGCCCTG TCGCCGGTAT GGGGCATGAT CAGACGAGCC GCGCCGCGCG

116351 TTGGGCCCTG TACAGCTCGC GCGAATTGAC CCTAGGAGGC CGCCACGCGC

116401 CCGAGTTTTG CGTTCGTCGC TGGTCGTCGG GCGCCAAAGC CCCGGACGGC

116451 TGTTCGGTCG AACGAACGGC CACGACAGTG GCATAGGTTG GGGGGTGGTC

1704 U_L/IR_L Deletion

116501 CGACATAGCC TCGGCGTACG TCGGGAGGCC CGACAAGAGG TCCCTTGTGA

116551 TGTCGGGTGG GGCCACAAGC CTGGTTTCCG GAAGAAACAG GGGGGTTGCC

VmwIE63 could not be detected at either the polypeptide or RNA levels under immediate early conditions (MacLean and Brown, 1987a). It seems likely that either unknown sequences downstream from the motif YGTGTTYY could be playing a combined role in the efficient expression of IE2 mRNA or the possibility of a point mutation within the IE2 gene cannot be ruled out.

The above findings indicate that the termination point of the deletions (i) could be entirely arbitrary within the sequence between the 3'end of UL54 and the U_L/IR_L junction or (ii) may depened on the structure and conformation of the DNA with particular regions being more "accessible". The deletions in HSV-1 reported so far usually involve at least one of the repeats and may also involve unique sequences. This indicates that the repeats may act as hotspots for illegitimate recombination (MacLean and Brown, 1987b).

From the sequence analysis it is evident that 1704, 1705 and 1706 (1) have not deleted at precisely the same end points, (2) the IR_L deletions of 1704 and 1705 could be related, (3) R_L deletions are not dependent on tandem reiterations, (4) 1706 could have arisen by illegitimate recombination as proposed, (5) U_L deletions (repeat extension) are controlled by the essential nature of the genes (genomes with UL1 deletions never being isolated) and (6) the deletion start/stop point within non-essential DNA is probably arbitrary.

When Steiner *et al* (1989) demonstrated that 1704 failed to make LATs as detected by Nothern blotting and *in situ* hybridisation in addition to reactivating slowly from latency, the precise extent of the deletion with respect to the LATs and their promoter region had not been determined; the assumption being made that the absence of detectable LATs could only be due to the deletions affecting the

transcripts and/ or promoter region. Sequence analysis of 1704 has shown that in U_L/IR_L, 170bp of UL56 has been retained and the deletion does not affect IE1 whose 3' end is at np 120882. The 5' end of the LATs is at np 119461 which means that 799bp of the transcripts has been deleted. Wechsler *et al* (1988) showed that the LAT promoter region was located between 662-940bp upstream of its 5' end (np 118575-118775) and is therefore totally absent in the IR_L region of 1704. In TR_L the LAT transcript whose 5' end is at np 6910 is not removed but the deletion between 7202-8144 np completely removes the promoter region i.e 7596-7796 np. 1704 has therefore no LAT promoters but retains one complete copy of the LATs and 2/3 of the other copy.

The findings using the mouse footpad model of latency confirm the results using the mouse eye model in that at equivalent input doses to 17^+, the absence of LATs in 1704 causes a delay in reactivation *in vitro* and in the footpad model the frequency of reactivation was also significantly reduced. However this appears to be dose dependent. On increasing the input doses from 10^5 to 10^7 pfu/mouse, the kinetics of reactivation are more akin to those of wild type. The percentage of ganglia reactivating (30%) is much higher than at an input dose of 10^5 pfu/mouse (3%) but only about half the value of 17^+ infected ganglia reactivating. At an input dose of 10^7 pfu compared to 10^5 pfu it is assumed that either (1) latency is established in more neurons/ganglia or (2) more virus molecules/neuron establish latency or that (1) plus (2) pertain. This would lead to the frequency of reactivation being higher and to the kinetics being faster due to the number of genomes reactivating rising above the detection threshold earlier. This would suggest that the process may be independent of the presence or absence of the LATs *per se* but could be codependent on the gene dosage of one or

more other HSV genes. On the other hand if reactivation is dependent on a cellular factor initiating transcription and this process is facilitated by the LATs although they are not an absolute requirement then the higher number of genomes present, the greater chance of reactivation occurring and virus being detectable.

The isolation of 1704R, the rescuant with a rate of reactivation and frequency of reactivation intermediate between that of 17^+ and 1704 would suggest that 1704R has a secondary undetected mutation precluding it reverting fully to wild type behaviour. Similar results have also been found with 1704R in the mouse eye model of explant reactivation (N.W.Fraser, personal communication). However, when 1704R was compared to 1704 and 17^+ in *in vivo* reactivation using the rabbit eye model it was found that 17^+ and 1704R behaved similarly, whereas the frequency and kinetics of reactivation of 1704 was significantly impaired (Trousdale *et al.*, in press). These apparently contradictory results are not immediately explicable. It will be necessary to isolate another 1704 rescuant and test its latency phenotype before definitive conclusion can be drawn.

The latency results with 1705 mimic those of 17^+. Although 1706 infected ganglia had similar reactivation kinetics to 17^+ and 1705, the percentage of ganglia reactivating was considerably reduced (16% compared to 50%). As the deletion/insertion in 1706 does not affect the LATs or LAT promoter, it is assumed that 1706 is less efficient possibly due to its growth impairment. *In vitro* at low moi (1:1000), 1706 is 12-24 h delayed in growth compared to 17^+ and 1705 over a 72 h period (MacLean and Brown, 1987b). This impairment is also marked *in vivo* and hence may have an effect on latency reactivation.

Before studying pathogenicity of the variants in the mouse

model system it was essential to determine the phenotype of the parental wild type virus. Therefore we have evaluated the neurovirulence of individual plaque stocks isolated from the elite stock of 17 syn$^+$ to determine the base line for evaluating deletion variants 1704, 1705 and 1706 and to further investigate any variation in pathogenicity among individual plaques in the elite stock of HSV-1 strain 17 syn$^+$. Although a proportion (up to 24%) of viable virus with divergent genomic structures in the population of HSV-2 strain HG52 showed deletions in R_L (Harland and Brown, 1985; 1988; Brown and Harland, 1987), the heterogeneity in the neurovirulence of individual plaque stocks of the HSV-2 strain HG52 elite stock has been documented without detectable variations in the genomes of the plaques inoculated intracranially in BALB/c mice. (Taha *et al.*, 1988).

The nine individual stocks isolated from the elite stock of 17$^+$ showed no differences in their LD_{50} values following intracranial inoculation of 3 week old BALB/c mice. All the plaques have LD_{50}'s similar to the parental 17 syn$^+$. Restriction endonuclease analysis of DNAs of the isolated plaques was carried out showing no differences in the size and distribution of the fragments. The restriction endonuclease analysis however can not detect any point mutation, small deletions and/or insertions less than 150bp in length in the genome which could be possible sources of variation in the pathogenic phenotype of the virus in the mouse model.

Intracranial inoculation of 3 week old BALB/c mice showed that the LD_{50} for 1705 was similar to the wild type virus but the difference with 1704 and 1706 compared to wild type was approximately one log and two logs respectively. Following footpad inoculation 1705 behaved as wild type but 1704 and 1706 were unable to kill any of the animals even at the highest possible infective dose of 10^7 pfu/mouse. 1704 and 1706 failed to grow with

wild type virus kinetics and were undetectable by day four in the peripheral nervous system (DRG) which could explain the inability of these viruses to kill the animal following foot-pad inoculation. The impairment of the growth *in vitro* of 1706 has already been observed (MacLean and Brown, 1987b). Possible explanations for this defect in 1706 are:

(i) Deletion of genes UL55 and UL56. Recently it has been observed in transient gene expression assays that UL55 and UL56 play a role in transactivation and inhibition of immediate early gene expression (Block *et al.*, 1991). This possibility can be ruled out because the UL55 gene is deleted in both the variants 1704 and 1705. (ii) Deletion of the sequences downstream from the 3' end of IE2. Although neither the 3' end nor the poly A site of IE2 is deleted, the deletion in 1706 stops 80bp downstream of the 3' end. The variant 1706 expresses half of the wild type level of VmwIE63 polypeptide (MacLean, 1988). As discussed earlier it is possible that a consensus sequence other than YGTGTTYY (McLauchlan *et al.*, 1985) could be important in processing mRNA from the IE2 gene. The variant 1705 has a deletion starting 183bp downstream from the 3' end of the IE2 gene, however it expresses a similar amount of VmwIE63 as 1706, therefore the amount of VmwIE63 made by these two variants can not explain the differences in the LD_{50} values.

(iii) Double dose of all or one of the genes UL1, 2, 3 and 4. These genes might have an inhibitory effect on the gene regulation system of the virus thus rendering it growth impaired.

and (iv) Small deletions, insertions and/or point mutations elsewhere in the genome can not be ruled out.

The variant 1705 consistently behaved as the wild type following intracranial and footpad inoculations despite a 4735bp deletion in U_L/IR_L. It has been shown that intraperitoneal virulence

of HSV-1 is associated with the *Bam*HI *b* fragment (Ben-Hur *et al.*, 1989). The HSV-1 strain HFEM is deleted within the *Bam*HI *b* fragment from 0.762-0.789 map units, approximately 4.1kb. The strain HFEM is avirulent following intraperitoneal inoculation of tree shrews (Rosen and Darai., 1985) and in mice (Rosen *et al.*, 1986). Marker rescue of the *Bam*HI *b* fragment from the virulent HSV-1 strain F into HFEM resulted in the return of IP virulence in tree shrews but not in mice. The end points of the deletion in the strain HFEM corresponds to the residues 117107 and 120640 of strain 17 syn^+. The only functional elements which the deletion is removing are the presumed control sequences for the gene UL56 (Perry and McGeoch, 1988). The variant 1705 has a 600bp larger deletion than HFEM involving the UL56 gene, within the *Bam*HI *b* fragment. Intraperitoneal inoculation of 3 week old BALB/c mice with 1705 showed consistent parental wild type behaviour. This suggests (i) intraperitoneal virulence is not associated with the *Bam*HI *b* fragment in HSV-1 strain 17^+ and (ii) IP virulence is host dependent because the rescuant of HFEM remains avirulent for mice and (iii) IP pathogenicity in the variant 1705 is multifocal with other amplifying loci rendering it virulent despite the deletion.

Various factors affect the outcome of *in vivo* experiments including seasonal variation in animal house temperature and the resultant stress this produces in the animals. It has been shown that reactivation from latency and peripheral replication of virus is more efficient when the temperature of animal house does not go below 70°F (L.Robertson, personal communication). Other factors like age and route of inoculation have already been discussed (Caspary *et al.*, 1980; Kohl and Loo, 1980).

FUTURE PROSPECTS

Information provided in this thesis provides a sound basis for future work on the variants 1704, $1704LP^-$, 1705 and 1706. Since the genes UL55 and UL56 are deleted in 1705 and 1706, these viruses may be useful in eliciting the functions encoded by these two genes. The products of UL55 and UL56 are non-essential in BHK-21 C13 cells therefore future investigation must involve different cell types. The effects of gene duplication (UL1, 2, 3 and 4) and rearrangement in 1706 may possibly be responsible for its altered biological behaviour but this needs further investigation. It is possible that diploid genes in 1706 result in their protein products being over-expressed which may be detrimental to other protein products essential for virus growth.

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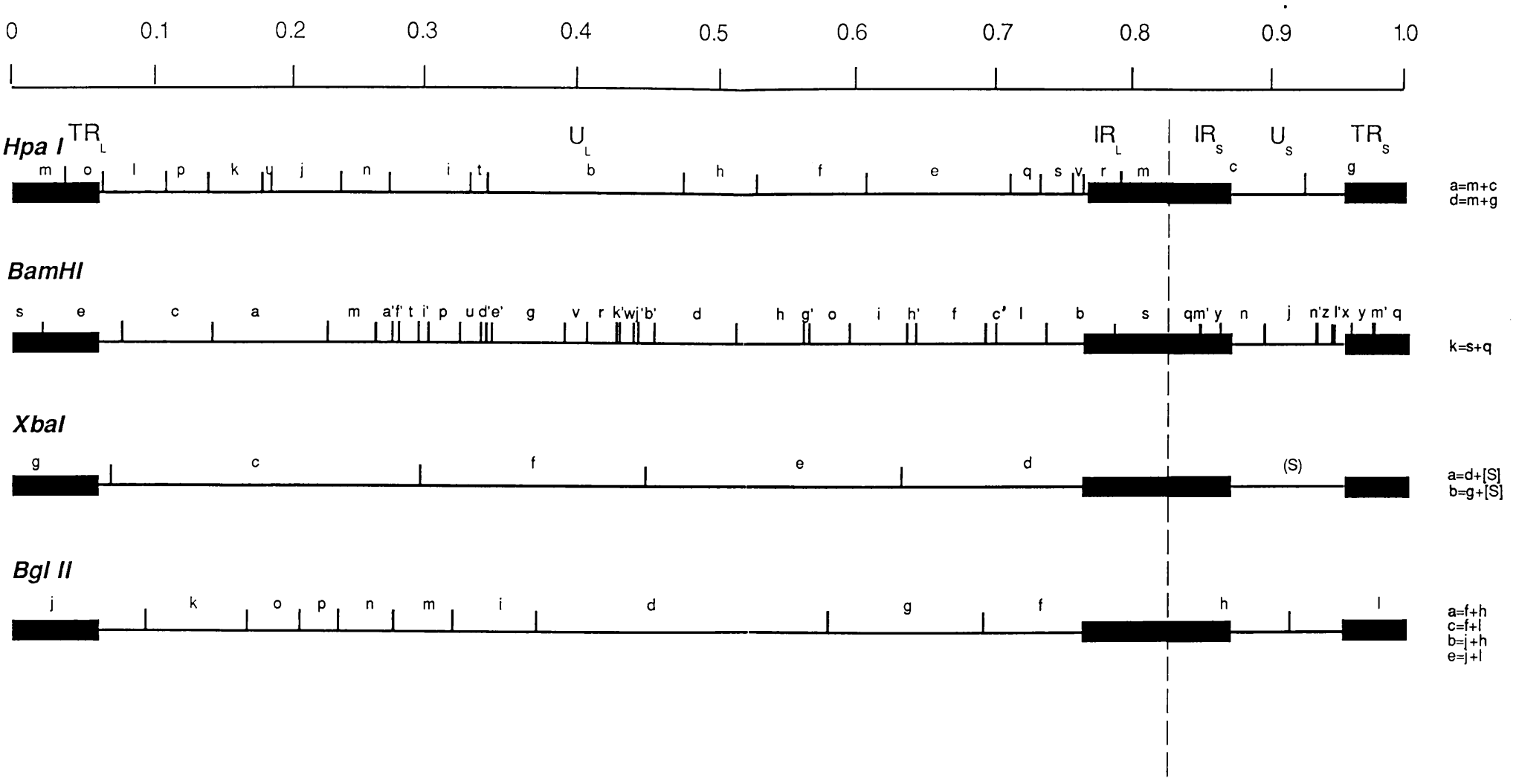
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d=m+g
BamHI
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XbaI
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Bgl II
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e=j+l