ANALYSIS OF NEUROVIRULENCE IN THE MOUSE MODEL SYSTEM USING DELETION VARIANTS OF HERPES SIMPLEX VIRUS TYPE 2 (HSV-2)

by

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SUMMARY

The aim of the work described in this thesis was to identify gene (s) involved in determining the neurovirulence of HSV-2 strain HG52 in the mouse model system using deletion variants. The availability of variants with deletions in specific regions of the genome afforded the unique opportunity to determine the possible role of specific sequences in virulence. The phenotype of the parental wild type virus has also been determined by examining the neurovirulence of individual plaque stocks to identify the baseline from which to evaluate the deletion variants.

Twenty well separated plaques were picked from the elite stock of HG52 and passaged twice at $37^{\circ}C$. Restriction endonuclease analysis of the DNA from each of the twenty plaque stocks showed no differences in the size of fragments and distribution of the sites. To determine their neurovirulence, ten of the individual plaque stocks were selected randomly for mice inoculation. Following intracranial inoculation of 3 week old BALB/c mice, the plaque stocks segregated into three classes of neurovirulence on the basis of their LD_{50} values; high ($<10^{3}$ pfu/mouse), intermediate (10^{3}-10^{4} pfu/mouse) and low virulence ($>10^{5}$ pfu/mouse). The particle : pfu ratios of the plaque stocks were within the acceptable range for HSV-2. Restriction endonuclease analysis of viruses reisolated from the brains of infected mice showed no apparent difference in their DNA profiles compared to the initial infecting viruses.

Two plaque stocks of high, one of intermediate and one

of low virulence were selected on the basis of their LD_{50} values and particle : pfu ratios. These stocks retained their original LD_{50} values compared to the non-plaque purified elite stock of HG52 ($<10^2$ pfu/mouse) when retested in mice. Following intraperitoneal inoculation, the selected plaque stocks showed differences in their LD_{50} values comparable to the differences seen following intracranial inoculation.

The selected plaque stocks grew as well as the parental HG52 in one step growth experiments in BHK-21 Cl3 cells. However, they showed differences in the growth kinetics in vivo in mouse brain where the high virulence stocks showed comparable growth to HG52, while the intermediate and low virulence stocks grew less well.

The selected plaque stocks were passaged five times in BHK-21 Cl3 cells and ten plaques from each were picked and stocks grown. Restriction endonuclease analysis of their DNA showed no differences compared to the wild type HG52. Following intracranial inoculation of the virus stock derived from the fifth passage, those derived from intermediate or low virulence virus remained stably of intermediate or low virulence, while those derived from high virulence virus showed in some cases a shift to intermediate levels of virulence.

These results clearly demonstrate virulence heterogeneity within the elite stock of HSV-2 strain HG52. The LD_{50} value of high virulence virus was chosen as a baseline from which to evaluate the virulence of deletion variants

A number of deletion variants of HSV-2 strain HG52 with deletions ranging in size from 1.5 to 9 kb have been tested

for virulence following intracranial inoculation of 3 week old BALB/c mice. The variants were segregated into three categories of virulence on the basis of their LD_{50} values; avirulent ($>10^7$ pfu/mouse), reduced (10^5-10^6 pfu/mouse) and attenuated (10^4 pfu/mouse) compared to $<10^2$ pfu/mouse for the elite stock of HG52.

Analysis of the variants with deletions in U_S/TR_S indicated that the US4, US10, US11 and US12 genes have a role in neurovirulence. Deletion of one copy of ori_S and one copy of the IE3 gene has a minor effect on neurovirulence.

Analysis of the variants with deletions in IR_L/TR_L regions of the genome implies that deletion of one copy of the IE1 gene and part of LAT transcripts in IR_L has a minor effect on neurovirulence. The analysis demonstrated that none of the deleted genes appear to be a unique determinant of neurovirulence with the exception of the DNA sequences between 0-0.02 and 0.81-0.83 m.u.

The variant JH2604 whose genome is deleted by 1.5 kb in both copies of the BamHI v fragment between 0-0.02 and 0.81-0.83 m.u. in TR_L and IR_L respectively was avirulent for mice following intracranial and footpad inoculation with LD_{50} values of $>10^7$ and $>10^8$ pfu/mouse respectively compared to $<10^2$ pfu/mouse for the elite stock of HG52. Therefore, the variant JH2604 is at least 6 logs less neurovirulent than the wild type virus. The variant JH2604 grew as well as the wild type virus in vitro in BHK-21 C13 and 3T6 cells, but it failed to grow in mouse brain in vivo demonstrating that the lack of neurovirulence was due to inefficient replication in mouse brain. The variant JH2604 synthesises TK as efficiently as the wild type virus and its infected

cell polypeptide profile shows no detectable differences.

The lack of growth in mouse brain in vivo of JH2604 has been confirmed by neuropathological studies which showed that JH2604 did not cause necrotising encephalitis but there was infiltration of lymphocytes and perivascular cuffing around the blood vessels localised to the site of inoculation. The wild type HG52 infected brains showed necrotising encephalitis throughout. Using polyclonal anti-HSV antibodies in immunohistochemical studies, viral antigens were seen along the needle track in JH2604 infected brains, while they were widely distributed in HG52 infected brains.

Correction of the deletion in JH2604 by cotransfection in vitro using large wild type XbaI fragments resulted in the isolation of recombinants which gave LD_{50} values comparable to those of individual plaque stocks of the parental HG52. Correction of the deletion by the small wild type BamHI q fragment using in vivo selection in mouse brain resulted in recombinant viruses which displayed wild type LD_{50} values.

Introduction of the deletion into the wild type genome by in vitro cotransfection resulted in recombinants which on intracranial inoculation of mice were avirulent. These results imply that the sequences within BamHI q most probably within both copies of the BamHI v fragment between 0-0.02 and 0.81-0.83 m.u. in TR_L and IR_L are required for maximum demonstration of virulence in HSV-2 strain HG52.

Cloning of the deleted fragment of JH2604 in pAT153 and restriction enzyme and Southern blot analysis of the positive clones confirmed that the deletion was approximately 1.5 kb in both copies of the BamHI v fragment

with apparently identical end points. Sequencing analysis
revealed that the deletion in each copy of the BamHI v
fragment was 1488 bp and was contained within the
XhoI-HincII fragment within BamHI v. It includes a complete
17 bp DR1 element, four copies of a 19 bp reiterated
sequence and terminates 522 bp upstream of the IE1 gene.
These results clearly demonstrate that the deleted sequence
has a function in vivo which may suggest that the region is
protein coding.

ABBREVIATIONS

A	adenosine
ABC	avidin biotinylated horseradish peroxidase complex
ACV	acyclovir
.AIDS	acquired immunodeficiency syndrome
APS	ammonium persulphate
ATP	adenosine triphosphate
BCIG	5-chloro-4-bromo-3 indolyl-β-D-galactoside
BHK	baby hamster kidney cells
BMV	bovine mammilitis virus
bp	base pairs
CAM	chorioallantoic membrane
CCV	channel catfish virus
Ci	curies
CIP	calf intestinal phosphatase
cm	centimetre
cpe	cytopathic effect
CSF	cerebrospinal fluid
dATP	deoxyadenosine triphosphate
DBP	DNA binding protein
dCTP	deoxycytidine triphosphate
ddNTP	dideoxynucleoside triphosphate
dGTP	deoxyguanosine triphosphate
DMEM10	Dulbecco's Modified Eagle's medium containing 10% calf serum
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DR	direct repeat

dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
dUTPase	deoxyuridine triphosphatase
E	early
EBV	Epstein-Barr virus
E.coli	Escherichia coli
EDTA	sodium ethylene diamine tetra-acetic acid
EHu5	Eagle's medium containing 5% human serum
EHV	equine herpes virus
EMC5	Eagle's medium containing 1.5% methyl cellulose
Emet/5C2	Eagle's medium containing 20% the normal concentration of methionine plus 2% calf serum
ETC10	Eagle's medium containing 10% calf serum
Fc	fragment crystalisable
G	guanosine
g	gram
h	hour
H & E	haematoxylin and eosin
HCMV	human cytomegalovirus
HHV	human herpesvirus
HSV	herpes simplex virus
HVS	herpesvirus saimiri
ICP	infected cell polypeptide
IE	immediate early
IFN	interferons
IgG	immunoglobulin G
IPTG	isopropyl-D-thiogalactoside
IR_L	inverted long repeat
IR_S	inverted short repeat
K	kilodalton
kb	kilobase

L	late
l	litre
LAT	latency associated transcripts
LD_{50}	50% lethal dose
M	molar
<u>mar</u>	monoclonal antibody resistant
mCi	millicuries
MCMV	murine cytomegalovirus
MCP	major capsid protein
MDV	Mareks disease virus
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mm	millimetre
moi	multiplicity of infection
Mr	molecular weight
mRNA	messenger ribonucleic acid
m.u	map units
ng	nanogram
NP40	Nonidet P40
OD	optical density
ORF	open reading frame
ori	origin of viral DNA replication
oz	ounce
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
pfu	plaque forming units
PIC	phosphate free Eagle's medium containing 1% calf serum

PRV	pseudorabies virus
RF	replicative form
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
RR	ribonucleotide reductase
RT	room temperature
SDS	sodium dodecyl sulphate
SSC	trisodium citrate and sodium chloride
$\underline{syn}+$	non-syncytial
T	thymidine
TBE	Tris, boric acid and EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TIF	$\underline{trans}$-inducing factor
TK	thymidine kinase
TK^-	thymidine kinase negative
TK^+	thymidine kinase positive
$\underline{ts}$	temperature sensitive
TR_L	terminal long repeat
TR_S	terminal short repeat
3T3	mouse embryo fibroblast cells clone 3
3T6	mouse embryo fibroblast cells clone 6
U_L	long unique
U_S	short unique
UV	ultraviolet
V	voltage
Vmw	molecular weight of viral-induced polypeptide
v/v	volume per volume
VZV	varicella zoster virus
W	watt

w/v	weight per volume
w/w	weight per weight
X	times
°C	degree centigrade
uCi	microcuries
ug	microgram
ul	microlitre
uM	micromolar
%	percentage
<	less than
>	higher than

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CHAPTER 1

INTRODUCTION

The subject of this thesis is the identification of genes involved in neurovirulence of herpes simplex virus type 2 (HSV-2) using HSV-2 deletion variants. The introduction provides background information on the general biology and molecular biology of HSV with particular reference to advances in the understanding of virulence and pathogenesis.

1.1 Herpesviridae family and its classification

At least 80 viruses which comprise the family, herpesviridae, have been isolated from a wide variety of animal species and man. The members of the herpesviridae can be identified by the presence of double stranded DNA in the core of the virion, an icosahedral capsid containing 162 capsomeres which is assembled in the nucleus and an envelope derived from the nuclear membrane (Roizman and Batterson, 1986).

The herpesvirion consists of four structural elements (Figure 1.1) (i) an electron-opaque core containing DNA (ii) an icosahedral capsid containing 162 capsomeres (iii) an electron-dense amorphous proteinaceous layer (the tegument) distributed around the capsid and (iv) an outer membrane, or envelope, which surrounds the capsid and tegument (Wildy et al., 1960; Roizman and Furlong, 1974). Herpesviruses can not be differentiated from each other by electron microscopic examination but are differentiated with respect to virion antigenic properties, biological properties and composition, size and arrangement of their genomes (Roizman, 1982).

Figure 1.1

Schematic diagram of a HSV virion. The virion consists
of four structural elements; the core, capsid, tegument
and envelope. The viral DNA is within the core and is
spooled around a cylindrical protein mass. The virion
envelope contains a number of glycoproteins, visible on
electron microscopy as spikes protruding from the
envelope. The capsid contains the capsomeres.

Core

DNA

Capsid

Tegument

Envelope

Glycoprotein
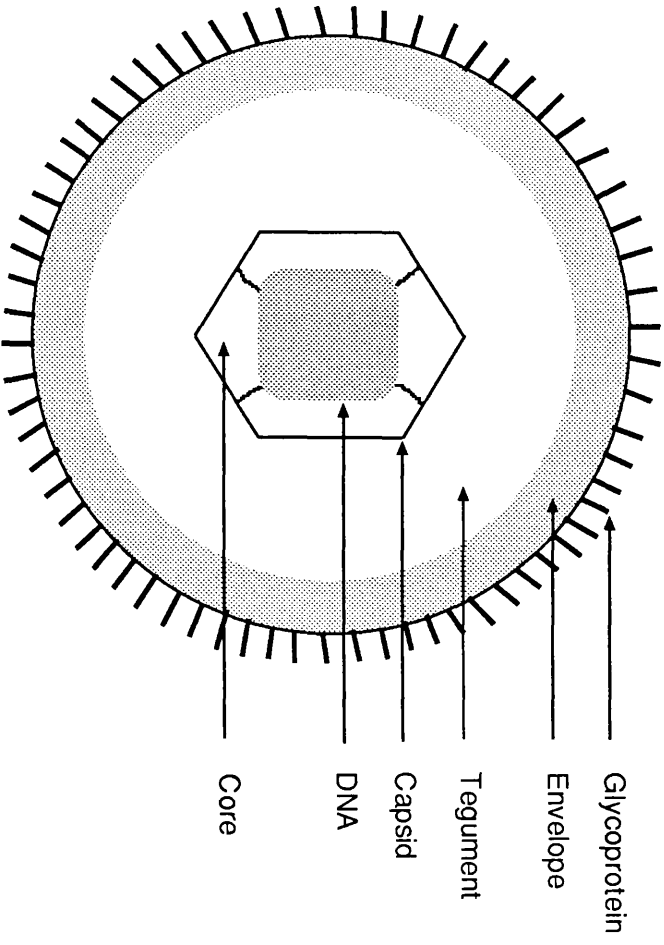
1.1.1 Classification on the basis of biological properties

Using biological properties, the members of the family herpesviridae have been classified into three subfamilies, alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. The biological properties include host range, cytopathogenicity, duration of the replicative cycle and characteristics of the latent infection (Matthews, 1982).

Members of alphaherpesvirinae have a short replicative cycle and grow rapidly in tissue culture resulting in mass destruction of infected cells. They have also the capacity to establish latent infections . This subfamily contains herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), bovine mammilitis virus (BMV), pseudorabies virus (PRV), varicella zoster virus (VZV) and equine herpes virus-1 (EHV-1).

Betaherpesvirinae have restricted host range, long replicative cycles and grow slowly in tissue culture resulting in enlargement of infected cells. The virus can be maintained in a latent form in secretory glands, lymphoreticular cells, kidney and other tissue. This subfamily contains human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV).

Gammaherpesvirinae have limited host range, replicate in lymphoid cells and some cause lytic infections in some types of epithelioid and fibroblastic cells. Latent infections can be established in lymphoid tissue. This subfamily contains herpes virus saimiri (HVS), Epstein-Barr virus (EBV), Mareks disease virus (MDV) and herpes virus ateles.

1.1.2 Classification on the basis of genomic structure

There are three characteristic features of herpesvirus genomes; base composition, size of the genome and arrangement of the reiterated sequences. Molecular weights (Mr) range from 80 to 150 x 10^6, base composition from 32 to 75% G+C and the different sequence arrangements are illustrated in Figure 1.2. On the basis of genomic structure, the viral genomes fall into five groups (Roizman, 1982).

Group A

This group is represented by channel catfish virus (CCV) and their genomes contain a single set of reiterated sequences in the same orientation at the termini (Chousterman et al., 1979).

Group B

The genomes of the members of this group contain multiple reiterations of the same set of sequences at both termini in the same orientation and is represented by HVS (Bornkamm et al., 1976).

Group C

Group C genomes contain multiple reiterations of one set of sequences at both termini in the same orientation and variable numbers of internal tandem reiterations of different sequences. This group is represented by EBV (Raab-Traub et al., 1980).

Group D

Figure 1.2

Schematic diagram of the genomic arrangement of channel

catfish virus (CCV), herpesvirus saimiri (HVS),

Epstein-Barr virus (EBV), pseudorabies virus (PRV) and

herpes simplex virus (HSV) representing subgroups A, B,

C, D and E respectively of the family Herpesviridae.

Lines represent unique sequences (long and short).

Repeat sequences larger than 1kb are shown in open boxes

and those of small reiterated sequences are shown in

shaded boxes.   Arrowheads above boxes indicate the

orientation of the repeat sequences.   Letters a_n and b_n

signify multiple tandem repeat sequences.   The small

terminal direct and internal inverted repeats of HSV

('a' sequence) are indicated.   The type of genomes and

number of isomers are also indicated.
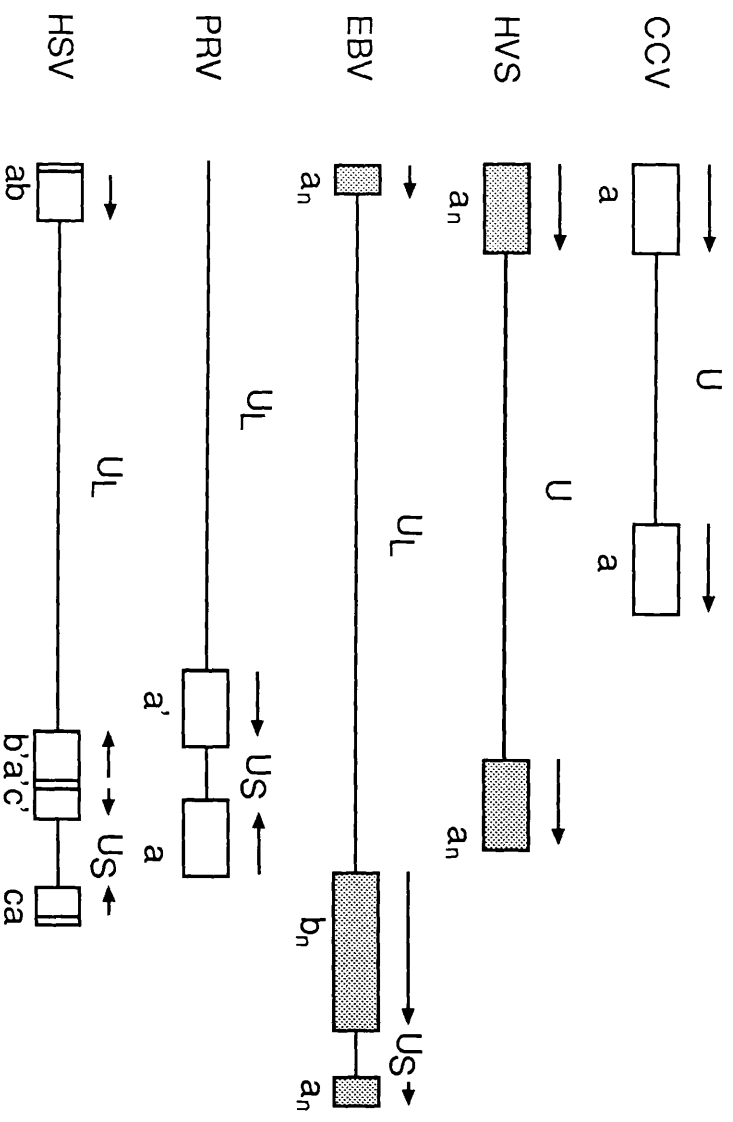
	Genome type	isomer
CCV	A	1
HVS	B	1
EBV	C	1
PRV	D	2
HSV	E	4

This group is represented by PRV (Ben-Porat et al., 1979) and their genomes contain a single set of reiterated sequences from one terminus inverted internally plus a subset of reiterated sequences at the other terminus in the same orientation.

Group E

Genomes of group E viruses contain a single set of sequences from both termini reiterated internally in inverted form as well as a subset of terminal sequences reiterated at both termini in the same orientation. This group is represented by HSV.

1.2 Human herpesviruses

There are six known human herpesviruses which are HSV-1, HSV-2, VZV, EBV, HCMV (Roizman, 1982) and human herpesvirus 6 (HHV-6) (Salahuddin et al., 1986).

Infection with HSV is commonly associated with lesions of mucous membranes where HSV-1 causes mouth and lip lesions (cold sores), while HSV-2 classically is encountered in genital tract infections. Other diseases caused by HSV include eye infection, encephalitis and meningitis (see section 1.10). A characteristic feature of HSV following primary infection at the peripheral site is the establishment of latency in neurons of the peripheral nervous system and reactivation upon stimulation (see section 1.9).

VZV infection causes chicken pox during primary infection whereas in adults the disease shingles is more severe and is a consequence of reactivation from latency (Gleb, 1985).

Infection with HCMV is usually subclinical, but it
causes severe problems in immunocompromised patients
especially those on transplant therapy and with acquired
immunodeficiency syndrome (AIDS) (Hamilton, 1982).

EBV infects B-lymphocytes, is implicated in causing
infectious mononucleosis and is strongly associated with
Burkitts lymphoma and nasopharyngeal carcinoma (Epstein et
al., 1964).

HHV-6 was first described in 1986 and was then termed
human B-lymphotropic virus (Salahuddin et al., 1986). It is
associated with exanthem subitum, a transient childhood
illness (Yamanishi et al., 1988).

1.3 Genomic structure of HSV

1.3.1 General properties of the HSV genome

The HSV genome is a linear double stranded DNA molecule.
The genome of HSV-1 strain 17 has been sequenced and
contains 152260 base pairs (McGeoch et al., 1985; 1986;
1988; Perry and McGeoch, 1988). The DNA consists of two
covalently joined segments, designated as the long (L) and
short (S) regions (Figure 1.3.A) (Roizman, 1979). The L
region consists of a long unique sequence (U_L) flanked by a
pair of oppositely oriented repeated elements termed long
repeat (R_L) with the terminal and internal copies designated
(TR_L) and (IR_L) respectively. Similarly the S region
consists of a short unique (U_S), a short terminal repeat
(TR_S) and a short internal repeat (IR_S) region. The TR_L/IR_L
sequences flanking the U_L segment are designated ab and b'a'
while IR_S/TR_S flanking U_S are designated a'c' and ca
respectively. The sequences of R_L and R_S are distinct,
except for the 'a' sequence, at least one further copy of

Figure 1.3

A. The structure of the HSV-1 genome. The long region

(L) is composed of unique long (U_L) bounded by a

terminal sequence (TR_L) which is repeated internally in

an inverted orientation (IR_L). Similarly, the short

region (S) is composed of a unique region (U_S) bounded

by a terminal sequence (TR_S) which is repeated

internally in an inverted orientation (IR_S). The 'a'

sequence at the termini of the L and S regions and in

inverted orientation at the L/S junction (a') is also

shown. The remaining sequences within TR_L/IR_L and

TR_S/IR_S are designated b/b' and c/c' respectively.

B. The four genome isomers

   P   =   prototype.

   I_L =   inversion of the long region (L).

   I_S =   inversion of the short region (S).

   I_{SL} =   inversion of both short and long regions.

A
ab

TR_L   U_L   IR_L IR_S U_S TR_S

b'a'c'

ca

L   S

A
P
I_L
I_S

B
P
I_L
I_S
ISL
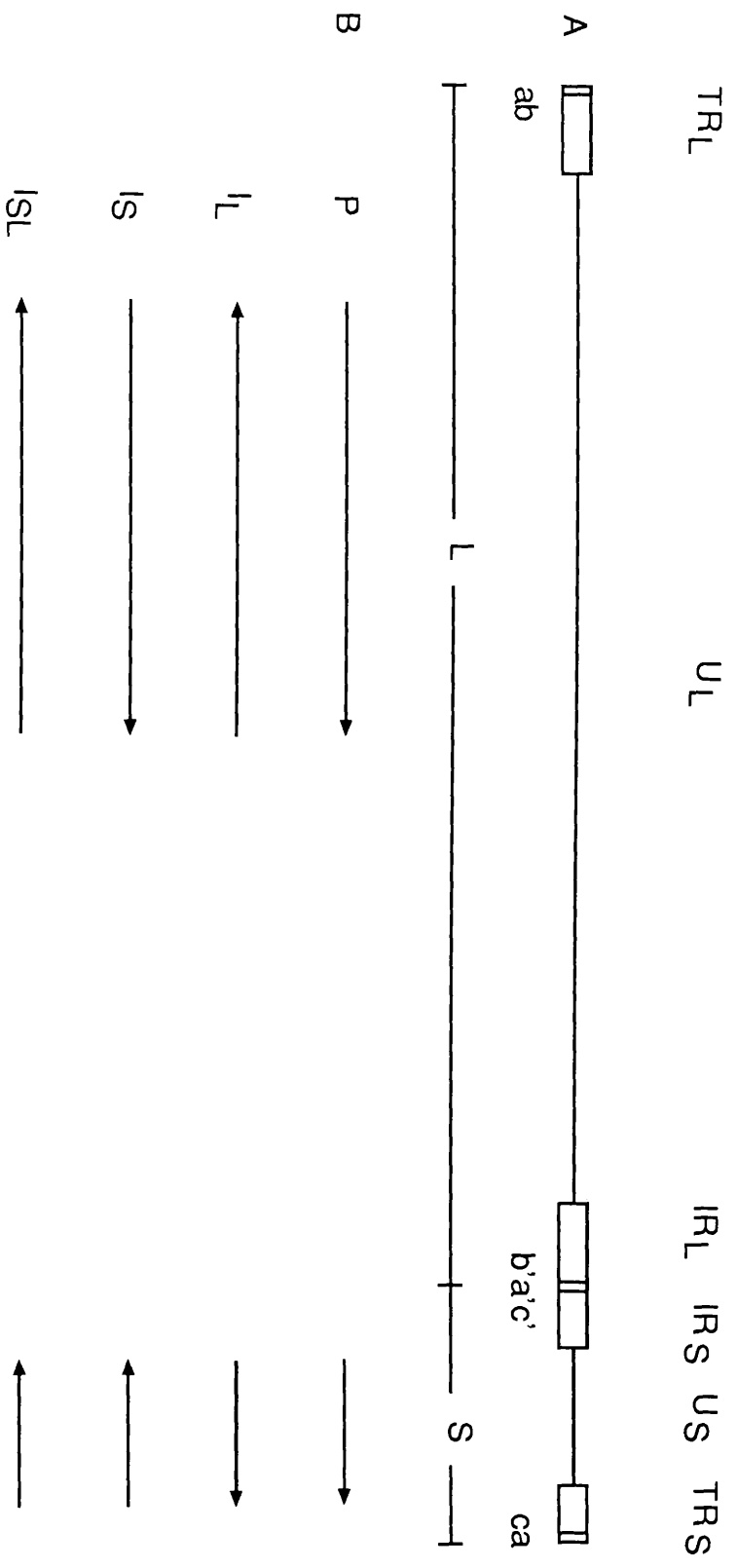
which is found at the L/S joint in the orientation opposite to the terminal copies (Roizman, 1979; Davison and Wilkie, 1981). Each terminus possesses an overhanging residue with its 3'-hydroxyl (OH) group free and lacking a complementary residue on the opposite strand (Mocarski and Roizman, 1982).

Preparations of HSV DNA contain equimolar amounts of four sequence-oriented isomers, in which U_L and U_S are independently in one of two possible orientations with respect to the joint between L and S (Roizman, 1979). The isomers are shown in Figure 1.3.B and are designated as prototype (P), inversion of the S region (I_S), inversion of the L region (I_L) and inversion of both S and L regions (I_{SL}).

HSV-1 DNA has an overall base composition of 68.3% G+C (McGeoch et al., 1988) which is not uniform throughout the genome e.g the 6.6 kb R_S elements have a G+C value of 79.5% (McGeoch et al., 1986). In the HSV genome there are families of short, directly repeated sequences ranging in size from 10 to 100 bp (McGeoch, 1989). The copy number of these repeats differs between virus stocks (Rixon et al., 1984). Three of these repeats are located within the coding region of the US7 gene and in the intergenic region of the US9 and US10 genes (McGeoch et al., 1985). The other repeats present in the S region are (i) in the introns of IE mRNA4 and 5 (Murchie and McGeoch, 1982) and (ii) downstream of the IE mRNA3 (Rixon et al., 1984). There are also other repeats in the 'a' sequence (Davison and Wilkie, 1981; Mocarski and Roizman, 1981) upstream and downstream of the IE mRNA1 (Perry et al., 1986). The only repeat family in the U_L region is present within the coding region of the UL36 gene (McGeoch et al., 1988).

1.3.2 Organisation of HSV genes

The open reading frames (ORF) corresponding to the proposed HSV-1 genes in Glasgow strain 17 are shown in Figure 1.4. It has been shown that U_L contains 56 genes termed UL1 to UL56 (McGeoch et al., 1988), while U_S contains 12 genes designated US1 to US12 (McGeoch et al., 1985). There is also one gene in each copy of R_L and R_S, giving a total of 72 genes encoding for 70 distinct proteins. Chou and Roizman (1986) and Ackermann et al. (1986) have postulated another gene upstream of the IE1 gene and in the same orientation, but their interpretation has been questioned (Perry and McGeoch, 1988). In addition, there is a 3500 bp region in R_L downstream of IE1, whose functional potential is obscure. The latency associated transcripts (LAT), which are the major virus-specific transcripts in latently infected neurons lie in R_L downstream of the IE1 gene (Stevens et al., 1987; Wagner et al., 1988; see section 1.9).

The genes in HSV-1 are classified into three categories; those with control functions; those involved in DNA replication and associated functions and those encoding structural and assembly proteins. During HSV infection, the IE genes are first expressed. Five IE genes have been identified, IE1, IE2 (UL54), IE3, IE4 (US1) and IE5 (US12) encoding for VmwIE110, VmwIE63, VmwIE175, VmwIE68 and VmwIE12 respectively (see section 1.5.2). The functions of the products of the first three IE genes are transcription and regulation, controlling the expression of later classes of genes and autoregulation of the IE genes (Everett, 1987; see section 1.5.2). The functions of the products of the

Figure 1.4

Organization of the genes of HSV-1, represented on four

successive lines of 40 kbp each. Locations of the

reading frames are shown by arrows and the splicing

within the coding regions is indicated. Genes UL1-UL56

are shown on the top three lines as 1-56 and genes

US1-US12 are shown in the bottom line as 1-12.

Locations of origins of DNA replication are indicated by

vertical arrows. This Figure is taken from McGeoch

(1989).

IE110

IE110

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

20 21 22 23 24 25 26 27 28 29 oriL 30 31 32 33 34 35 36

37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56

IE175

oriS

IE175

oriS

1 2 3 4 5 6 7 8 9 10 12 11 oriS
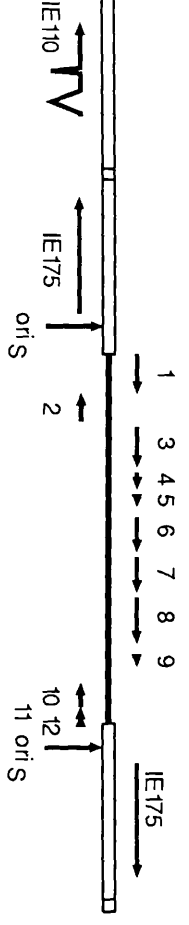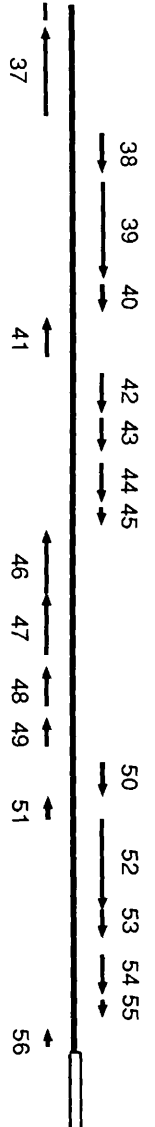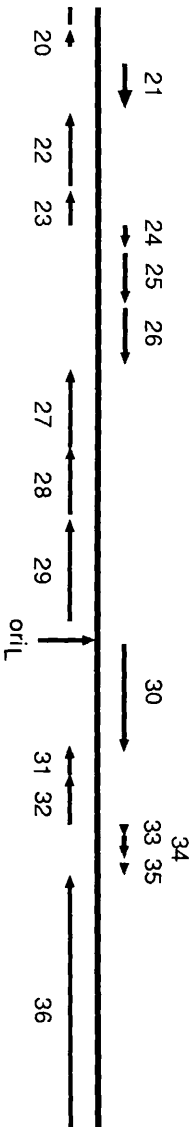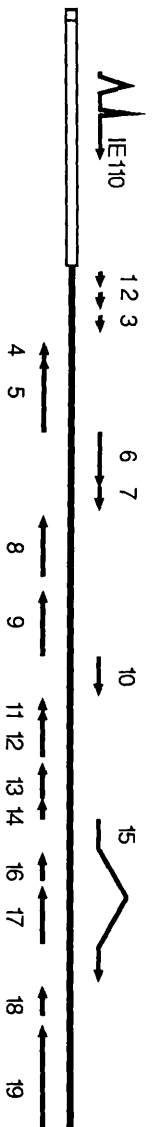
IE4 and IE5 genes remain unknown.

The Vmw65 trans-inducing factor (Vmw65 TIF) encoded by the UL48 gene (Campbell et al., 1984), has a clear regulatory role. It acts in combination with one or more cellular proteins to activate IE gene expression through the upstream sequences of these genes (O'Hare and Goding, 1988; Preston et al., 1988). Another regulatory protein, is the product of UL41, which acts by shutting off host cell macromolecular synthesis by degradation of mRNA (Kwong et al., 1988). Two other genes, US3 and UL13, which have recently been identified and proposed to encode protein kinases may have a role in control and modulation of HSV infection.

The second class of genes include those whose products are involved in nucleotide or nucleoside metabolism, DNA replication and DNA metabolism and repair. Of those involved in nucleotide and nucleoside metabolism, thymidine kinase (TK) has been extensively studied (see section 1.8.4.a). More recently ribonucleotide reductase (see section 1.8.4.a) encoded by the UL39 and UL40 genes (Preston et al., 1984; Frame et al., 1985) and deoxyuridine triphosphatase (dUTPase) encoded by the UL50 gene (Preston and Fisher, 1984) have been identified and analysed. Seven genes were found to be necessary for DNA replication; UL5, UL8, UL9, UL29, UL30, UL42 and UL52 (see section 1.6.3). The deoxyribonuclease encoded by the UL12 gene has an unknown role in replication. Finally the gene UL2 encodes the DNA repair enzyme, uracil-DNA glycosylase (Worrad and Caradonna, 1988; Mullaney et al., 1989).

Other elements of the HSV genome which have specific roles in DNA replication are (1) the origins of DNA

replication; ori$_L$, located between genes UL29 and UL30 and two copies of ori$_S$ in R$_S$ between genes IE3 and IE4 in IR$_S$ and IE3 and IE5 in TR$_S$ (see section 1.6.3) and (2) the 'a' sequence which contains elements involved in cleavage of concatameric DNA as part of the process of packaging of DNA into nascent virions (see section 1.4.2).

The third class of genes consists of those encoding structural proteins and proteins involved in assembly of virions. Herpes virus particles are complex assemblies containing at least 30 different species of protein (Dargan, 1986). Twenty two genes have been identified as specifying structural or assembly proteins. The major capsid protein (MCP) is encoded by gene UL19 (Costa et al., 1984; Davison and Scott, 1986), although the capsid contains at least seven other proteins whose genes have not been identified (McGeoch, 1989). The major tegument protein is encoded by the UL48 gene (Campbell et al., 1984) and other tegument proteins are encoded by the UL36, US9 and US10 genes (Batterson et al., 1983; Frame et al., 1986a; Rixon and McGeoch, 1984).

Seven viral glycoproteins have been identified (see section 1.8.4.d) and these are gH (encoded by gene UL22), gB (UL27), gC (UL44), gG (US4), gD (US6), gI (US7) and gE encoded by the US8 gene (McGeoch, 1989).

1.3.3 Relationship between HSV-1 and HSV-2 genomes

HSV-1 and HSV-2 are closely related viruses and show many serological cross reactions. DNA hybridisation studies have shown that most of the genome sequences are highly related (Davison and Wilkie, 1983). It is likely that the genetic organisation of HSV-2 is indistinguishable from that

of HSV-1. Around 33 kb of the HSV-2 genome has been

sequenced. This includes the 'a' sequence, ori_L, ori_S, the

equivalents of the HSV-1 genes, UL2, UL11, UL12, UL23, UL27,

UL39, UL40, UL44, UL45, US2, US7, US12 and parts of the

UL30, UL54, US1, US8, US10 and IE3 genes (McGeoch, 1989).

Direct comparison of the coding sequences of corresponding

genes has shown 70-80% identities (McGeoch et al., 1987).

The greatest differences were shown in the non-coding

sequences which are highly divergent especially in parts of

the major repeats (Davison and Wilkie, 1981; Whitton and

Clements, 1984). A difference was found between the two

genomes in their US4 genes, encoding gG (McGeoch et al.,

1987). HSV-1 has a deletion of 1460 bp in its US4 coding

sequence relative to HSV-2, therefore gG of HSV-1 is of

smaller Mr than that of HSV-2.

1.4 The HSV 'a' sequence

1.4.1 General properties of the 'a' sequence

The HSV genome contains a 200-500 bp sequence called the

'a' sequence, situated at the junction between the L and S

components and repeated in an inverted orientation at the

termini of the genome (Wadsworth et al., 1975). Multiple

copies of the 'a' sequence can be present at the L terminus

and the L-S junction, but only one copy is present at the S

terminus (Wagner and Summers, 1978; Locker and Frenkel,

1979; Davison and Wilkie, 1981; Mocarski and Roizman, 1981).

The size variation of the 'a' sequence between different

strains has been shown by DNA sequencing of the 'a' sequence

(Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982;

Mocarski et al., 1985; Varmuza and Smiley, 1985).

The 'a' sequence of HSV-1 strain F is shown in Figure

1.5 (Mocarski and Roizman, 1982). It is arranged as a
head-to-tail dimer which has one copy of the direct repeat
(DR1) at the point of fusion. The 'a' sequence consists of
(1) a 20 bp terminal repeat designated DR1 (2) a 64 bp
unique sequence designated Ub (3) 22 repeats of a 12 bp
sequence designated DR2 (4) three repeats of a 37 bp
sequence designated DR4 and containing 11 of the 12
nucleotides of DR2 (5) a unique 58 bp sequence designated Uc
and (6) a second copy of DR1. Variability of the copy
number of DR elements accounts for most of the observed 'a'
sequence polymorphism in different strains. For example,
HSV-2 strain HG52 has only one copy of DR2 (Davison and
Wilkie, 1981) while HSV-1 strain F has 22 copies (Mocarski
and Roizman, 1982). Similarly HSV-1 strain 17 and HSV-2
strain HG52 contain one copy of DR4 homology, which is a
part of Uc (Davison and Wilkie, 1981). Some strains of
HSV-1 like Justin contain an extra direct repeat called DR3
which is present in two copies (Mocarski and Roizman, 1981,
1982; Mocarski et al., 1985). Although there is variation
in the copy number of the DR2 and DR4 elements, some regions
of the 'a' sequence are highly conserved in different
strains. These are within Ub and Uc and are represented by
a short well conserved sequence about 20 bp in length
located approximately 40 bp and 35 bp respectively from the
ends of the 'a' sequence (Davison and Wilkie, 1981; Deiss et
al., 1986).

1.4.2 Functions of the 'a' sequence

The 'a' sequence has a number of functions associated
with genome organisation and viral DNA replication. These
functions are described in the following section.

Figure 1.5

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

DR1 =   20 bp elements present as a direct repeat at
           the ends of the 'a' sequence.

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

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

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

Uc =   a unique sequence of 58 bp located towards the
           c' sequence.

TR_L   U_L   IR_L IR_S U_S TR_S
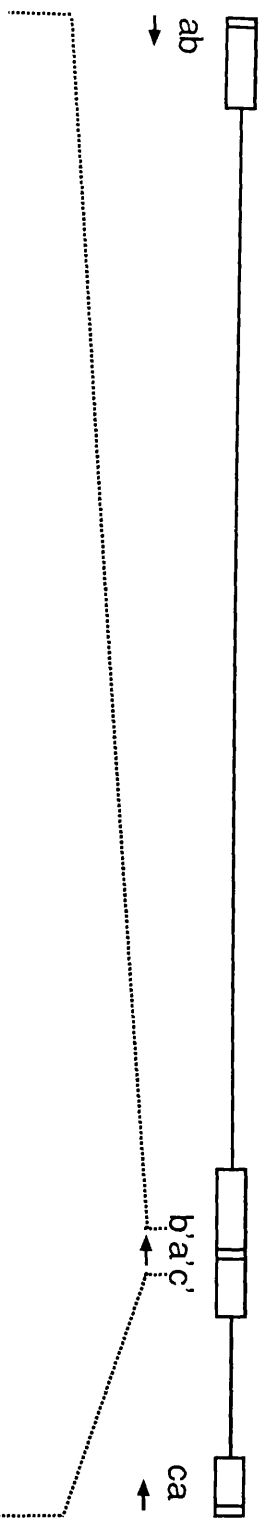
ab   b'a'c'   ca

b'   DR1   Ub   DR2   DR4   Uc   DR1   c'

(1) Circularisation of the genome

Circularisation of the HSV genome soon after infection is believed to be controlled by the 'a' sequence (Davison and Wilkie, 1983; Poffenberger et al., 1983). Circularisation of the genome occurs by ligation of the two termini which posses a single base overhang on the opposite strands (Mocarski and Roizman, 1982).

(2) Isomerisation of the genome

HSV DNA contains equimolar amount of four isomers differing in the orientation of U_L and U_S (see section 1.3.1). Inversion of the genome at the L-S junction occurs by site specific recombination mediated by the 'a' sequence (Mocarski et al., 1980; Mocarski and Roizman, 1981; Smiley et al., 1981). The role of the 'a' sequence in inversion and isomerisation of the genome has been demonstrated by Chou and Roizman (1985) who carried out detailed deletion analysis of the 'a' sequence. They found that deletion of the DR4 sequence results in impairment of genomic inversion, while deletion of DR2 and DR4 sequences abolished inversion. This demonstrates the presence of a cis-acting signal for site specific recombination and inversion in the DR2 and DR4 elements. The authors however did not exclude the role of DR1 in inversion, since deletion of both DR1 elements resulted in an extremely low frequency of inversion (Varmuza and Smiley, 1985).

(3) Cleavage and packaging of the genome

Linear viral DNA molecules circularise soon after infection and newly replicated viral DNA molecules consist

of large head-to-tail concatamers (Ben-Porat et al., 1976; Jacob et al., 1979) which are subsequently cleaved and packaged into capsids (Vlazney et al., 1982). The HSV 'a' sequence has been shown to contain cis-acting signals for cleavage/packaging of viral genomes and plasmid constructs containing an HSV DNA origin of replication can replicate but are only packaged if they contain an 'a' sequence (Stow et al., 1983; Deiss and Frenkel, 1986). Two separate signals designated Pac1 and Pac2, located in the Ub and Uc regions respectively of the 'a' sequence, were essential for cleavage/packaging of viral genomes (Varmuza and Smiley, 1985; Deiss et al., 1986). Recently Nasseri and Mocarski (1988), using a transient cleavage assay, have shown that a 179 bp fragment (containing Uc - DR1 - Ub) from the junction of two tandem 'a' sequence carries all the sequence elements necessary for cleavage recognition and encapsidation.

(4) Promoter activity

It has been reported that the Ub region of the 'a' sequence of HSV-1 strain F contains the promoter of a gene encoding a polypeptide ICP34.5 whose coding region is in the long repeat in the same orientation as the IE1 gene (Ackermann et al., 1986; Chou and Roizman, 1986). The gene encoding ICP34.5 lacks TATA consensus and instead there is TATA homology (TTTAAA). The 5' terminus of the transcript maps to DR1. The function of this gene is not clear and its sensitivity to phosphonoacetate suggests that it belongs to the late category genes. DNA sequence analysis of the corresponding region of HSV-1 strain 17 has not identified a similar ORF (Perry and McGeoch, 1988).

(5) Protein binding

The HSV 'a' sequence encodes several cis-acting sites involved in circularisation, inversion, cleavage and packaging of DNA and expression of mRNA. It would be expected that specific viral or host proteins or both interact with the DNA at the specific cis sites to mediate such functions. Wu et al. (1979) using the electron microscope, have shown that a small polypeptide binds to both termini and to L-S junctions of DNA extracted from virions. Similarly Dalziel and Marsden (1984) have shown that the late polypeptides (21K and 22K) encoded by gene US11 (Rixon and McGeoch, 1984) interact with the 'a' sequence of HSV-1 in vitro. These polypeptides were shown to be strong binding proteins (MacLean et al., 1987). The function of the interaction with the 'a' sequence is still not clear, since viruses lacking the US11 gene are viable in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

Recently Chou and Roizman (1989) have reported that, the virus-specific DNAase as well as two proteins with Mr of >250K and 140K, form complexes with both Pac1 and DR1 regions of the 'a' sequence.

1.5 HSV transcription

Transcription of viral DNA takes place in the nucleus by the host cell polymerase II (Costanzo et al., 1977). Viral RNAs are capped at their 5' ends, methylated at A residues and polyadenylated although non-polyadenylated RNA can be isolated (Bachenheimer and Roizman, 1972; Bartoski and Roizman, 1976; Moss et al., 1977; Stringer et al., 1977). A relatively small proportion of HSV mRNAs are spliced; these

include mRNAs of IE1, IE4, IE5 (Rixon and Clements, 1982; Watson et al., 1981; Perry et al., 1986), UL15 (McGeoch et al., 1988) and the mRNA of gC (Frink et al., 1983). The abundance and the stability of the various HSV-1 mRNAs vary and generally mRNAs of IE genes appear more stable than those of late genes (Wolf and Roizman, 1978). Following transcription, HSV mRNAs are transported to the cytoplasm, bind to ribosomes and are translated into proteins (Wagner and Roizman, 1969).

1.5.1 HSV temporal regulation

HSV encodes 70 predicted genes (McGeoch et al., 1988) which are expressed as three temporally regulated classes of proteins, immediate early (IE or alpha), early (E or beta) and late (L or gamma) according to their order of synthesis (Honess and Roizman, 1974; Clements et al., 1977; Jones and Roizman, 1979). Synthesis of these proteins is sequentially ordered in a cascade fashion. IE proteins induce E proteins and the latter shut off the expression of IE proteins and induce the expression of L proteins. The L proteins in turn shut off the expression of E proteins, and immediately after the next round of infection, induce the expression of IE proteins. Regulation of viral gene expression would appear to be predominantly at the level of transcription.

1.5.2 Immediate early (IE) gene expression

IE genes are the first to be expressed during viral infection and are first detected very early (1 h post infection), reach peak rates at approximately 2 to 4 h following infection and then decrease. Their corresponding RNAs are produced in the absence of viral protein synthesis

(Honess and Roizman, 1974). There are five IE genes in HSV-1, their transcript locations and orientations on the viral genome are shown in Figure 1.6. These are IE1, IE2, IE3, IE4 and IE5 coding for polypeptide products VmwIE110 (ICP0), VmwIE63 (ICP27), VmwIE175 (ICP4), VmwIE68 (ICP22) and VmwIE12 (ICP47) respectively (Clements et al., 1979; Preston, 1979a; Easton and Clements, 1980; Wagner, 1985; Everett, 1987).

Three of the five IE transcripts are spliced. The IE4 and IE5 transcripts are derived from identical promoters located in the repeated sequences of the S region of the viral genome. The splice sites of their RNAs are also in the repeated sequences while their coding regions are different (Watson et al., 1981; Rixon and Clements, 1982). The transcript of the IE1 gene is also spliced and lies totally within the repeated sequences of the L region and is therefore diploid (Perry et al., 1986). The transcript of the IE3 gene is unspliced, diploid and transcribed entirely within TR_S (Rixon et al., 1982). The only IE gene that is not associated with the repeated sequences is IE2 whose unspliced RNA lies in U_L (Whitton et al., 1983).

Most of the IE polypeptides are phosphorylated and found predominantly in the nucleus of infected cells. The exceptional IE polypeptide is VmwIE12 which is not phosphorylated and is found in the cytoplasm (Preston, 1979a; Hay and Hay, 1980; Marsden et al., 1982; Ackermann et al., 1984). The IE1 and IE3 gene products have been shown to be potent transactivators of early and late gene promoters in transient expression assays (Everett, 1984, 1987; O'Hare and Goding, 1988). The IE2 gene product (VmwIE63) has also been implicated in the regulation of

Figure 1.6

The genome structure of HSV-1 (prototype) showing the map locations and orientations of the immediate early (IE) mRNAs (numbered 1 to 5). The spliced mRNAs are indicated.

0   0.1 0.7   0.8   0.9   1.0

TRL   UL   IRL IRS   US   TRS

1   2   1   3   4   5   3
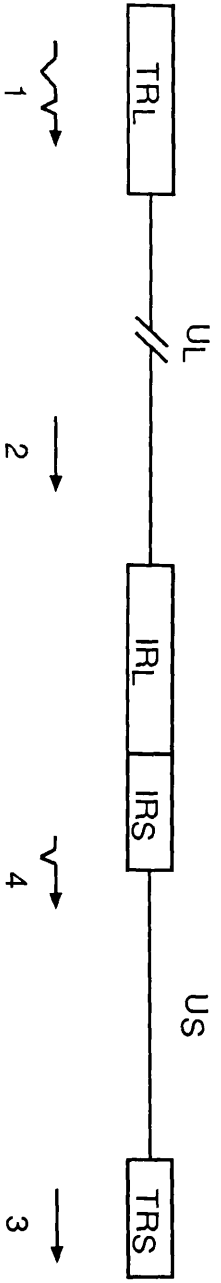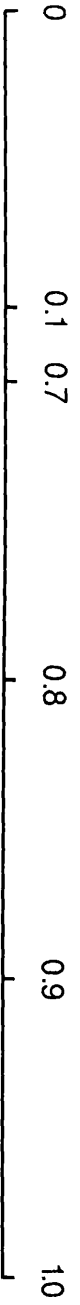
viral promoters (Everett, 1986; Rice and Knipe, 1988; Sekulovich et al., 1988).

Analysis of ts mutants demonstrated that the gene products of both IE2 (VmwIE63) and IE3 (VmwIE175) are essential for productive infection and that VmwIE175 is required for early and late gene expression while VmwIE63 appears to be required after the onset of early gene expression and DNA replication (Preston, 1979b; Dixon and Schaffer, 1980; Watson and Clements, 1980; Sacks et al., 1985). Viable mutants with deletions in IE1 show growth restriction in certain cell types at low multiplicity of infection (moi) and normal growth at high moi (Stow and Stow, 1986; Sacks and Schaffer, 1987). Deletion mutants in IE4 confer a host range phenotype to the virus (Post and Roizman, 1981; Sears et al., 1985). The IE5 gene has been shown to be non essential for the growth of HSV in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

All the IE genes contain a characteristic cis-acting element, TAATGARAT, which interacts with the major tegument protein (Vmw65 TIF) resulting in stimulation of IE gene expression (Mackem and Roizman, 1982; Batterson and Roizman, 1983; Cordingley et al., 1983; Campbell et al., 1984; Preston et al., 1984). Mutations within the gene coding for Vmw65 TIF abolish its ability to stimulate IE gene expression (Ace et al., 1989).

1.5.3 Early (E) gene expression

The early or beta genes are distributed in both the L and S regions of the HSV genome. Their transcription begins following the synthesis of functional IE polypeptides

(Honess and Roizman, 1974). The synthesis of the E gene products starts at 3 h post infection, reaches peak rates of synthesis about 5 to 7 h post infection after which it declines with time (Honess and Roizman, 1974).

The E genes have been subdivided into beta 1 and beta 2 on the basis of their requirement of IE polypeptides (Pereira et al., 1977; Roizman and Batterson, 1985). The functions of several E polypeptides appear to be related to the synthesis of viral DNA. The beta 1 polypeptides are exemplified by the major DNA binding protein (DBP) and the large subunit of ribonucleotide reductase (RR1).

The beta 2 polypeptides include the viral TK and the DNA polymerase. Some early polpeptides require DNA synthesis for maximal expression and gD is an example of such a protein (Gibson and Spear, 1983; Johnson et al., 1986).

1.5.4 Late (L) gene expression

The late or gamma gene products can be detected by 2 to 3 h post infection and reach their maximum peaks by 10 to 16 h post infection parallel to DNA synthesis which starts about 2 h post infection and reaches its maximum peak by 8 h post infection (Wilkie, 1973; Roizman, 1979).

The late genes can be subdivided into two classes, the gamma 1 (leaky late) and gamma 2 (true late), depending on their DNA replication requirement (Roizman and Batterson, 1985; Wagner, 1985). In the absence of DNA synthesis, the expression of gamma 1 genes is reduced but still detectable, while those of gamma 2 is reduced to barely detectable levels (Powell et al., 1975; Johnson et al., 1986; Godowski

and Knipe, 1985). Examples of the leaky late genes are UL19, UL27 encoding the MCP and gB respectively, while UL44 and US11 encoding gC and 21K/22K proteins are examples of true late genes.

The regulation of late gene expression is not fully understood. Some have been shown to require IE products for maximum trans-activation of their promoters (Everett, 1987).

1.6 The HSV replicative cycle

The replicative cycle of HSV starts with the initiation of infection in which the virus envelope attaches to cell receptors resulting in fusion with plasma membranes and release of the capsid which is then transported to the nuclear pore where DNA is released into the nucleus. Transcription, DNA replication and assembly of capsids takes place in the nucleus. The steps of the replicative cycle are discussed below.

1.6.1 Attachment, penetration and uncoating

Attachment of the virus to the host cell surface takes place through specific receptors on the cell surface (Vahlne et al., 1978). The nature of these receptors has not been identified. It appears that HSV-1 and HSV-2 have different receptors. Infection of cells with one virus strain causes interference with the attachment of a second virus strain of homologous but not heterologous serotype (Vahlne et al., 1979). Following attachment, the virus enters the cells either by endocytosis (Hummeler et al., 1969) or by fusion of the viral envelope and plasma membrane (Morgan et al., 1969). The latter is more likely to be the case since it has been demonstrated that the viral envelope fragment

crystalisable (Fc) receptors can be detected on the cell surface following penetration (Para et al., 1980). The identification of virus-encoded gB (Manservigi et al., 1977) which is involved in membrane fusion supports the fusion mode of entry of HSV into cells. Temperature-sensitive mutants (ts) which are defective in the gB gene can attach but are unable to penetrate the cells (Sarmiento et al., 1979).

Following penetration, the naked viral particle is transported via the cytoplasm (Batterson and Roizman, 1983) to the nuclear membrane where the capsid is degraded and the viral DNA released. The DNA enters the nucleus through the nuclear pores (Hummeler et al., 1969) where transcription occurs using the cellular polymerase II (see section 1.5).

1.6.2 Suppression of cellular macromolecular synthesis

Infection with HSV results in a decrease in the host cell DNA, RNA and protein synthesis. An immediate consequence of infection is the disaggregation of polyribosomes which is mediated by a component of the infecting virion. This process results in inhibition of host protein synthesis (Sydiskis and Roizman, 1966; Fenwick and Walker, 1978; Nishioka and Silverstein, 1978). The virion component is also involved in inhibition of host mRNA. These events called early shut off of protein synthesis, are more rapid in HSV-2 infected cells than in HSV-1 infected cells (Fenwick and Clark, 1982; Fenwick and McMenamin, 1984). Delayed inhibition of host protein synthesis depends on virus gene expression which results in the degradation of host transcripts (Nishioka and Silverstein, 1978; Hill et al., 1983). The virion function

involved in shut off of host protein synthesis has been
mapped to the region between 0.52-0.59 map units (m.u.) on
the HSV-2 strain HG52 genome (Morse et al., 1978; Fenwick et
al., 1979) and to 0.604-0.606 m.u. on the HSV-1 strain KOS
genome (Kwong et al., 1988). In addition, transfer of the
UL41 equivalent gene from HSV-2 strain G (strong shut off)
into the HSV-1 strain 17 genome (naturally weak shut off)
results in enhancement of the shut off function of strain 17
(R.D. Everett, personal communication). The mechanism by
which HSV decreases the rate of host cell DNA synthesis
remains unclear.

1.6.3 DNA replication

Replication of HSV is a complex process which is not
fully understood. Soon after infection, viral DNA
circularises by ligation of the termini in the nucleus
(Davison and Wilkie, 1983). DNA synthesis starts at 3 h
post infection, reaches a maximum between 9-11 h and is
completed by 16 h post infection (Rixon, 1977).

Electron microscope analysis of viral DNA molecules
before the onset and during the first round of DNA
replication showed the presence of single stranded ends and
circular molecules (Jean and Ben-Porat, 1976; Jacob and
Roizman, 1977). At later times in infection, DNA molecules
with internal eyes and forks, circular molecules with linear
tails and large tangles of DNA are seen (Ben-Porat et al.,
1976; Hirsch et al., 1977; Jacob and Roizman, 1977; Jean et
al., 1977; Ben-Porat and Rixon, 1979). It has been shown
that the number of terminal fragments detectable in
replicating viral DNA was reduced compared to virion DNA
(Jongeneel and Bachenheimer, 1981). This data suggested

that circular DNA molecules replicate by a rolling circle mechanism yielding head-to-tail concatameric DNA (Jacob et al., 1979).

Two approaches have indicated that the products of seven genes (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) are involved in viral DNA replication. The first approach, using genetic analysis of HSV-1 mutants, has shown that seven complementation groups containing mutants with defects in DNA synthesis map to these genes (Chartrand et al., 1979; Conley et al., 1981; Purifoy and Powell, 1981; Matz et al., 1983; Weller et al., 1983, 1987; Carmichael et al., 1988; Goldstein and Weller, 1988; Marchetti et al., 1988). The second approach, using a plasmid amplification assay for HSV-1 origin-dependent DNA replication, has demonstrated that the products of these seven genes are both necessary and sufficient for DNA synthesis (Challberg, 1986; McGeoch et al., 1988; Wu et al., 1988). Three of these seven DNA replication genes are UL29 which encodes for the major single strand DNA binding protein (Conley et al., 1981; Quinn and McGeoch, 1985), UL30 which encodes the viral DNA polymerase (Gibbs et al., 1985; Quinn and McGeoch, 1985) and UL42 which encodes a 65K double stranded DNA binding protein, DBP, (Marsden et al., 1987; Parris et al., 1988). Recently Weir et al. (1989), using a gel retardation assay, showed that the UL9 gene product binds specifically to a viral origin of replication. These results are consistent with the findings of Olivo et al. (1988) who showed that the UL9 gene specifies an origin binding protein. The UL5 gene product is a part of the DNA helicase in infected cells (Zhu and Weller, 1988). The DNA helicase has been shown to consist of three polypeptides with Mr of 120.000, 97.000 and

70.000 which are the products of genes UL52, UL5 and UL8
required for replication of a plasmid containing HSV-1 ori$_S$
(Crute et al., 1989). Furthermore, the DNA primase (Holmes
et al., 1988) has been found to be tightly associated with
the DNA helicase forming a helicase-primase complex (Crute
et al., 1989). The relationship between the two enzymes
remains unclear.

The cis-acting elements needed for replication (viral
origins of DNA replication) are specified by two distinct
but related sequences. One (ori$_L$) lies close to the centre
of the U$_L$ region while the other (ori$_S$) is present within
the short repeats, TR$_S$ and IR$_S$ (Vlazney and Frenkel, 1981;
Spaete and Frenkel, 1982; Stow, 1982; Weller et al., 1985).
It has been shown that a DNA fragment of 90 bp specifies a
functional ori$_S$ (Stow and McMonagle, 1983; Deb and Doelberg,
1988; Lockshon and Galloway, 1988) and includes a 45 bp near
perfect palindrome with a central A+T rich region. Elias
and Lehman (1988) have demonstrated the presence of two
specific binding sites within ori$_S$.

The ori$_L$ is located in U$_L$ between 0.360 and 0.419 m.u.
However, when fragments spanning these sequences were cloned
into plasmid vectors, between 100-650 bp were deleted. The
deleted fragments have no origin activity (Spaete and
Frenkel, 1982). Successful cloning of ori$_L$ in an undeleted
form was achieved in yeast plasmid vectors (Weller et al.,
1985). Sequence analysis of ori$_L$ from HSV-1 KOS (Weller et
al., 1985), HSV-1 ANG (Gray and Kaerner, 1984) and HSV-1
strain 17 (Quinn and McGeoch, 1985) has shown that ori$_L$
contains a large palindrome (144 bp) which is responsible
for the deletion and cloning instability. The palindrome
constitutes the minimum requirement of ori$_L$ (McGeoch, 1987)

with a high degree of homology to ori_S. Sequence analysis

of HSV-2 ori_L showed a strong degree of homology to the

HSV-1 sequence especially its palindrome (Lockshon and

Galloway, 1986). The significance of why the HSV genome has

three origins of replication and their contribution to DNA

replication is unknown. Mutant viruses lacking either one

copy of ori_S (Longnecker and Roizman, 1986; Brown and

Harland, 1987) or ori_L (Polvino-Bodnar et al., 1987), grow

normally in vitro.

1.6.4 Assembly and maturation

Replicating viral DNA forms a pool of concatameric

molecules which are cleaved into unit lengths and packaged

into empty capsids in the nucleus (see section 1.4..2). The

nuclear capsid containing the DNA attaches to the inner

nuclear membrane and acquires an envelope by budding into

the perinuclear space (Dargan, 1986; Roizman and Batterson,

1986). It has been reported that herpesviruses can acquire

their envelopes by budding into the cytoplasmic vacuoles or

Golgi membranes (Nii, 1971; Haguenau and Michelson-Fiske,

1975). Virus particles maturing from different sites may

exhibit morphological differences in their envelopes. It

has been noted that HSV particles derived from nuclear

membranes are circular and electron-dense, while those

derived from cytoplasmic membranes have projections or

globular structures on the surface (Nii, 1971).

Viruses egress from infected cells by a reverse

phagocytosis process which is thought to happen when virus

particles acquire envelopes from the cytoplasmic membrane.

These particles bud into cytoplasmic vacuoles and are

transported via the cytoplasm to the plasma membrane.

Fusion of the vacuolar membrane with the plasma membrane extrude the enveloped viruses into the extracellular space (Nii, 1971; Katsumoto et al., 1981). Those virus particles that acquire envelopes from the inner nuclear membrane move along the reticulo-endothelial system and then either pass directly into the extracellular space or extrude by vacuole formation and reverse phagocytosis (Nii, 1971; Fong et al., 1973).

1.7 HSV-induced polypeptides

Following HSV infection, approximately 50 HSV-induced polypeptides have been identified on SDS-PAGE (Powell and Courtney, 1975; Marsden et al., 1976). Over 230 distinct polypeptides have been recognised using two dimensional PAGE (Haarr and Marsden, 1981). This greater number may be due in part to the resolution of families of peptide species related through post translational modification. The occurrence of post translational modification is reflected by the failure of some in vitro synthesised viral polypeptides to comigrate with their in vivo synthesised counterparts on SDS-PAGE (Preston, 1977). Four main post translational events occur comprising glycosylation (Haarr and Marsden, 1981; Palfreyman et al., 1983; Hope and Marsden, 1983), phosphorylation (Marsden et al., 1978; Wilcox et al., 1980), sulphation (Hope et al., 1982; Hope and Marsden, 1983) and cleavage (Eisenberg et al., 1984; Balachandran and Hutt-Fletcher,1985). However, other forms of HSV polypeptide modification have been reported e.g fatty acylation which includes palmitylation of several surface glycoproteins (Schmidt, 1982) and myristylation of the HSV-1 UL11 gene product (MacLean et al., 1989).

1.7.1 HSV-induced enzymes

During the course of infection, many viral enzymes are expressed by HSV. Most of them are involved in nucleic acid metabolism and some of them are associated with virus particles. These enzymes are briefly described below.

The HSV-induced DNA polymerase, which is distinguishable from the host cell polymerase (Keir and Gold, 1963; Keir et al., 1966), has a Mr of 136.272 in HSV-1 strain 17 as deduced from DNA sequence data (Quinn and McGeoch, 1985). It has been mapped to the UL30 gene between 0.40-0.42 m.u. of the genome and been shown to be essential for virus DNA replication (Hay and Subak-Sharpe, 1976; Chartrand et al., 1979, 1980). The HSV DNA polymerase has an associated 3' to 5' exonuclease activity (Knopf, 1979).

The thymidine kinase (TK) encoded by HSV has been mapped to the UL23 gene between 0.300-0.309 m.u. and sequenced (McKnight, 1980, Wagner et al., 1981). This enzyme phosphorylates thymidine and deoxycytidine (Jamieson and Subak-Sharpe, 1974). TK-negative viruses have been shown to have reduced pathogenicity (see section 1.8.4.a).

The alkaline exonuclease induced by HSV has been mapped to 0.145-0.185 m.u. (Moss et al., 1979; Preston and Cordingley, 1982) and shown to be essential for DNA synthesis (Francke et al., 1978; Moss et al., 1979). This enzyme possesses an associated endonuclease activity (Hoffmann and Cheng, 1979).

DNA topoisomerases have been described in HSV virions (Muller et al., 1985). These enzymes catalyse the breakage and rejoining of phosphodiester bonds in DNA and may be involved in DNA replication, transcription and recombination

(Gellert, 1981).

Uracil-DNA glycosylase, a class of enzyme involved in
DNA repair (Lindahl, 1979), is responsible for removing
uracil residues in the DNA. The pathways which result in
the presence of uracil in DNA are the incorporation of dUMP
into it during replication (Bessman et al., 1958) and
deamination of cytosine (Shapiro et al., 1973). The
induction of viral DNA-uracil glycosylase activity has been
reported in cells infected with HSV-1 and HSV-2 (Caradonna
and Cheng, 1981). Isolation of an HSV-2 cDNA which encodes
this enzyme has been described (Caradonna et al., 1987).
The latter authors concluded that uracil-DNA glycosylase
                                   created
acts in removing uracil residues₍by deamination of cytosine
rather than by misincorporation of dUMP residues. Recently
the HSV-1 UL2 gene, has been shown to encode uracil-DNA
glycosylase and is dispensible for virus growth in tissue
culture (Mullaney et al., 1989). Moreover, viruses with
double insertion mutations in deoxyuridine triphosphatase
(dUTPase) and uracil-DNA glycosylase grow normally in vitro
(J. Mullaney, personal communication).

The HSV-induced ribonucleotide reductase (see section
1.8.4.a) has been described in both HSV-1 (Cohen, 1972) and
HSV-2 (Cohen et al., 1974). This enzyme has been mapped to
genes UL39 and UL40 between 0.580-0.585 m.u. (Preston et
al., 1984) and consists of two subunits, large (RR1) and
small (RR2) which form a complex that is essential for its
activity (Frame et al., 1985; Bacchetti et al., 1986).
Synthetic oligopeptides corresponding to the carboxy
terminus of RR2 inhibit ribonucleotide reductase activity in
vitro (Frame et al., 1985). This lack of enzyme activity is
believed to be due to inhibition of the complex formation

between RR1 and RR2 (Cohen et al., 1986; Dutia et al., 1986).

Deoxyuridine triphosphatase (dUTPase) catalyses the conversion of dUTP to dUMP and pyrophosphate and has been mapped to gene UL50 between 0.69-0.70 m.u. (Wohlrab et al., 1982; Preston and Fisher, 1984). Virus mutants lacking dUTPase activity have been shown to grow normally in vitro (Fisher and Preston, 1986).

HSV-1 and HSV-2 have been shown to encode protein
kinases by US3∧genes (McGeoch, 1987) which are homologous with the members of the protein kinase family of eukaryotes (McGeoch and Davison, 1986). The role of protein kinase in HSV infection has not been determined although it is not essential for virus growth in tissue culture (Longnecker and Roizman, 1987).

1.7.2 HSV DNA binding proteins

Approximately 16 HSV-1 induced polypeptides and 12 HSV-2 induced polypeptides have DNA binding properties (Bayliss et al., 1975; Powell and Purifoy, 1976). Some of these like the major DBP (Powell et al., 1981) and the 65K DBP (Vaughan et al., 1985) bind to DNA directly. VmwIE175 has been shown to be a DNA binding protein (Hay and Hay, 1980; Faber and Wilcox, 1986). In contrast, Freeman and Powell (1982) showed that VmwIE175 binds to DNA indirectly by association with the host cell DNA binding protein.

Several HSV-1 DNA binding proteins have enzymatic activities including DNA polymerase and alkaline exonuclease (see section 1.7.1) and have been shown to interact directly with DNA (Powell and Purifoy, 1977; Banks et al., 1983). The 21K protein has been shown to bind to the 'a' sequence

(see section 1.4.2). The functions of the other DNA binding
proteins are not yet known.

1.8 Virulence of Herpes simplex virus
1.8.1 Introduction

Herpes simplex virus (HSV) is a neurotropic human
herpesvirus responsible for a variety of clinical conditions
ranging from mild cutaneous lesions (cold sores) to the
very rare fatal encephalitis. In many cases shedding may be
asymptomatic. After acute infection, the virus travels via
the axons of sensory neurons and establishes a latent
infection in the dorsal root ganglia of the peripheral
nervous system (Stevens and Cook, 1971). There is also
evidence for latency in the central nervous system (Knotts
et al., 1973). Following reactivation and travel down the
axons infectious virus is produced at the periphery which in
some cases may result in disease that can range in severity
from benign cutaneous lesions to acute fatal encephalitis
(Baringer and Swoveland, 1973; Johnson, 1982). A variety of
animal models e.g rabbits, mice, guinea pigs and tree shrews
have been used to study HSV pathogenesis. Laboratory
strains and clinical isolates with differing virulence for
animal hosts exist (Dix et al., 1983) and strain specific
differences within a given virus type may in part account
for the variability of the resulting disease patterns
(Wander et al., 1980).

Two types of HSV have been identified and classified on
the basis of neutralisation (Nahmias and Dowdle, 1968) and
restriction endonuclease patterns (Lonsdale et al., 1979;
Buchman et al., 1981). In addition to these differences,
functional difference such as variation in tissue culture

cytopathic effect (cpe) has been demonstrated (Ejercito et al., 1968).

1.8.2 Neurovirulence and neuroinvasiveness

Although neurovirulence and neuroinvasiveness are in part related, they can be distinguished. The molecular events that determine the neurovirulence and neuroinvasiveness of HSV are largely unknown. Neurovirulence of a virus can be defined as the capacity to replicate following direct inoculation and to cause disease in the nervous system. Neuroinvasiveness relates to the capacity of virus to enter and move through the nervous system following inoculation at the peripheral site. All neuroinvasive viruses are also neurovirulent; the converse, however, is not true (Stevens, 1987).

Virulence is determined by calculating the 50% lethal dose (LD_{50}) for example using the formula of Reed and Muench (1938), following inoculation of experimental animals. The LD_{50} value for a given virus in a particular inbred strain of animal is also dependent on the age of the animal (Kohl and Loo, 1980; Zawatsky et al., 1982; Ben-Hur et al., 1983) and the route of inoculation (Caspary et al., 1980; Dix et al., 1983).

1.8.3 Extrinsic factors influencing the virulence of HSV

The interaction between HSV and the host is complex and heavily influenced by the genotype of both virus and host. The outcome of HSV infection, which ranges from subclinical to fatal, depends on many factors acting in concert. The factors determining the outcome of HSV infection are not fully understood and the relationship between them is

unclear. These factors are listed in Table 1.1.

The in vivo roles of specific immunological processes (humoral and cell-mediated) in modulating the course of primary and recurrent HSV infection have not been fully elucidated. It is apparent that both processes are effective defence mechanisms which may act together to clear acute HSV infection at peripheral sites. HSV infection stimulates the production of antibodies which participate in virus neutralisation, antibody-dependent complement mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (Norrild, 1985). Humoral immunity has been reported to play a role in restriction and spread of virus from peripheral sites to sites within the nervous system (McKendall et al., 1979). Knowledge regarding cell-mediated immunity has been derived from murine models of experimental herpesvirus infection. During HSV infection of mice a number of cell-mediated immune responses are stimulated which result in virus clearance from tissue. These include macrophages, natural killer cells (Lopez, 1985) and a variety of T-lymphocyte subpopulations which mediate cytotoxicity, delayed type hypersensitivity and helper or suppressor functions (Nash and Wildy, 1983; Nash et al., 1985).

Interferons (IFN) a group of proteins with a wide range of biological activities (Lengyel, 1982) play a central role in determining resistance to HSV infection (Lopez, 1985). There is increasing evidence that the antiviral activity of IFN is based on a number of different antiviral mechanisms which may operate independently of each other and may affect different stages of the virus growth cycle (Friedman, 1977; Jacobsen, 1986). Recent work on HSV infection in IFN

Table 1.1 Factors involved in determining the outcome of HSV
infection

I. Host factors   II. Viral factors

A. Immunological   1. Interference

   1. Antigen specific   2. Strain of virus

   a. T-lymphocytes

   b. Antibodies

   2. Antigen non-specific

   a. Interferon

   b. Macrophages

   c. Natural killer cells

B. Non-immunological

   1. Barrier (skin, mucous membrane)

   2. Age of the host

   3. Route of inoculation

treated cells shows that IFN inhibits the onset of HSV IE transcription (Mittnacht et al., 1988).

Non-immunological host factors such as the integrity of the skin and mucous membranes are also involved in resistance to HSV infection. Recent work on Langerhan s cells, which are present in the epidermis of the skin, suggests that in mice these cells play an important role in defence against HSV infection and an increase in their density in the skin was correlated with increased resistance to infection with HSV (Sprecher and Becker, 1986, 1987). Langerhan s cells have also been shown to be highly efficient as antigen presenting cells (Braathen et al., 1980) and to be able to interact with T-lymphocytes in local lymph nodes (Silverberg et al., 1976).

Many studies have demonstrated that virus strain, route of inoculation, age of host, all have effects on virulence of HSV (Lopez, 1975; Caspary et al., 1980; Kohl and Loo, 1980; Zawatsky et al., 1982; Ben-Hur et al., 1983; Dix et al., 1983; Sprecher and Becker, 1987). It has also been reported that serial passage of virus in vivo (Kaerner et al., 1983), in vitro (Goodman and Stevens, 1986) and at body temperature i.e 37.5°C (Thompson and Stevens, 1983a) could increase the virulence of HSV.

Viral interference is a very important determinant of viral infection and is defined as a phenomenon in which the presence of an initial or primary virus infection prevents the infection or replication by a secondary (superinfecting or challenge) virus. The mechanism by which this occurs is not well understood. Stow (1985) suggested that the block may result from either the presence of defective viral particles or competiton for host proteins necessary for

viral DNA replication.

1.8.4 Viral genes determining virulence of HSV

HSV may rarely induce a life threatening encephalitis in humans (Rawls, 1985) and there is a continuing interest in understanding the properties of the virus relating to this syndrome. To define various aspects of the disease, experimental animals such as mice, rabbits, guinea pigs and rats have been used as models. Genetic analysis designed to identify specific neurovirulence and neuroinvasiveness associated genes is limited by the availability of suitable mutant viruses. Many laboratories are studying HSV genes and gene products associated with virulence using deletion mutants, monoclonal antibody resistance (mar) mutants, temperature sensistive (ts) mutants, drug resistance mutants and intertypic recombinants. Genes and loci modifying virulence of HSV are shown in Figure 1.7.A and B and described in the following sections.

1.8.4.a Viral enzymes

HSV encodes a thymidine kinase (TK) which is distinct from mammalian TK (Kit, 1979) and facilitates HSV replication in non-dividing cells (Jamieson et al., 1974). Virus mutants (TK$^-$) deficient in the production of this enzyme replicate well in actively growing cell cultures and poorly in serum starved cells(Jamieson et al., 1974; Field and Wildy, 1978). This led to the speculation that such mutants might be less virulent in vivo and it has been reported that TK$^-$ mutants of HSV-2 are less virulent than wild type (TK$^+$) HSV-2 (Marcialis et al., 1975; Field and Wildy, 1978; Stanberry et al., 1985). Similarly TK$^-$ mutants

Figure 1.7

A. Genes and loci in U_L, TR_L and IR_L involved in
virulence of HSV. The top line shows the fraction map
units (m.u.) of the genome from 0 to 0.83. The
locations of different genes and loci are indicated on
the viral genome.

B. Genes in IR_S, U_S and TR_S involved in virulence of
HSV. The top line shows the fraction m.u. of the viral
genome from 0.83 to 1.0. The locations and orientations
of the genes are indicated on the viral genome.

A

0   0.1   0.2   0.3   0.4   0.5   0.6   0.7   0.8

TR_L

U_L

IR_L

TK   gB   POL   RR   gC   Vmw65_TIF

IE1

0-0.02

0.11-0.14

0.32-0.42

0.31-0.44

0.4-0.44

0.25-0.53

0.3-0.45

0.49-0.64

0.71-0.74

0.81-0.83

0.76-0.789

0.79-0.83

0.71-0.83

B

0.83 0.9 0.95 1.0

IRs Us TRs

VmwIE68

US2

gG

gD

gE

PK

US11

VmwIE12
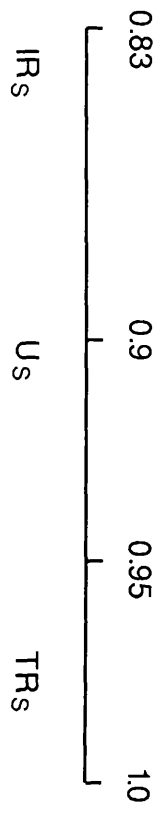
of HSV-1 have been shown to have reduced pathogenicity in animal models of herpes encephalitis (Field and Wildy, 1978), herpes keratitis (Marcialis et al., 1975; Price and Khan, 1981) and herpes labialis (Klein et al., 1981). Therefore viral TK would appear to influence HSV virulence, although the possibility that there are additional defects in these virus stocks arising from unrecognised mutations can not be excluded. The mechanism by which virus coded TK influences the pathogenicity of HSV may relate to reduced virus replication in neural tissue (Field and Wildy, 1978; Tenser et al., 1979; Klein et al., 1981; Price and Khan, 1981; McDermott et al., 1984). During maturation, nerve cells stop expressing cellular TK and it is likely that TK^{+} viruses are able to replicate in such cells since these viruses are able to produce enough of their own TK to multiply in the central nervous system (Gordon et al., 1983).

The TK enzyme is required for DNA synthesis through phosphorylation of thymidine to thymidine monophosphate. This function makes a good target for antiviral drugs. The common anti-HSV drug used for prophylaxis and therapy of HSV infections is acyclovir (ACV). It was initially feared that ACV resistant HSV strains might become a serious problem, analogous to penicillin resistant bacteria. ACV behaves as a nucleoside analogue which is selectively phosphorylated by HSV specific TK. The monophosphate form of ACV is then converted to the diphosphate and subsequently by cellular kinases to the triphosphate which is a potent inhibitor of HSV DNA polymerase. Three mechanisms of resistance to ACV have been described (i) loss of TK activity, (ii) alteration in TK substrate specificity and (iii) alteration of DNA

polymerase (Coen and Schaffer, 1980; Crumpacker et al.,
1980; Schippers and Crumpacker, 1980; Darby et al., 1981;
Field et al., 1982; Larder et al., 1983; Schinazi et al.,
1986; Ellis et al., 1987; Kit et al., 1987). Mutants with
an altered TK which are resistant to ACV may have reduced
virulence (Darby et al., 1981; Klein et al., 1981; Sibrack
et al., 1982). In one case, an ACV resistant mutant with an
alteration in TK was shown to be highly virulent for mice
(Sakuma et al., 1988). Mutants containing a drug resistance
mutation in the viral DNA polymerase gene were attenuated
for pathogenicity after intracranial inoculation and
cataracts were reported as a common sequel (Field and Coen,
1986). Modulation of pathogenicity was also seen using
flank and ear inoculation (Larder et al., 1986).

Ribonucleotide reductase (RR) catalyses the reduction
of ribonucleotides to deoxyribonucleotides
(Thelander and Reichard, 1979). The HSV enzyme consists of
two subunits named large (RR1) and small (RR2) (Cohen, 1972;
Dutia, 1983; Preston et al., 1984; Cohen et al., 1985; Frame
et al., 1985). It has been shown that RR is essential for
virus growth in tissue culture (Preston et al., 1984).
Recent findings by Goldstein and Weller (1988) utilising
viral RR mutants have substantiated the theory that the HSV
enzyme is non-essential for virus growth in vitro. It has
been shown that the HSV-1 ts mutants in RR, ts 1207 and ts
1222 (Preston et al., 1988) have reduced pathogenicity for
mice following intracranial and intraperitoneal inoculation
(Cameron et al., 1988). Jacobson et al. (1989) showed that
a mutant containing a deletion in the RR1 gene is severely
impaired in growth during acute infection of the eye and
trigeminal ganglia in mice. In contrast, Turk et al. (1989)

showed that two mutants containing a deletion in HSV-1 RR1 produce lesions in guinea pigs as severe as those of wild type strains following intradermal inoculation.

1.8.4.b Vmw65 trans-inducing factor (TIF)

A distinguishing feature of the IE genes is the presence of the cis-acting element, TAATGARAT, in their 5' regulatory regions. This element responds to the HSV-1 virion polypeptide Vmw65 TIF resulting in stimulation of transcription from the IE promotors (Mackem and Roizman, 1982; Batterson and Roizman, 1983; Cordingley et al., 1983; Campell et al., 1984; Kristie and Roizman, 1984; Gaff ney et al., 1985; Pellett et al., 1985; O'Hare and Hayward, 1987). Recent work by Ace et al. (1989) has shown that a HSV-1 mutant unable to transinduce IE gene expression has reduced virulence for mice following intracranial and intraperitoneal inoculation.

1.8.4.c Temperature sensisitive (ts) mutants

Most of the ts mutants of HSV have been generated using a variety of mutagens (Schaffer et al., 1970, 1974; Timbury, 1971; Brown et al., 1973; Esparza et al., 1974; Manservigi, 1974). It has been shown that some ts mutants of HSV have reduced virulence for guinea pigs (Anderson et al., 1980) and for mice (Lofgren et al., 1977). Temperature-sensitive mutants have been valuable in understanding the molecular events of the virus replication cycle in vitro.

1.8.4.d Glycoproteins

The genome of HSV-1 encodes at least seven antigenically distinct glycoproteins; gB, gC, gD (Spear, 1976), gE (Baucke

and Spear, 1976), gG (Marsden et al., 1984; Roizman et al., 1984), gH (Buckmaster et al., 1984) and gI (Longnecker et al., 1987). HSV-2 counterparts for gB, gC, gD, gE and gG have also been described based on biochemical or immunological criteria or analysis of DNA sequence data (Pereira et al., 1981; Balachandram et al., 1982; Eisenberg et al., 1982; Hope et al., 1982; Para et al., 1982; Zezulak and Spear, 1983; Zweig et al.., 1983; Dowbenko and Lasky, 1984; Swain et al., 1985; McGeoch et al., 1987). No HSV-2 counterpart of gH has yet been identified. Virus-specific glycoproteins are structural components of the virion envelope and are expressed on the surface of infected cells where they may serve as targets for immune surveillance (Norrild, 1985; Dix, 1987). The known HSV-1 glycoproteins and their biological and immunological functions are summarised in Table 1.2 and their map locations is indicated in Figure 1.8.

Studies with ts and deletion mutants in various glycoproteins indicated that gC, gE, gG and gI are dispensible for growth of virus 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; Longnecker et al., 1987; Weber et al., 1987; Harland and Brown, 1988; Schranz et al., 1989) whereas gB, gD and gH have been shown to be essential (Manservigi et al., 1977; Little et al., 1981; Gompels and Minson, 1986). Studies involving HSV-1 ts mutants in gB have suggested a role for gB in the entry of virus into cells (Sarmiento et al., 1979; Little et al., 1981) and cell-cell fusion (Manservigi et al., 1977; Haffey and Spear, 1980). Furthermore evidence supporting the role of gB and

Table 1.2 Known biological and immunological properties of HSV-1 glycoproteins

Glycoprotein	Biological properties	Immunological properties
gB	required for infectivity and involved in cell fusion	induces neutralising antibody and helper T-lymphocytes
gC	involved in cell fusion	induces delayed type hypersensitivity, cytotoxic T-lymphocytes and neutralising antibody; binds to C3b component of complement
gD	required for infectivity and involved in cell fusion	induces neutralising antibody and delayed type hypersensitivity
gE	not determined	binds to Fc portion of IgG
gG	not determined	induces neutralising antibody and delayed type hypersensitivity
gH	involved in cell fusion and egress	induces neutralising antibody
gI	not determined	binds to Fc portion of IgG

Figure 1.8

Locations of the genes encoding HSV-1 glycoproteins.

The HSV-1 genome is represented in the prototype

orientation with fractional genomic length (m.u.) above

the genome. The approximate map locations of the

glycoproteins are indicated by shaded boxes.

(Adapted from Marsden, 1987).
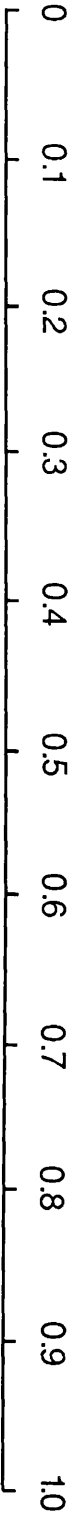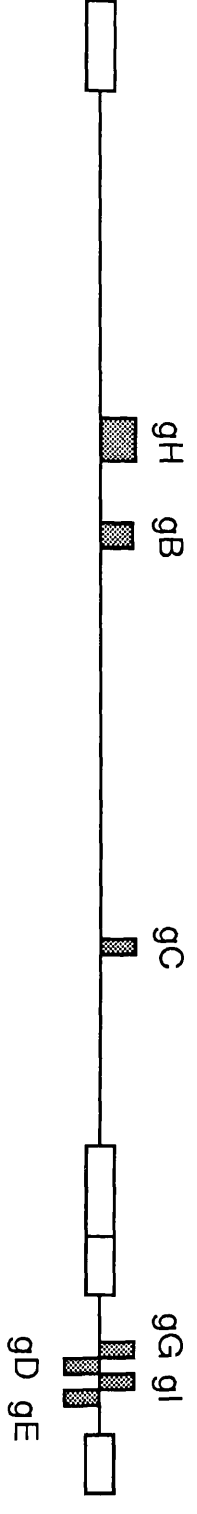
to a lesser extent gD in virus attachment to the cell and cell-cell fusion has come from experiments with reconstituted viral glycoproteins (Johnson et al., 1984) and monoclonal antibodies (Nobel et al., 1983; Fuller and Spear, 1985). Similar experiments have implicated gH in cell fusion (Gompels and Minson, 1986). Two antigenic sites in gB have been identified and shown to be linked with the ability of gB to contribute to virus infectivity (Highlander et al., 1989).

The role of gH in egress of virus from infected cells has been shown using a monoclonal antibody against gH which inhibits plaque formation suggesting the involvement of gH in cell-cell spread of infectious virus (Buckmaster et al., 1984). It has been reported recently that monoclonal antibodies specific to gH of HSV block viral penetration without inhibiting adsorption of virus to cells (Fuller et al., 1989).

HSV-1 gE (Bauke and Spear, 1979; Para et al., 1982) and gI (Johnson and Feenstra, 1987) have been associated with virus specific binding activity for the Fc component of immunoglobulin G (IgG). Evidence that binding of non-immune IgG to infected cell membranes can protect the cells from immune cytolysis (Adler et al., 1978) suggests that such binding may modify immune recognition of virus infected cells in vivo.

Glycoprotein C plays a role in the induction of humoral and cell-mediated immune responses during infection (Oakes et al., 1980; Glorioso et al., 1985; Kumel et al., 1985; Marlin et al., 1985; Nash et al., 1985; Rosenthal et al., 1987) and has the unique property of functioning as a receptor for the C3b component of complement (Friedman et

al., 1984). This activity has been shown to interfere with activation of the alternative complement pathway (Fries et al., 1986).

Glycoproteins have been identified as virulence factors in studies of other viruses such as rabies (Dietzschold et al., 1983) and murine corona virus (Fleming et al., 1986). Among herpesviruses gI and gIII, pseudorabies virus structural components have been reported to influence viral neurovirulence (Mettenleiter et al., 1987, 1988). Although HSV encodes at least seven glycoproteins, their roles in neurovirulence remain largely unexplored.

The ability of gC to interfere with activation of the complement pathway suggested that gC may act as an important virulence determinant by protecting the virus from complement-mediated antiviral host defence mechanisms. Intravaginal inoculation of mice with a gC-2 negative (gC$^-$2) mutant produced local inflammation followed by lethal spread of virus infection into the nervous system in an identical manner to that produced by the parental HSV-2 strain 333 (Johnson et al., 1986). Similarly direct inoculation of a gC$^-$2 mutant into the central nervous system produced lethal neurological disease. These results support the impression that gC-2 is not involved in virulence. On the other hand, HSV-2 marC mutants were found to be reduced in pathogenicity for the nervous system (Kumel et al., 1985). HSV-1 strain MP which is gC$^-$1 (Hoggan and Roizman, 1959; Holland et al., 1984) was found to be highly virulent for mice following intracranial or footpad inoculation (Dix et al., 1983). Recently Sunstrum et al. (1988) have tested eight additional gC$^-$1 mutants derived from HSV-1 strain KOS 321 and shown that these mutants differ widely in their ability to induce

lethal encephalitis in mice following intracranial
inoculation. It was concluded that gC-1 is not a virulence
determinant in the mouse model of HSV-1 induced
encephalitis. It has been suggested that it is unlikely
that either gC-1 or gC-2 play an essential role in the entry
of virus into epithelial or neural cells, either in mice or
humans (Johnson et al., 1986).

MarB or marD mutants with single epitope changes have no
effect on pathogenicity, while multi-marB mutants show
significantly reduced pathogenicity. This difference may be
due to an unrecognised mutation other than the ts phenotype
of multi-marB mutants which alters pathogenicity (Kumel et
al., 1985).

The involvement of gE and gG in virulence has been shown
using genetically engineered deletion mutants of HSV-1
strain F (Meignier et al., 1988). The LD_{50} of a gG^- was 10^4
fold higher than the wild type, while that of a gE^- mutant
was <100 fold higher following intracranial inoculation of
mice. These results indicated that gG and to a lesser
extent gE have important roles in virulence. In addition
Weber et al. (1987) have shown that a HSV-1 mutant carrying
a chain terminating Tn5 insertion in the gene encoding gG
has reduced neurovirulence in mice. It has been shown
recently that mutants of HSV-2 strain HG52 in the US4 gene
encoding gG were reduced in virulence for mice following
intracranial inoculation (see section 3.2.2).

The role of gB in pathogenicity was demonstrated by
transfer of the gB gene from HSV-1 strain KOS, which has
been shown to be apathogenic for mice following
intraperitoneal inoculation (Schroder et al., 1983), to
HSV-1 strain ANG path, which is pathogenic for mice when

inoculated by the same route (Kaerner et al., 1983), resulting in recombinant virus which was apathogenic for mice (Weise et al., 1987).

1.8.4.e Other loci

Many groups have identified regions in the HSV genome involved in virulence using different animal model systems. Thompson and Stevens (1983b) used the HSV-1 intertypic recombinant RE6 which was generated by marker rescue of ts E syn[+] with wild type HG52 fragments (Marsden et al., 1978). They showed that RE6 was completely avirulent for mice following intracranial inoculation and concluded that RE6 was at least 10 million fold less neurovirulent than the parental wild type viruses. In an other study, Thompson et al. (1983) confirmed the avirulent phenotype of RE6 and localised the HSV-1 region which restored neurovirulence to between 0.71-0.83 m.u. Cleavage of the wild type HindIII c fragment (0.64-0.87 m.u.) with EcoRI eliminated its capacity to rescue RE6 and neurovirulence was restored by the addition of the HSV-1 BamHI l fragment (0.71-0.74 m.u.) that spans an EcoRI site at 0.72 m.u. (Thompson et al., 1985). Similarly Ben-Hur et al. (1987) following intracranial inoculation of mice concluded that a neurovirulence function resided in the BamHI l fragment (0.7-0.738 m.u.) of HSV-1 strain F and that the function was closely linked to the viral gene for cell fusion. The latter correlated with the report by Yamada et al. (1986), who examined several HSV-1 variants (all derived from the Miyama strain) and concluded that viral pathogenicity correlated with a higher cell fusion index and large plaque size.

Another intertypic recombinant RS6, which was generated

by cotransfection of unit length $\underline{ts}$ S of 17 $\underline{syn}^+$ DNA and
XbaI generated DNA fragments derived from HG52 (Stow and
Wilkie, 1978), was also studied in pathogenicity
experiments. Javier $\underline{et\ al}$. (1987) have shown that the HSV-1
intertypic recombinant RS6 was approximately 10^5 times less
neurovirulent for mice following intracranial inoculation
than either of its wild type parental viruses. The
avirulent phenotype of RS6 was due to failure to replicate
in mouse brain. Cotransfection experiments of RS6 DNA with
EcoRI $\underline{e}$ + $\underline{k}$ fragments (0.72-0.87 m.u.) of 17 $\underline{syn}^+$ generated
recombinant viruses with variable degrees of neurovirulence.
The relevant region between 0.72-0.87 m.u. was tentatively
delimited to 0.79-0.83 m.u. DNA analysis of the recombinant
viruses showed that the highly neurovirulent recombinants
had type 2 DNA replaced with type 1 DNA in the two regions
between 0.11-0.14 m.u. and 0.79-0.83 m.u. One exceptional
recombinant designated R13-1 was avirulent and had type 2
DNA replaced by type 1 DNA in the region between 0.79-0.83
m.u. These results suggested that there was at least
another region (0.11-0.14 m.u.) involved in HSV
neurovirulence. The same group (Javier $\underline{et\ al}$., 1988a) have
shown that the R13-1 recombinant was 10^3 fold less
neurovirulent than either of the wild type parental viruses
despite a comparable efficiency of replication in mouse
brain to that of the parental viruses. Marker rescue of
unit lengh R13-1 DNA and cloned type 1 EcoRI $\underline{d}$ fragments
(0.079-0.192 m.u.) spanning the region from 0.11-0.14 m.u.
generated viruses with the wild type neurovirulence
phenotype.

Another region of the HSV genome has been implicated in
neuroinvasiveness. Thompson $\underline{et\ al}$. (1986) have shown that a

cosmid clone containing the HindIII a fragment (0.25-0.53 m.u.) of strain 17 syn[+] in contransfection with full lengh KOS DNA, known to be less neurovirulent than most other HSV-1 strains (Dix et al., 1983), yielded recombinants with enhanced neuroinvasiveness for mice following footpad inoculation. Javier et al. (1986) reported that simultaneous inoculation of mice on the rear footpad with two non-neuroinvasive viruses (HSV-1 strains ANG and KOS) resulted in the deaths of 62% of animals. Studying the events resposible for pathogenesis of this virus mixture Sedarati et al. (1988) investigated the replication capacity and spread of virus within specific tissue and found that the amount of neuroinvasive virus mixture had increased within the spinal cords and brains of infected mice compared to ANG and KOS strains which are defective in this capacity. Their findings suggested that the neuroinvasiveness of the virus mixture was a consequences of complementation as well as the generation and selection of neuroinvasive recombinants in the spinal cords of mice.

Recent work by Goodman et al. (1989) has shown that HSV-1 strain ANG DNA contains at least two distinct neuroinvasive regions designated INV-1 (0.49-0.64 m.u.) and INV-II (0.32-0.42 m.u.) containing functions which can be transferred by DNA cotransfection to confer mesodermal invasiveness and virulence to the avirulent HSV-1 17 syn[+] on the chorioallantoic membrane (CAM) of chicken embryos. The genes in the region INV-1 (0.49-0.64 m.u.) include the virion function associated with shutoff of host cell protein synthesis (Morse et al., 1978). The 5' end of the gC gene also maps within this region and gC is highly type divergent in terms of both sequence and antigenicity (Spear,

1985). In addition the genes encoding RR and ICP10 reside within this region (Schaffer et al., 1987). The region INV-II (0.32-0.42 m.u.) contains genes encoding gB (Bzik et al., 1984) and its associated syn 3 mutation locus (Ruyechan et al., 1979).

Sedarati and Stevens (1987) have examined the biological properties of HSV-1 strains F, HF and HFEM compared to HSV-1 17 syn$^+$. They found that the three strains of HSV (F, HF and HFEM) were defective in replication in mouse cells, had reduced neurovirulence for mice following intracranial inoculation and were completely avirulent following footpad inoculation. In coinfection experiments, strain F complemented strains HF and HFEM but the latter two strains did not complement each other. All three strains were found to complement the non-neuroinvasive KOS emphasizing the multigenic nature of HSV virulence.

Centifanto-Fitzgerald et al. (1982), studying the patterns of ocular disease produced by HSV using the rabbit eye model system, identified a region of the HSV-1 genome between 0.71-0.83 m.u. involved in stromal disease. In addition a study by Oakes et al. (1986) using intertypic recombinants, has shown that HSV-1 DNA sequences between 0.31-0.44 m.u. were necessary for recombinant viruses to spread from the cornea to the central nervous system of mice following ocular inoculation. Similarly Day et al. (1987) showed that a region important in DNA replication between 0.4-0.44 m.u. of HSV-1 strain 17 syn$^+$ was involved in spread of virus from the cornea to the central nervous system following ocular inoculation of mice. This region contains ori$_L$, DNA polymerase and a part of the major DBP (Quinn and McGeoch, 1985; Weller et al., 1985).

The tree shrew has also been used as an animal model to define HSV genes involved in virulence. The HSV-1 strain HFEM, which has a deletion of about 4.1 kb between 0.76-0.79 m.u., is apathogenic for tree shrews after intraperitoneal inoculation (Rosen and Darai, 1985). Pathogenicity of strain HFEM was restored by cotransfection of its DNA and the BamHI b fragment (0.733-0.809 m.u.) from the pathogenic HSV-1 strain F (Rosen et al., 1985). Similarly insertion of the MluI DNA fragment (0.7615-0.789 m.u.) from HSV-1 strain F restored the intraperitoneal pathogenicity to strain HFEM (Rosen et al., 1986). A similar DNA fragment (MluI 0.761-0.786 m.u.), spanning the HpaI p sequences of the pathogenic strain F, has been shown to restore the intraperitoneal pathogenicity of HFEM for mice (Becker et al., 1986). Recent work on the neurovirulence of seven deletion mutants of HSV-1 showed that all were attenuated for virulence in mice following intracranial inoculation (Meignier et al., 1988). The genes deleted from six of these map in the S component of HSV-1 DNA and were IE4, US2, US3, gG, gE, US11 and IE5.

Recently Taha et al. (1989a; and this thesis), have shown that a variant (JH2604) of HSV-2 strain HG52, whose genome is deleted by approximately 1.5 kb within both copies of the BamHI v fragment between 0-0.02 and 0.81-0.83 m.u. (Harland and Brown, 1985), is completely non-neurovirulent for mice following intracranial inoculation. Correction of the deletion by wild type XbaI (e, f or g) or BamHI g fragments resulted in recombinants with wild type neurovirulence. Introduction of the JH2604 deletion back into the wild type genome resulted in non-neurovirulent recombinants, demonstrating that the deleted sequences in

the variant JH2604 determine neurovirulence. Sequence
analysis has shown that the variant JH2604 is deleted by
1488 bp in both the IR_L and TR_L copy of BamHI $\underline{v}$ (Taha $\underline{et}$
$\underline{al}$., 1989b). A similar region has recently been identified
in HSV-1 strain 17 as being important in the determination
of virulence in mice (Thompson $\underline{et\ al}$., 1989).

1.9 <u>Latency of HSV</u>

    Unlike many other human viruses which are eliminated by
host mediated defence mechanisms soon after primary
infection, HSV has evolved a complex strategy of replication
and survival whereby it may remain within neural or
non-neural tissues for long periods of time in a latent
state thereby evading the immune response. The latent phase
of HSV infection is defined as isolation of infectious virus
from explant cultures after a period of time but failure to
detect infectious virus from cell homogenates of These tissues or
organs immediately post explantation.

    The focal point of most clinical disease arising from
HSV is the ability of the virus to interact with components
of the peripheral nervous system. Following primary
infection and active replication at peripheral sites, virus
attaches to the sensory nerve terminals (Vahlne $\underline{et\ al}$.,
1978), enters them and travels centripetally via neural
routes to sensory ganglia (Cook and Stevens, 1973). It is
within the neurons of the sensory ganglia that virus becomes
latent (Cook $\underline{et\ al}$., 1974; Stevens, 1975). HSV has been
recovered in humans and animals from explanted nervous
tissue, and adrenal glands (Nesburn $\underline{et\ al}$., 1972; Knotts $\underline{et}$
$\underline{al}$., 1973; Cook and Stevens, 1976; Price $\underline{et\ al}$., 1975;
Warren $\underline{et\ al}$., 1978). Evidence is accumulating which

suggests that peripheral non-neural sites of herpesvirus latency in humans and experimentally infected animals also exist. HSV-1 has been isolated in the absence of detectable clinical lesions from the ear skin, which was the initial site of inoculation, in 8% of mice latently infected with the virus though this was initially interpreted as reactivation from dorsal root ganglia (Hill et al,. 1980). HSV-2 and HSV-1 have both been recovered independently from the footpad as well as from the dorsal root ganglia of latently infected mice (Al-Saadi et al., 1983; Clements and Subak-Sharpe, 1983, 1988; Subak-Sharpe et al., 1984a, 1984b; Al-Saadi et al., 1988). In the guinea pig both HSV-1 and HSV-2 have been isolated from the site of primary infection (footpad and vagina) long after inoculation (Scriba, 1976, 1981; Donnenberg et al., 1980). However, in the guinea pig spontaneous reactivation is very common. 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 unsucessful (Rustigian et al., 1966) apart from the isolation from human corneas removed prior to corneal transplantation, due to chronic stromal keratitis (Shimeld et al., 1982; Tullo et al., 1985; Cook, 1988).

The mechanism of initiation, maintenance and reactivation of latent HSV has been extensively studied (Wildy et al., 1982; Hill, 1985) but is still not fully undestood. The molecular investigation of viral genes expressed during the latent state has been difficult since only a minority of cells in neural tissue harbour latent virus (Kennedy et al., 1983). During the latent phase, the viral genome appears to be present in circular or

concatameric forms rather than the linear molecules found in intact virions (Rock and Fraser, 1983). During the latent phase there have been no confirmed claims of the detection of any HSV gene expression. Recently HSV-specific transcripts have been detected during HSV-1 latency in ganglia of seropositive patients (Croen et al., 1987; Steiner et al., 1988; Stevens et al., 1988) and in experimentally infected mice (Deatly et al., 1987; Puga and Notkins, 1987; Spivack and Fraser, 1987; Stevens et al., 1987; Wagner et al., 1988) and rabbits (Rock et al., 1987; Wechsler et al., 1988).

The HSV-1 transcripts present in sensory ganglia of latently infected animals and humans originate from the long repeat region of the viral genome which are present as two copies per genome. At least three viral transcripts (latency associated transcripts- LAT), 2.0, 1.5 and 1.45 kb are present during HSV-1 latency in humans and experimentally infected animals (mice and rabbits) and map to a 3.0 kb region contained within the BamHI b and e fragments in the R_L region. These RNAs partially overlap the 3' terminus of the IE1 gene but are transcribed in the opposite direction (Rock et al., 1987; Spivack and Fraser, 1987; Stevens et al., 1987; Deatly et al., 1988; Steiner et al., 1988; Wagner et al., 1988; Wechsler et al., 1988).

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, most of the transcripts present during latency are not polyadenylated (Wagner et al., 1988). The LATs might modulate latency via control of IE1 gene expression, possibly by interacting with the DNA

sequence of the gene or with IElmRNA (Croen et al., 1987; Rock et al., 1987; Stevens et al., 1987). However, HSV deletion variants that lack all or part of the LATs are latency competent in laboratory animals (Javier et al., 1988b; Sedarati et al., 1989; Steiner et al., 1989). Thus these transcripts, the only HSV specified products so far detected in latently infected neurons, are not essential for the latency process in neurons, at least in animals.

Several studies have suggested that mutants of HSV-1 with defective TK have a reduced ability to establish latent infections in vivo. Moreover, studies using genetically engineered TK$^-$ deletion mutants have shown that the mutants can not establish either acute or latent ganglionic infection in mice (McDermott et al., 1984; Tenser and Edris, 1987). Similarly, an HSV-1 TK$^-$ mutant was shown to be latency negative in mice but positive in rabbits suggesting that the host cell may be a determinant in the establishment of latent infection (Meignier et al., 1988). In contrast, Efstathiou et al. (1989) have demonstrated by superinfection of explanted mouse ganglia with wild type HSV-1, that a TK$^-$ deletion mutant was able to establish latent ganglionic infection suggesting that the virus-encoded TK is not essential for establishment of latent infection. Recently Leist et al. (1989) have reported that an HSV-1 variant harboring a deletion in the TK gene can establish latent infections in mice spinal ganglia. That variant could not be reactivated when spinal ganglia were cultured in vitro suggesting that the expression of the viral TK gene plays no major role in establishment of the latent state but it has a role in reactivation from explanted ganglia. Similarly Tenser et al. (1989) concluded that although HSV-1 TK$^-$

mutants were able to establish latent infections in mouse ganglia, they failed to reactivate from explanted ganglia suggesting a role for TK in the reactivation process. Using an _in vitro_ system to study latency, Russel and Preston (1986) have shown that infection of human foetal lung (HFL) cells with HSV-2 at the superoptimal temperature of $42^{\circ}C$ results in a latent state which is stable on downshift of cultures to $37^{\circ}C$. The latent virus can be reactivated by superinfection with HSV or CMV but not with adenovirus. Using this approach, Russel _et al_. (1987) have demonstrated that the superinfection of HSV-1 dll403, which has a large deletion in the IE1 gene coding for VmwIE110 (Stow and Stow,1986), can not reactivate latent HSV-2 suggesting a role for the IE1 gene product in reactivation. Recently, Harris _et al_. (1989) using two mutants, have shown that adenovirus recombinants expressing HSV-1 VmwIE110 reactivate latent HSV-2 _in vitro_. The HSV-1 mutant which possesses a deletion in the carboxy-terminal region of VmwIE110 reactivated latent HSV-2, whereas the other HSV-1 mutant which has a deletion in the second exon did not, suggesting that VmwIE110 alone is required for reactivation of latent HSV-2 _in vitro._ Contrary to this, Clements and Stow (1989) have shown that dll403, which has reduced virulence for mice, is able to establish a latent infection in mice sensory ganglia following footpad inoculation and the latent virus can reactivate from explanted ganglia suggesting that the gene product of the IE1 gene (VmwIE110) is dispensible for establishment and maintenance of latency and reactivation from the latent state.

1.10 HSV infection of the nervous system

1.10.1 HSV-1 encephalitis

The most important neurological disease caused by HSV-1 is an acute encephalitis which is the commonest type of fatal sporadic acute encephalitis occuring in man (Finelli, 1975). Beyond the fact that HSV-1 infection spreads within the brain causing progressive destruction of both neurons and glial cells, little is known of the pathogenesis of herpes encephalitis. The route of infection is likely to be an important factor in determining the topography of herpetic brain infection. HSV-1 can directly invade the brain of immunologically normal adults and produce an acute haemorrhagic necrotising encephalitis which is accompanied by high mortality and severe morbidity (Whitley et al., 1982a).

Two theories currently exist to explain the origin of the virus responsible for this devastating disease. Davis and Johnson (1979) speculated that HSV-1 encephalitis of adults represents reactivation of a latent endogenous virus from the trigeminal ganglia. Support for this hypothesis has been supplied by Whitley et al. (1982b) who demonstrated that brain and oral isolates recovered from five of eight patients with simultaneous encephalitis and oral lesions were identical when compared by DNA restriction enzyme analysis. Alternatively the rather consistent striking localisation of tissue destruction to the subfrontal and medial temporal regions of the brain led Johnson and Mims (1968) to speculate that this disease is a primary infection caused by an exogenous virus which gains access to the central nervous system by the olfactory route. Evidence obtained from experimental animal models of focal HSV-1 encephalitis produced by virus inoculation of the olfactory

bulb or nasal mucosa would tend to support this hypothesis
(Anderson and Field, 1983; Schlitt et al., 1986; Stroop and
Schaefer, 1986).

On the other hand, a number of animal studies have
demonstrated that after inoculation of HSV-1 by a variety of
routes there is a predilection for certain brain regions
suggesting a selective vulnerability of these regions
(Anderson and Field, 1983; Tomlinson and Esiri, 1983;
McFarland and Hotchin, 1987).

1.10.2 HSV-2 encephalitis and meningitis

HSV-2 may produce severe neurological infections in
infants infected at the time of birth who develop
disseminated herpetic infection with hepatic and adrenal
necrosis and diffuse encephalitis (Johnson, 1982). In
adolescents and adults, primary infection is occasionally
complicated by an acute self-limiting benign meningitis
often associated with outbreaks of genital disease (Corey,
1984). The pathogenesis of HSV-2 meningitis is uncertain.
Unlike HSV-1, HSV-2 can be recovered from both cerebrospinal
fluid (CSF) and blood (Craig and Nahmias, 1973) of adults
suffering from aseptic meningitis. Whether all cases of
this illness are associated with viraemia remains to be
determined. Nevertheless, it is generally accepted that
HSV-2 meningitis is a consequence of reactivation from
sacral ganglia of virus which subsequently seeds the CSF.
Complications of HSV-2 meningitis include intermittent or
permanent paralysis associated with transverse myelitis of
the spinal cord.

An exception to the more benign nature of HSV-2 in
adults is seen in patients with immunodeficiency. Fatal

disseminated herpesvirus infections, including encephalitis
(Sutton et al., 1974) and severe even fatal meningo-
encephalitis associated with HSV-2 as well as HSV-1 have
been reported in patients undergoing intensive
immunosuppressive therapy (Johnson, 1982).

1.11 Pathology of HSV encephalitis

There is marked necrosis in HSV encephalitis with
petechial haemorrhage accounting for the earlier term, acute
necrotising encephalitis. Microscopically, haemorrhagic
necrosis with mononuclear inclusion bodies in the neurons
and glial cells is seen (Drachman and Adams, 1962). Viral
antigen distribution in human cases of herpes encephalitis
was analysed using immunoperoxidase techniques (Esiri,
1982). The analysis showed that HSV-1 antigens were
detected in the medial and inferior temporal lobes,
hippocampus, amygdaloid nuclei, olfactory cortex, insula and
cingulate gyrus. In a number of cases antigens were
detected in glial cells of the olfactory tracts. The grey
matter was predominantly affected, but antigens and
inflammation extended also into the white matter. Viral
antigens could be visualised early in the disease and by the
fourth week, antigens were largely cleared and replaced by
inflammatory cells. Using a combination of immunoperoxidase
staining and in situ hybridisation it has been possible to
show localisation of viral antigens and viral nucleic acid
in the same cell allowing the distinction between productive
and non-productive viral infections (Kennedy et al., 1988).
Viral antigens and HSV RNA were detected in the same
astrocytes, glial cells and macrophages.

In experimentally infected animals, viral antigen

distribution in the central nervous system depends on the portal of virus entry (Anderson and Field, 1983; Chrisp et al., 1989) and the animal species (Stroop and Schaefer, 1986). Hayashi et al. (1986) have reported hydrocephalus in mice following intracranial inoculation with HSV-1. These brains showed dilation of the ventricles with irregular walls devoid of ependymal lining cells and proliferation of subependymal astrocytes. Mild mononuclear cell infiltration was also commonly seen in the subarachnoid space of hydrocephalic mice.

1.12 HSV and ocular diseases

HSV is endemic in the world with 60-70% of children aged 5 years and 90% of adults having neutralising antibody against HSV (Smith et al., 1967). However, only 20-30% are estimated to manifest clinical disease and less than 1% exhibit ocular disease (Kaufman et al., 1983). The virus is the most common cause of corneal ulcer and corneal blindness in developing countries (Foster and Duncan, 1981). The two types of HSV are implicated in ocular disease. Approximately 98% of non-neonatal ocular infection is caused by HSV-1 (Neumann-Haefelin et al., 1978) while 70-80% of neonatal ocular infection is from HSV-2 (Nahmias et al., 1970).

HSV ocular disease occurs as primary or recurrent infections. The most common manifestations of primary ocular disease are the infection of the eyelids and conjuctivi which are characterised by vesicle formation on the lid and acute follicular conjuctivitis respectively. Epithelial keratitis (involvement of the cornea) in the primary HSV infection, which is a self limiting disease,

usually follows the onset of follicular conjuctivitis resulting in keratoconjuctivitis (Grayson, 1983). In neonates, HSV ocular manifestations include keratoconjuctivitis and retinitis. Unilateral or bilateral cataracts associated with HSV neonatal infection is thought to develop secondary to uveitis or from direct viral invasion of the lens (Gibis and Burde, 1971).

The clinical manifestations of recurrent HSV ocular disease include blepharitis, conjuctivitis and keratitis. The latter differs from that in primary HSV ocular infection in that keratitis extends to the stroma and endothelium resulting in permanent visual loss (Verdier and Krachmer, 1984).

Using animal model systems, such as rabbits, mice and guinea pigs for experimental HSV ocular infection, has provided valuable information on the pathogenesis of the disease (Nesburn et al., 1967; Hill et al., 1975; Wander et al., 1987).

CHAPTER 2

MATERIALS

Cells

Baby hamster kidney clone 13 (BHK-21 Cl3) cells (Macpherson and Stoker, 1962) and BALB/c mouse embryo fibroblast (3T3) cells (Flow Laboratories) were used.

Viruses

Herpes simplex virus type 2 (HSV-2) strain HG52 (Timbury, 1971) and plaque purified stocks of HG52 (Taha et al., 1988) were used throughout this study. The HSV-2 (HG52) deletion variants used were JH2603, JH2604, JH2605, JH2606, JH2607, JH2608, JH2609, JH2610 and JH2611 (Harland and Brown, 1988), characterised (Harland and Brown, 1985) under their previous designation of HG52XD86, HG52XD192, HG52XD85/5, HG52XD85/4, HG52X163X12, HG52X163X14, HG52X163X21, HG52X163X3 and HG52X163X3X53 respectively. Herpes simplex virus type 1 (HSV-1) Glasgow strain 17 syn+ (Brown et al., 1973) and the HSV-2 thymidine kinase negative mutant (TK^{-7}), obtained from D. Dargan, were also used.

Tissue culture media

BHK-21 Cl3 cells were cultured in Eagle's medium (Gibco Biocult.) containing twice the normal concentration of vitamins and amino acids, 100 units/ml penicillin, 100 ug/ml streptomycin, 5% (v/v) tryptose phosphate broth and 10% (v/v) calf serum (ETC10). 3T3 cells were grown in Dulbecco's Modified Eagle's medium (Flow Laboratories) containing 10% (v/v) newborn bovine serum and 2mM L-glutamine (DMEM10). Other media used during this study were;

Emet/5C2   Eagle's medium containing 20% the normal
                concentration of methionine plus 2% calf
                serum

PIC   phosphate free Eagle's medium containing
                1% calf serum

EHu5   Eagle's medium containing 5% human serum

EMC5   Eagle's medium containing 1.5% methyl
                cellulose and 5% calf serum

Agar and bacterial growth media

2X YT broth   85mM NaCl, 1% (v/v) Difco bactotryptone,
                1% 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

Plasmids

Plasmid pAT153 (Twigg and Sherratt, 1980) was used as
the cloning vector and the pGZ1 plasmid containing the HG52
BamHl g fragment (0.81-0.85 m.u.) was supplied by A.
Davison.

Sera

Normal goat serum and biotinylated anti-rabbit IgG were
supplied by Vector Laboratories (USA). Polyvalent rabbit
antiserum to HSV was supplied by Dako Ltd.

Experimental animals

Three week old BALB/c mice, both sexes, (Bantin and
Kingman) were used in this study.

Stains

Giemsa stain

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

Eosin

0.5% solution of eosin Y in distilled water.

Haematoxylin

1g haematoxylin, 50g potassium alum, 0.2g sodium iodate, 1g citric acid and 50g chloral hydrate dissolved in 1000ml water with gentle heat.

Enzymes

Restriction endonucleases were obtained from Bethesda Research Laboratories (BRL). E. coli polymerase I (Klenow), T4 DNA ligase, calf intestinal phosphatase (CIP) were supplied by Boehringer Corporation Limited. Protein kinase and lysozyme were purchased from Sigma Chemicals Company.

Radiochemicals

All radiochemicals were obtained from Amersham International plc at indicated specific activity;

$[\alpha - ^{32}P]$ dNTP'S	3000 Ci/mmol
^{35}S-methionine	800 Ci/mmol
^{32}P-orthophosphate	200 mCi/mmol
$[Me-^{3}H]$ thymidine	60 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.

Tris-saline

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

10% Formal buffered saline

A stock solution of 40% formaldehyde to which 9% NaCl has been added. The working solution (10%) consists of one part of stock to nine parts water kept over marble chips for buffering.

Trypsin

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

Versene

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

Trypsin-Versene

One volume of 0.25% trypsin plus four volumes versene.

Saturated phenol

Phenol was saturated by mixing 2:1 with phenol saturation buffer (10mM Tris-HCl pH 7.5, 10mM EDTA, 100mM NaCl).

Phenol : chloroform (1 : 1)

This is a 1 : 1 mixture of phenol and chloroform.

Chloroform : isoamyl alcohol (24 : 1)

This is a 24 : 1 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 8.0.

Restriction enzyme buffers

The buffers used were as recommended by BRL or Maniatis (1982).

Chemicals

M13 single strand primer, unlabelled nucleotides, 2', 3' dideoxyribonucleoside triphosphates were supplied by Pharmacia Ltd. Wacker silicone was provided by Wacker-Chemical Company, Munich. Repelcote (2% solution of dimethyldichlorosilane in 1, 1, 1,- trichloroethane) was supplied by Hopkin and Williams, England. Avidin biotinylated horseradish peroxidase complex (ABC) was obtained from Vector Laboratories USA. DNA blotting membrane (Hybond[N]) was purchased from Amersham International

plc.   Other chemicals were supplied by BDH Chemicals UK or
Sigma Chemicals Company and were of analytical grade.

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METHODS

2.1 Growth of cells

BHK-21 Cl3 cells were grown in 80 oz roller bottles containing 100ml ETC10 at $37^{o}C$ in an atmosphere of 5% CO_2 in air. Confluent cells were harvested by washing twice with versene and once with trypsin-versene and resuspended in 20ml ETC10. The cells from one roller bottle ($3x10^8$) are enough to seed 10 bottles ($2x10^6$). 3T3 cells were grown in a similar way using DMEM10 and were split 1 in 3.

2.2 Cell storage

Cells were harvested as described in section 2-1, pelleted and resuspended in ETC10 (for BHK-21 Cl3 cells) or DMEM10 (for 3T3 cells) containing 10% (v/v) DMSO. Cells were aliquoted into black cap vials at a concentration of 10^7/ml and frozen slowly to $-140^{o}C$. Cells were recovered by thawing quickly and resuspended in ETC10 or DMEM10.

2.3 Growth of virus stocks

Virus stocks were propagated in BHK-21 Cl3 cells grown in 80 oz roller bottles. The growth medium was removed and the cells were infected at a multiplicity of infection (moi) of 0.003 plaque forming units (pfu) per cell, in 20ml ETC5. After 3-4 days incubation at $31^{o}C$, when extensive cytopathic effect (cpe) had developed, the cells were shaken into the medium and pelleted at 2000 rpm for 10 min at $4^{o}C$ in a Fison's Coolspin centrifuge. The pellet was resuspended in 5ml of supernatant medium, sonicated in a bath sonicator (50W at $4^{o}C$) and centrifuged as before. The cell debris pellet was discarded and the supernatant was the cell

associated virus stock. The original supernatant from the 2000 rpm spin was centrifuged at 12000 rpm for 2 h at 4°C in a Sorval GSA rotor and the pellet resuspended in 5ml of the supernatant was sonicated. This was termed supernatant virus. Virus stocks were aliquoted in 1ml volumes and stored at -70°C. Stocks were checked for sterility and titrated at 37°C or 31°C.

2.4 Sterility 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 5 days. The cell and virus stocks were considered sterile if no contamination was detected on the plates.

2.5 Titration of virus stocks

Serial ten-fold dilutions of virus stocks were made in PBS/calf serum and 0.1ml of each dilution was added to 80% confluent BHK-21 Cl3 cells in 50mm petri-dishes from which the growth medium had been removed. After absorption at 37°C for 1 h, monolayers were overlaid with 4ml of EHu5 or EMC5 and incubated at 37°C for 2 days or 31°C for 3 days. Monolayers were fixed and stained with Giemsa at room temperature (RT) for 30 min and plaques were counted using a dissecting microscope.

2.6 Preparation of virion DNA (HSV)

Virion DNA was prepared as described by Wilkie (1973) and Stow and Wilkie (1976). Virus infected BHK-21 Cl3 cells in 80 oz roller bottles (section 2.3) were harvested after exhibiting extensive cpe and pelleted at 2000 rpm for 10 min

in a Fison's Coolspin centrifuge. The supernatant was kept
on ice and the cell pellet resuspended in RSB (10mM KCl,
1.5mM MgCl$_2$, 10mM Tris-HCl pH 7.5) containing 0.5% (v/v)
Nonidet P40 (NP40), was incubated on ice for 10 min and
centrifuged at 2000 rpm for 3 min to remove the nuclei and
cell debris. The pellet from this step was resuspended in
RSB/NP40 and treated as above. The two cytoplasmic
supernatants were pooled with the clarified virus infected
cells medium and centrifuged at 12000 rpm in a Sorval GSA
rotor for 2 h at 4°C. The virus pellet was resuspended by
sonication in 8ml of NTE (10mM Tris-HCl pH 7.5, 10mM NaCl,
1mM EDTA) and lysed by addition of SDS and EDTA to a final
concentration of 2% (w/v) and 10mM respectively. Virus DNA
was gently extracted twice with an equal volume of saturated
phenol and once with chloroform : isoamyl alcohol (24 : 1
v/v) and precipitated by the addition of two volumes of
ethanol. The DNA was pelleted at 2000 rpm for 10 min, dried
in a vacuum desiccator and redissolved in water containing
50 ug/ml RNase A. The DNA was quantitated by agarose gel
electrophoresis using a standard DNA of known concentration.

2.7 Virus particle counts

An aliquot of a virus stock was mixed with an equal
volume of latex beads and the same volume of phosphotungstic
acid. A sample was spotted on a parlodium grid, air dried
and visualised under the electron microscope. The number of
virus particles and latex beads was counted and the
concentration of particles in the virus stock calculated.

2.8 Purification of single plaque isolates

Virus stocks were titrated as described (section 2.5)

and cell monolayers with the fewest plaques (5-10 plaques) were washed twice with PBS/calf serum. Individual separated plaques were picked into 500ul PBS/calf serum, sonicated and stored at -70°C. To grow a plate stock, a BHK-21 Cl3 monolayer was infected with 100ul of the plaque solution and incubated at 37°C until the cpe was complete. The monolayer was harvested using rubber policemen, sonicated and stored at -70°C. Individual plaques were purified an additional three times prior to further analysis. To passage the plaque stocks, BHK-21 Cl3 monolayers in 50mm petri-dishes were inoculated at low moi and incubated at 37°C. Cells were harvested, sonicated and released 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 3T3 or BHK-21 Cl3 cells in 35mm petri-dishes were infected at a moi of 5 pfu/cell and incubated at 37°C or 38.5°C for 1 h. Cells were washed twice with PBS/calf serum, overlaid with 2ml of ETC5 and incubated at 37°C or 38.5°C. Samples were harvested at 0, 2, 4, 6, 8, 12, and 24 h post infection, sonicated and virus yields titrated on BHK-21 Cl3 cells at 37°C.

In multi-step growth experiments, cells were infected at a moi of 0.002 pfu/cell. At 0, 24, 48, 72, and 96 h post infection, cells were harvested, sonicated and titrated as before. Twenty four hour yield experiments were carried out by infecting cells at a moi of 5 pfu/cell. Cells were harvested after 24 h and virus titrated as above.

2.10 Virus growth properties in vivo

Virus growth experiments in vivo were carried out essentially as described by Thompson et al. (1983). Virus stocks (10^5 pfu/mouse) were inoculated into the left cerebral hemisphere of 3 week old BALB/c mice of both sexes (section 2.11). At 0, 12, 24, 48, 72, 96, 120, and 144 h post infection, two surviving mice from each time point were sacrificed and their brains were removed aseptically and homogenised in 1ml PBS/calf serum using a Dounce homogeniser (Quick Fit, England). 0.2ml of the resulting suspension was titrated for infectious virus on BHK-21 Cl3 cells at 37°C as described (section 2.5).

2.11 Animal inoculation

Three week old BALB/c mice of both sexes were used. Mice were anaesthetised with ether and 0.025ml of the appropriate virus dilution in PBS/calf 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 10^2-10^7 pfu/animal). The virus stocks were always retitrated on BHK-21 Cl3 cells on the day of inoculation to determine the precise quantity of virus inoculated. Mice were observed daily for 21 days after inoculation and their clinical states 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 day 3 and 21. Using the intraperitoneal or footpad routes, mice were inoculated with 0.1ml of appropriate virus dilutions and the LD_{50} calculated as before.

2.12 Preparation and isolation of ^{32}P-labelled virus DNA

This method is a modification of that described by
Lonsdale (1979). BHK-21 Cl3 cells, in linbro wells in PIC
medium, were infected at a moi of 10 pfu/cell. After 1 h
absorption at 37°C, the virus was removed and the cells
washed with and maintained in PIC for 2 h at 31°C. Fifty
uCi of ^{32}P-orthophosphate was added per well and incubation
continued for a further 48 h at 31°C. Cells were lysed by
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 saturated phenol and precipitated
with two volumes of ethanol. The DNA was dried at 37°C for
10 min and redissolved in 200ul water. 20ul was digested
with appropriate restriction enzymes and run on agarose gels
(0.5%-1.2%) for 24 h at 40V. Gels were air dried and
autoradiographed using Kodak XS-1 film.

2.13 Thymidine kinase assay

The method used was a modification of that of Jamieson
and Subak-Sharpe (1974). BHK-21 Cl3 cells in 35mm
petri-dishes were infected at a moi of 20 pfu/cell. After
absorption for 1 h at 37°C, cells were overlaid with 2ml of
ETC5 and incubated for 6 h at 37°C. Cells were washed twice
with cold PBS, scraped into 1ml cold PBS and pelleted in a
microfuge for 2 min. The pellet was resuspended in 150ul
ice cold lysis buffer (20mM Tris-HCl pH 7.5, 2mM MgCl$_2$, 10mM
NaCl, 0.5% (v/v) NP40, 6.5mM 2-mercaptoethanol), maintained
on ice for 5 min, mixed briefly and placed on ice for a
further 5 min. The samples were centrifuged and the
supernatant retained and stored at -70°C. 5ul of extract
was mixed with the reaction buffer in a total volume of 50ul
(0.5M Na$_2$PO$_4$ pH 6.0, 100mM MgCl$_2$, 2mM dTTp, 100mM ATP, 5ul

aqueous [Me-^{3}H] thymidine 1 mCi/ml). After incubation for 1 h at 30°C, the reaction was stopped by the addition of EDTA and thymidine at a final concentration of 20mM and 1mM respectively. The samples were heated for 3 min at 100°C and placed on ice for 3 min. After centrifugation, 40ul of the supernatant was spotted onto DE81 discs and washed three times (10 min each at 37°C) with 4mM ammonium formate pH 6.0 and 10uM thymidine. After a further two washes with ethanol, the discs were dried and radioactivity due to [^{3}H] thymidine was determined using a scintillation counter.

2.14 Preparation and analysis of HSV infected cell polypeptides

The method was essentially that described by Marsden et al. (1976). Confluent BHK-21 Cl3 cells in 35mm petri-dishes were infected at a moi of 20 pfu/cell. After absorption for 1 h at 37°C, the monolayers were washed twice with Eagle'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 h incubation at 37°C, 100 uCi/plate of ^{35}S-methionine was added and incubation continued at 37°C for 24 h. 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, 0.31% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and after boiling for 5-10 min, were analysed by SDS-PAGE (described in section 2.15).

2.15 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Two glass plates separated by a 1.5mm thickness perspex

spacer, were cast vertically and sealed with teflon 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 bisacrylamide in resolving gel buffer. Ammonium persulphate (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 polymerisation, 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 stacking gel buffer (0.11mM Tris-HCl pH 6.7, 0.1% (w/v) SDS). APS and TEMED were added to the stacking gel solutions 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, 53mM glycine, 0.1% (w/v) SDS) at 60mA for 3-4 h (Marsden et al., 1976, 1978). Gels were fixed, stained for 1 h by shaking in methanol : water : acetic acid 50 : 43 : 7 in the presence of 0.2% (w/v) Coomassie Brilliant Blue R250. Gels were destained for 1-2 h in methanol : water : acetic acid 5 : 88 : 7, dried under vacuum and exposed for autoradiography at RT.

2.16 Agarose gel electrophoresis

Agarose gels (0.5%-1.2%) were prepared in 250ml 1X TBE

buffer (89mM Tris, 89mM boric acid, 2mM EDTA). The

solutions were poured onto glass plates (125cm x 85cm) in

which (12-15) tooth combs were placed. After setting, gels

were transferred to horizontal tanks containing 1X TBE. In

the case of non-radioactive samples, TBE contained 5 ug/ml

ethidium bromide. Gels were elecrophoresed at 50V

overnight.

2.17 Restriction enzyme digestion

Viral DNA was digested with appropriate restriction

enzymes (2-5 units/ug DNA), in the presence of 1X

recommended buffer, in a final volume of 40ul and incubated

at 37°C for 2-16 h. The reaction was stopped by the

addition of 20% restriction enzyme stop solution (RE stop)

(5X TBE, 100mM EDTA, 10% (w/v) Ficoll, 0.1% (w/v)

bromophenol blue) prior to electrophoresis (Maniatis et al.,

1982).

2.18 Elution of DNA fragments from agarose gels

The DNA was digested with appropriate restriction

enzymes and electrophoresed on agarose gels in the presence

of 5 ug/ml ethidium bromide. The gel was visualised under

long wave UV light and the appropriate fragment cut out

using a sharp scalpel. The isolated gel slice was placed in

an electrophoresis chamber containing 0.1X TBE. The DNA was

electroeluted from the agarose gel onto the dialysis

membrane of the electrophoresis chamber (preboiled in 0.1X

TBE for 10 min) at 200V for 1 h. The DNA was collected,

extracted with phenol : chloroform (1 : 1) and precipitated

at -20°C with one volume isopropyl alcohol and 0.1 volume

sodium acetate. DNA was washed with 70% ethanol, dried in a

vacuum desiccator and redissolved in water.

2.19 Transfection of viral DNA by calcium phosphate precipitation

Transfection was performed as described by Stow and Wilkie (1976). Calcium chloride (final concentration 130mM) was added to a solution containing 0.2-1ug DNA and 10 ug/ml calf thymus carrier DNA in HEBS buffer pH 7.05 (130mM NaCl, 4.9mM KCl, 1.6mM Na_2HPO_4, 5.5mM D-glucose, 21mM Hepes). The samples were left for 5 min at RT to allow a fine suspension to develop. This suspension was added to drained 80% confluent BHK-21 Cl3 cells in 50mm petri-dishes. After incubation at $37^{o}C$ for 45 min, cells were overlaid with ETC5. At 4 h post infection, cells were treated with 25% (v/v) DMSO in HEBS buffer for 4 min at RT. DMSO was then removed from monolayers by washing the cells twice with ETC5 after which incubation was continued at $37^{o}C$ for 3-4 days until extensive cpe had developped. The cells were scraped into the medium, disrupted by sonication and stored at $-70^{o}C$. The transfected plate stocks were titrated and single plaques isolated for further analysis as described before (section 2.8). For correction of the genomic deletion in the variants, wild type fragments spanning the deletion were added to the transfection mixture at a 5, 10, and 20 fold molar excess over the intact deletion variant genomes. When the cpe was complete, cells were harvested as described above. To introduce the deletion into the HG52 wild type genome, the fragments containing the deletion were added at 5, 10, and 20 fold molar excess with the intact wild type genomes.

2.20 Transfection and in vivo selection

The transfection procedure was described in section 2.19 and the method of in vivo selection used was a modification of that described by Thompson et al. (1983). Serial ten fold dilutions of transfected plate stocks were made in PBS/calf serum, and 0.025ml of each dilution was inoculated intracranially into the left cerebral hemisphere of 3 week old BALB/c mice (usually two mice per dilution). Mice were observed daily and those which died from encephalitis were dissected and their brains removed and homogenised in 1ml PBS/calf serum. For virus isolation, 0.2ml of the brain homogenate was inoculated onto BHK-21 Cl3 cells in 50mm petri-dishes at 37°C. Cells were harvested after extensive cpe had developed and the virus isolates were plaque purified three times (section 2.8) prior to further analysis.

2.21 Glycerol stocks of bacteria

Bacterial stocks were prepared from 10ml standing cultures grown overnight at 37°C in L-broth containing ampicillin (100 ug/ml). 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.

2.22 Construction of recombinant plasmids

Linearised plasmid vector pAT153 (Twigg and Sherratt, 1980) was treated with calf intestinal phosphatase at a concentration of 5 units/ug plasmid DNA. After incubation at 37°C for 1 h , the DNA was extracted twice with phenol : chloroform (1 : 1), once with chloroform and precipitated with two volumes of ethanol in the presence of 0.1 volume 3M

sodium acetate. The DNA pellet was washed with 70% ethanol, dried and resuspended in an appropriate amount of water to give 40 ng/ul. A 3-4 times molar excess of the purified HSV DNA fragment relative to the phosophatase treated vector (40ng) was ligated overnight at $15^{O}C$ in a 20ul ligation reaction containing 2 units of T4 DNA ligase and 1X ligase buffer (10mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM DTT, 1mM ATP).

2.23 Transformation of bacterial cells with plasmid DNA

This procedure was based on the method described by Cohen et al. (1972). Host bacterial cells, E. coli strain DH5 (Hanahan, 1985) were grown to an optical density at 630 (OD_{630}) of 0.3 and pelleted by centrifugation at 8000 rpm for 10 min at $4^{O}C$ in a Sorval SS34 rotor. The pellet was resuspended in a half volume of ice cold 50mM $CaCl_2$ and incubated on ice for 20 min. The cells were repelleted and resuspended in a tenth volume of ice cold 50mM $CaCl_2$. Plasmid DNA (20-100ng) was incubated with $CaCl_2$ shocked cells on ice for 1 h. The cells were heat shocked at $42^{O}C$ for 2 min and transferred to 1ml L-broth grown with shaking at $37^{O}C$ in an orbital shaker for 1 h. 100ul of each sample were spread on L-broth agar plates containing 100 ug/ml ampicillin and incubated overnight at $37^{O}C$. Single bacterial colonies were picked from the plates and analysed (section 2.24).

2.24 Small scale isolation of plasmid DNA

Bacteria from a single colony were grown overnight in an orbital shaker at $37^{O}C$ in 1.5ml L-broth containing 100 ug/ml ampicillin. The cells were pelleted at 10000 rpm for 2 h in

a microfuge and resuspended in 350ul STET buffer (8% (w/v) sucrose, 0.5% Triton X-100, 50mM EDTA pH 8.0, 10mM Tris-HCl pH 8.0) and 25ul of lysozyme (10 mg/ml) was added (Holmes and Quigley, 1981). This mixture was boiled for 1 min and centrifuged at 10000 rpm for 10 min. The supernatant containing the plasmid DNA was precipitated with an equal volume of isopropyl alcohol and 0.1 volume of 3M sodium acetate on ice for 15 min and subsequently pelleted at 10000 rpm for 5 min. The DNA pellet was washed with 70% ethanol, dried in a vacuum desiccator and redissolved in water containing 10 ug/ml RNase A.

2.25 Large scale isolation of plasmid DNA

The method is basically that described by Birnboim and Doly (1979) as modified by Maniatis et al. (1982). Single bacterial colonies from L-broth agar plates or 25ul from bacterial glycerol stocks were inoculated into 10ml L-broth containing 100 ug/ml ampicillin and incubated at $37^{O}C$ overnight in an orbital shaker. 2ml of the overnight culture was inoculated into 350ml L-broth and shaken overnight at $37^{O}C$. The bacteria were pelleted at 5000 rpm for 15 min at $4^{O}C$ in Sorval a GSA rotor. The culture pellet was resuspended in 6ml of freshly prepared (25mM Tris-HCl pH 8.0, 10mM EDTA, 15% (w/v) sucrose, 2 mg/ml lysozyme) and incubated on ice for 20 min. 12ml of freshly made 0.2M NaOH, 1% SDS (w/v) was added and incubation continued on ice for 10 min. Ice cold 3M sodium acetate pH 4.6 was added and incubation continued on ice for 20 min. The cell debris was pelleted at 15000 rpm for 15 min in a Sorval SS34 rotor at $4^{O}C$ and RNase A was added to the supernatant at a final concentration of 50 ug/ml. After

incubation at 37°C for 20 min, DNA was extracted twice with an equal volume of phenol : chloroform (1 : 1 v/v), once with an equal volume of chloroform and precipitated by the addition of 2 volumes of ethanol overnight at -20°C. The DNA pellet was washed with 70% ethanol, dried in a vacuum desiccator and resuspended in water. The DNA was quantitated by comparing to a known standard.

2.26 Transfer of DNA fragments to nitrocellulose

The procedure followed was that of Southern (1975). The agarose gel containing the separated DNA fragments was placed in gel soak I (200mM NaOH, 600mM NaCl) for 1 h to denature the DNA and neutralised in gel soak II (1M Tris-HCl pH 8.0, 0.59M NaCl) for 1 h. The gel was then transferred onto sheets of Whatman 3mm filter paper presoaked in 10X SSC (1X SSC is 15mM trisodium citrate, 150mM NaCl). A sheet of Hybond blotting membrane, cut to the exact size of the gel, was placed on top of the gel followed by a similar sized sheet of Whatman 3mm filter paper wetted with blotting buffer. A stack of absorbant paper towels was placed onto the Whatman filter paper and finally a weight was placed on top. After 12-24 h, the Hybond blotting membrane was air dried and UV cross-linked for 2-5 min at a wave length of 312nm (the DNA side down on a transilluminator).

2.27 Hybridisation procedure

The Hybond blotting membrane was prehybridised at 75°C for a minimum of 2 h in a sealed plastic bag containing hybridisation buffer [6X SSC, 6X Denhardt's buffer(1X Denhardt's is 0.02% polyvinyl-pyrrolidone, 0.02% Ficoll), 0.1 mg/ml salmon sperm DNA, 10mM Tris-HCl pH 7.5]. The

solution was replaced by fresh hybridisation buffer containing nick translated ^{32}P-labelled DNA (see section 2.28) and hybridisation was allowed to proceed for 48 h at 75°C. The Hybond blotting membrane was removed and washed 3 times, each for 45 min, at 60°C in washing buffer (2X SSC, 0.1% (w/v) SDS, 5mM Na$_2$HPO$_4$ pH 7.0). The membrane was air dried and placed in contact with Kodak XS-1 film at RT.

2.28 In vitro ^{32}P-labelling of DNA by nick translation

The method was as described by Rigby et al. (1977). DNA (0.5ug) to be used as a probe was labelled in a reaction containing 2 units of DNA polymerase I, 50mM Tris-HCl pH 7.8, 5mM MgCl$_2$, 10mM dTT, 10^{-4} mg/ml DNase, 10 ug/ml BSA, 2uCi [$\propto$-^{32}P] dCTP, 2uCi [$\propto$-^{32}P] dGTP, 0.2mM dATP and 0.2mM dTTP in a final volume of 30ul. Nick translation was carried out at 15°C for 2 h. The DNA was precipitated on dry ice for 15 min with 0.6 volume of isopropyl alcohol and 0.1 volume of 3M sodium acetate. The DNA pellet was resuspended in 30ul water and reprecipitated with isopropyl alcohol and sodium acetate. The resultant pellet was redissolved in 10ul water and 80% formamide and denatured by boiling at 100°C for 5 min.

2.29 Construction of recombinant M13

The double stranded replicative form (RE) of bacteriophage M13 mp18 and mp19 (Norrander et al., 1983) was prepared as for plasmids (section 2.25). The fragments were prepared for insertion into M13 by digestion of pAT153 containing the inserts with HincII-XhoI. The fragments were eluted from the gel as described (section 2.18). The vector was linearised with SmaI-SalI and 40ng was incubated with a

120-160ng of the DNA insert with 2 units of T4 DNA ligase in ligase buffer (see section 2.22) for 24 h at 15^OC (Sanger et al., 1980).

2.30 Transfection of bacterial cells with M13

E. coli strain JM101 (Messing, 1979) were grown in 2X YT broth to an OD_{630} of 0.3 and calcium shocked as described (section 2.23). The ligation mixture was added to 0.2ml aliquots of the calcium shocked cells and incubated on ice for 1 h. The sample was heated to 42^OC for 5 min and 0.2ml of an overnight standing culture of E. coli strain JM101 was added. 3ml of melted top agar at 42^OC containing 20ul of 2.5% isopropyl-D-thiogalactoside (IPTG) and 25ul of 2% 5-chloro-4-bromo-3-indolyl-β-D-galactoside (BCIG) in dimethyl formamide was added to the sample and the mixture poured onto 90mm L-broth agar plates and incubated at 37^OC overnight.

2.31 Growth and extraction of recombinant M13 clones

An overnight standing culture of E. coli strain JM101 was used to inoculate 2X YT broth (1 : 100). This was dispensed in 1.5ml aliquots into 25ml universal bottles. The recombinant M13 clones (colourless) plaques were tooth picked from the plates into the broth and incubated at 37^OC for 5-6 h with shaking. The solution was then transferred to an eppendorf tube and centrifuged at 10000 rpm for 5 min in a microfuge to pellet the bacteria. The supernatant (0.8ml) was transferred to another eppendorf tube and the phage was precipitated by addition of 200ul of 20% polyethylene glycol (PEG Mr 6000) in 2.5M NaCl for 30 min at RT. After centrifugation at 10000 rpm for 5 min, the

supernatant was removed. Following a brief centrifugation, any remaining supernatant was removed with a glass capillary tube. The phage pellet was resuspended in 100ul TE and extracted with 50ul phenol equilibrated with TE and precipitated by the addition of 2 volumes of ethanol and 0.1 volume 3M sodium acetate. The phage DNA was pelleted by centrifugation at 10000 rpm for 5 min, washed with 70% ethanol, dried thoroughly in a vacuum desiccator, redissolved in 50ul TE and stored at -70°C (Sanger et al., 1980).

2.32 Sequence analysis of recombinant M13 clones

Sequence analysis was carried out using the dideoxynucleotide chain termination method of Sanger et al. (1977). The single stranded DNA template (2ul) was annealed to 2.5ng of M13 single stranded primer in the presence of annealing buffer (10mM Tris-HCl pH 8.5, 10mM MgCl$_2$) in a volume of 10ul at 37°C for 30 min. To the annealed DNA, 2 units of Klenow DNA polymerase I was added and the mixture aliquoted in 2ul fractions into four wells of a U-bottomed microtiter plate corresponding to the specific T, C, G and A reactions of each clone. An equal volume of the nucleotide mixture containing dNTP's and specific ddNTP's (see Table 2.1), 1ul of 11.8uM dATP, 0.3uCi [α-^{32}P] dATP was added to each well and the reaction allowed to proceed for 15 min at RT. The reaction was chased by the addition of 2ul chase solution (0.5mM uniform mixture of all four dNTP's) and incubation was continued for 30 min at RT. The reaction was stopped by the addition of 2ul formyl dye mixture (0.1% bromophenol blue (w/v), 0.1% xylene cyanol (w/v) in deionised formamide). The plate was heated for 1 min at

Table 2.1. Nucleotide concentration in DNA sequencing reaction

Sequencing solutions dNTP's concentration (5mM)	dG-O	dA-O	dT-O	dC-O
dTTP	20	1	20	20
dCTP	20	20	1	20
7-deaza dGTP	20	20	20	1
10X TE	50	50	50	50
H_2O	540	370	370	370

Sequencing mix	dN-O mix	ddNTP's	H2O
T-sequence	250	250 (600uM ddTTP)	0
C-sequence	250	57.5 (140uM ddCTP)	197.5
G-sequence	250	77.5 (200uM ddGTP)	172.5
A-sequence	250	62.5 (140uM ddATP)	187.5

100°C and electrophoresis was carried out through polyacrylamide gels.

2.33 Electrophoresis and autoradiography of sequencing gels

Vertical plates 40 x 20 x 0.03cm in size were used and spacer, gel combs were cut from plasticard. The notched plate was treated with repelcote. The plain plate was treated with 0.5% (v/v) Wacker silicone in 0.3% (v/v) acetic acid and ethanol (Garoff and Ansorge, 1981), which bonds the acrylamide to the plate. Generally, electrophoresis was carried out through 6% acrylamide gels. In this system the top mix used was 0.5X TBE, 6% acrylamide (electran grade), 9M urea (McGeoch et al., 1986). APS 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 the gel rested in a nearly horizontal position until polymerisation was complete. The tape was removed from the bottom of the gel and the plates set with 0.5X TBE in both the top and bottom tank of the gel kit. Before loading the DNA samples, gel wells were flushed with 0.5X TBE and the gel was run at a constant power of 40W for 2 h. 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 the DNA and remove the urea from the gel. The gel was dried down on the plate in an oven at 125°C for 1-2 h and exposed against Kodak XS-1 film at RT.

2.34 Accumulation and handling of the sequence data

DNA sequence data was handled and interpreted using the Institute of Virology's VAX/VMS computer running the GCG soft ware (Devereux et al., 1984). The gel readings were

read and typed into the account using the EDIT programme which stores data from gel readings under a chosen file name. The data was converted into GCG sequences using the FROMSTADEN programme. The gel readings were compared against each other using the programme BESTFIT to determine overlapping. The gel readings were aligned and compared against known sequences currently held using the BESTFIT and GAP programmes.

2.35 Tissue preparation for histopathology

Infected brains from dissected mice were removed and fixed in 10% formal buffered saline. The brain tissues were cut into blocks, dehydrated in a graded series of ethanol (50%, 70%, 90% and 100%) and embedded in paraffin wax. Several sections (6-7 microns thick) from each block were cut, mounted, baked for 24 h at 37°C followed by 30-60 min at 60°C and stained with either haematoxylin and eosin (H & E) or by immunoperoxidase staining.

2.36 Immunohistochemistry

The method used was essentially that described by Kennedy et al. (1985). Paraffin embedded sections were deparaffinised and dehydrated through xylene (two times, 30 min each) and graded ethanol series (50%, 70%, 90% and 100%, 5 min each) respectively. Sections were washed for 10 min in PBS and incubated for 30 min with 0.3% hydrogen peroxide in methanol to stop the endogenous peroxidase activity. Following washing twice with PBS (10 min each), sections were incubated with blocking serum (3% normal goat serum in PBS) for 30 min. The excess of blocking serum was washed off and sections were incubated for 30 min with primary

antibody (polyvalent rabbit antiserum to HSV) diluted 1:100
in PBS. Sections were washed twice with PBS and incubated
with secondary antibody (biotinylated anti-rabbit IgG)
diluted 1:100 in PBS for 30 min. After washing twice with
PBS, sections were incubated with avidin biotinylated
horseradish peroxidase complex (ABC) for 30 min.
Visualisation of the reaction was carried out using 3, 3'-
diaminobenzidine tetrahydrochloride (0.5 mg/ml in PBS)
containing 0.03% hydrogen peroxide for 5 min until the
colour developed. Sections were washed with tap water,
counterstained in haematoxylin for 30-60 seconds, dehydrated
as before, mounted and examined under the light microscope.

CHAPTER 3

RESULTS

3.1 Virulence variation within the elite laboratory stock of HSV-2 strain HG52

3.1.1 Introduction

HSV is a neurotropic virus that spreads centripetally in the nervous system from the primary site of infection (Cook and Stevens, 1973). In humans, invasion of the nervous system frequently leads to a latent infection in sensory ganglia, and in rare instances fatal encephalitis (Baringer and Swoveland, 1973; Fenner et al., 1974; Johnson, 1982). The virus-host interaction is highly complex and influenced by the genotype of both virus and host. As direct study of HSV virulence in humans under experimental conditions is impossible, various animal model systems have been used. Previous work with animals has shown that most HSV strains, following different routes of inoculation, have the capacity to cause encephalitis and ultimately death. However, some isolates fail to kill the animals (Richards et al., 1981; Dix et al., 1983; Sedarati and Stevens, 1987). Heterogeneity of individual virus strains in functions required for replication of virus at the periphery and/or for spread to the central nervous system may be the cause of such differences. These functions might influence the pathogenesis of HSV at a variety of points; replication at the peripheral site, neural entry, spread within the nervous system and escape from immunological control. Though the intracranial route of inoculation may be criticised for being artificial, it has the clear advantage of bypassing many of the steps identified above as possibly subject to variation.

HSV-2 strains are normally more virulent than those of HSV-1 when inoculated intragenitally, intraperitoneally or intracranially into experimental animals (Plummer et al., 1968; McKendall, 1980; Richards et al., 1981; Scriba and Tatzber, 1981). However the HSV-2 strain HG52 is less virulent than several other HSV-2 strains (Thompson and Stevens, 1983a). It has been shown that the elite stock of HG52 contains variants with detectable deletions in the genome at a fequency of 24% (Harland and Brown, 1985). It should be emphasised that HSV-2 strain HG52 is not unique in this respect, genomic variation having also been observed in other laboratory strains e.g HSV-2 strain 168 (Esparaza et al., 1974; J. Harland, personal communication).

3.1.2 Isolation of single plaque stocks

In order to determine the basis for variation in neurovirulence we have evaluated the neurovirulence of single plaque stocks picked from HG52. The elite stock of HG52 was derived from an initial single plaque from a patient isolate which was plaque purified three times before being inoculated into a 20 oz bottle and finally two rounds of growth in 20 burrlers - all in BHK-21 Cl3 cells (Timbury, 1971). This elite laboratory stock of HSV-2 strain HG52 was plated out on BHK-21 Cl3 cells and twenty well separated plaques were picked at random from plates containing no more than 20 plaques. The virus had been grown in the presence of 5% human serum to neutralise released virus and to prevent the spread of plaques. The medium containing human serum was washed off thoroughly before picking plaques. The virus from each plaque was passaged twice at $37^{\circ}C$ in BHK-21 Cl3 cells (see method section 2.8), to increase the titre

before immediately growing a large scale stock at 31°C (see

method section 2.3). All the plaque stocks were of a

typical HSV-2 mixed syncytial/non-syncytial morphology. At

each passage the DNA from each of the twenty virus stocks

was subjected to restriction endonuclease digestion using

the enzymes Xbal, BglII, EcoRI, HindIII, HpaI and BamHI (see

method section 2.17). The results showed no apparent

differences in the sizes of fragments and distribution of

sites in the DNA of the twenty plaque stocks. The BamHI

profiles of four independent plaque stocks are shown in

Figure 3.1 as representative examples. The BamHI $\underline{g}$ and $\underline{z}$

bands which differed in size in individual plaque stocks are

located in the joint and repeat regions of the genome and

are known variable fragments (Davison and Wilkie, 1981).

3.1.3 Neurovirulence of single plaque stocks

To determine the neurovirulence of the individual plaque

stocks, ten were selected at random for mice inoculation.

Groups of six to eight 3 week old BALB/c mice were

inoculated with either 10^3, 10^4 or 10^5 pfu/mouse of each of

the plaque stocks intracranially (0.025ml of virus) into the

left cerebral hemisphere after anaesthetising (see method

section 2.11). In this series of experiments no mice died

before day three following inoculation. The 50% lethal dose

(LD_{50}) was calculated on the basis of deaths between days 3

and 21 according to the formula of Reed and Muench (1938).

The LD_{50} values of the plaque stocks are shown in Table 3.1.

The stocks were assigned to one of three classes of

neurovirulence depending on the LD_{50} values. Class I of

high virulence (LD_{50} <10^3 pfu/mouse) contains plaque stocks

17 and 14. Class II of intermediate virulence (LD_{50} 10^3-10^4

Figure 3.1

Autoradiographs of BamHI digests of viral DNA
^{32}P-labelled _in vivo_ (1.2% agarose) of plaque purified
stocks of HG52 (from left to right, 8, 12, 11 and 14).
Letters refere to specific fragments.
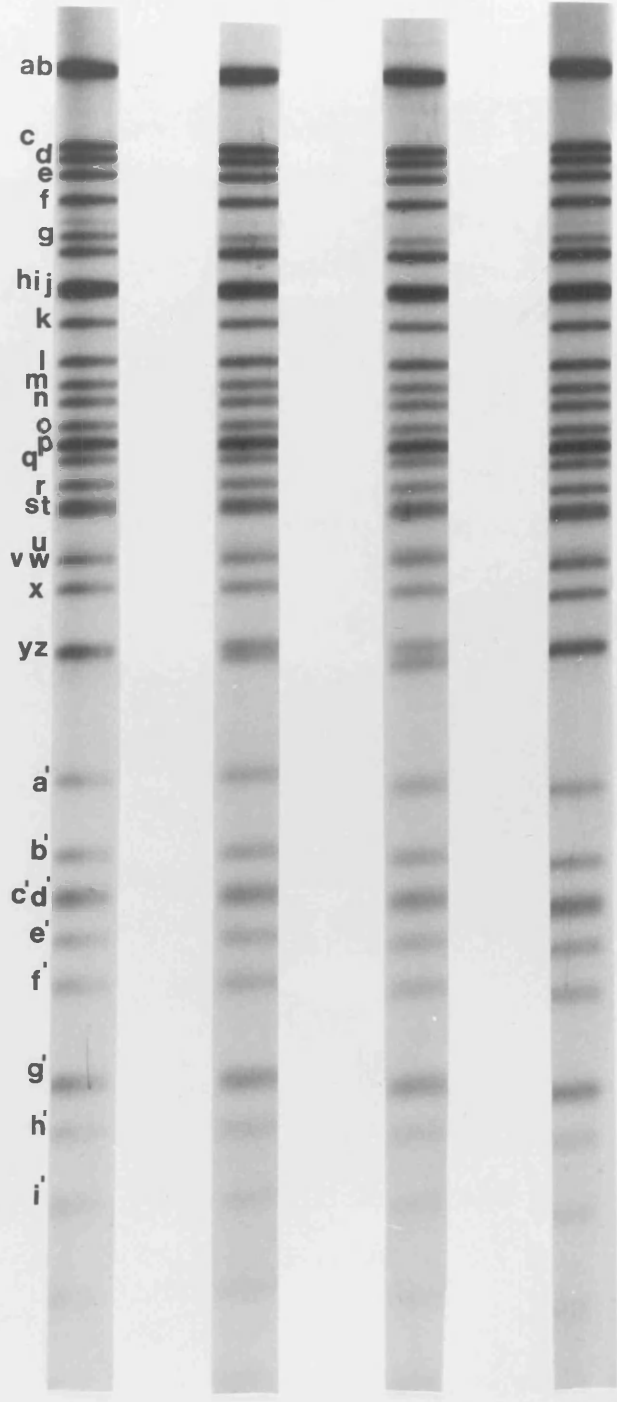
Table 3.1. LD_{50} values after intracranial inoculation of plaque purified stocks of HG52

Plaque number	Particle : pfu	LD_{50} (pfu/mouse)
1	24 : 1	4×10^4
2	10 : 1	7×10^4
3	50 : 1	2×10^4
4	294 : 1	1.5×10^4
7	235 : 1	1.5×10^4
8	14 : 1	3×10^5
11	281 : 1	1.5×10^4
12	168 : 1	10^4
14	9 : 1	$<10^3$
17	6 : 1	$<10^3$

3 week old BALB/c mice were used (6 mice/dose).

0.025ml of virus was inoculated.

pfu/mouse) contains plaque stocks 1, 2, 3, 4, 7, 11 and 12 and class III of low virulence (LD_{50} >10^5 pfu/mouse) contains plaque stock 8.

To determine whether the difference in the LD_{50} values of the plaque stocks was related to the number of viral particles within the stocks, particle counts were performed (see method section 2.7) and the particle : pfu ratios are presented in Table 3.1. All the plaque stocks had particle : pfu ratios within the acceptable range for HSV-2. There is no correlation between the particle : pfu ratio and virulence of the plaque stocks 2, 8, 14 and 17 all having ratios of 14 : 1 or less.

Eleven separate virus isolates were recovered from the brains of individual mice immediately after death. The mice had been inoculated with different plaque stocks at a variety of doses. Brain tissue was homogenised and 0.2ml of the homogenate inoculated onto BHK-21 Cl3 cells (see method section 2.10) from which virus stocks were made. The DNA from each of the eleven virus isolates was subjected to restriction endonuclease analysis using the enzymes Xbal, BglII, EcoRI, HindIII, HpaI and BamHI. There were no apparent differences in the restriction enzyme patterns of each virus isolate compared to the initial infecting viruses. The BamHI profiles of the DNA of five independent virus isolates are shown as representative examples in Figure 3.2.

Two plaque stocks categorised as being of high virulence (14 and 17), one of intermediate virulence (2) and one of low virulence (8) were chosen for further study on the basis of their LD_{50} values and particle : pfu ratios. To confirm their LD_{50} values and compare with that of the elite stock,

Figure 3.2

Autoradiographs of BamHI digests of viral DNA
^{32}P-labelled in vivo (1.2% agarose) of viruses recovered

from the brain tissue of dead mice (from left to right,

12, 7, 7, 14 and 17). Letters refer to specific

fragments.
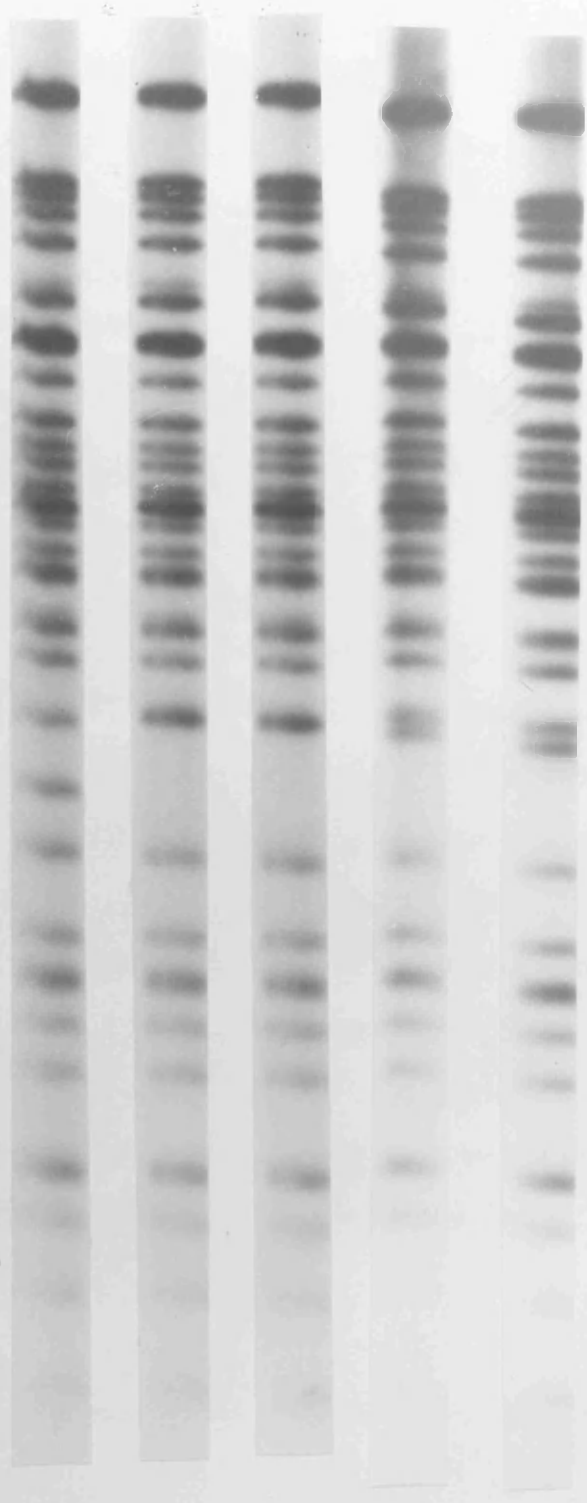
0.025ml of each of the above plaque stocks was inoculated intracranially into the left cerebral hemisphere of 2-3 week old BALB/c mice at doses ranging from 10^2-10^5 pfu/mouse and the LD_{50} values determined as before (see method section 2.11). The results are shown in Table 3.2 and are comparable with the results obtained in the first experiment (see Table 3.1). In addition the LD_{50} value of the elite stock of HG52 was determined and found to be $<10^2$ pfu/mouse.

The clinical signs produced in mice following intracranial inoculation were closely observed. Some mice became hunched with ruffled fur and ceased to be active with death following rapidly. These signs were seen within 2-3 days following inoculation with the high virulence stocks at doses of 10^4-10^5 pfu/mouse, but developed more slowly after inoculation with high virulence stocks at lower doses of 10^2-10^3 pfu/mouse or with virus stocks of low or intermediate virulence at doses of 10^2-10^5 pfu/mouse. The mice also showed a wide range of neurological signs, for example, fits and limb paralysis of varying severity. Some mice which showed severe neurological signs were killed according to the regulations for conducting animal experimentation.

3.1.4 Virulence analysis using the intraperitoneal route of infection

The LD_{50} values of the plaque stocks 14, 17, 2 and 8 were also examined using the intraperitoneal route of inoculation. Virus stocks were inoculated intraperitoneally at doses of 10^5-10^7 pfu/mouse (0.1ml of virus) into 3 week old BALB/c mice (see method section 2.11). HSV-1 strain 17 syn+ (Brown et al., 1973) was also used in this experiment

Table 3.2. LD_{50} values after intracranial inoculation of selected plaque purified stocks and the elite stock of HG52

Plaque number	LD_{50} (pfu/mouse)
2	2×10^4
8	$> 10^5$
14	$< 10^2$
17	5×10^2
HG52*	$< 10^2$

* Non-plaque purified elite stock.

2-3 week old BALB/c mice were used (6 mice/dose).

0.025ml of virus was inoculated.

as a positive control with a known LD_{50} value. The results

shown in Table 3.3 demonstrate a clear difference between

the LD_{50} value of the HSV-2 strain HG52 and the HSV-1 strain

17 <u>syn</u>[+]. The plaque stocks of HG52 (14, 17, 2 and 8) showed

differences in the LD_{50} value which were comparable to the

differences in the LD_{50} value following intracranial

inoculation. Virus stocks 14 and 17 had LD_{50} value of 10^5

pfu/mouse while stocks 2 and 8 were avirulent with LD_{50}

value of $>10^7$ pfu/mouse. An unexpected finding was

inversion of the dose response curves; though mice died at

low dose (10^5 pfu/mouse) none died at higher doses (10^6-10^7

pfu/mouse). These findings were seen only with the elite

stock of HG52 and the plaque purified stocks (14, 17, 2 and

8).

3.1.5 Replication efficiency in vitro and in vivo

The replication efficiencies of the plaque stocks (14,

17, 2 and 8) and the elite stock of HG52 were tested <u>in</u>

<u>vitro</u> and <u>in vivo</u>. One step growth experiments were carried

out in BHK-21 Cl3 cells at $37^{o}C$ (see method section 2.9) and

the results are shown in Figure 3-3. Each stock grew well

and there was no difference in the timing of the growth

cycle or the yield from any of the plaque stocks compared to

the elite stock of HG52.

The possibility that the observed differences in the

LD_{50} value of the individual plaque stocks was due to

differential abilities to replicate in mouse brain was

tested. The plaque stocks (14, 17, 2 and 8) and the elite

stock of HG52 were inoculated at doses of 10^5 pfu/mouse into

the left cerebral hemisphere of 3 week old BALB/c mice. At

daily intervals post inoculation, mice were sacrificed

Table 3.3. LD_{50} values after intraperitoneal inoculation of selected plaque purified stocks of HG52, elite stock of HG52 and HSV-1 strain 17 <u>syn</u>+

Plaque number	LD_{50} (pfu/mouse)
17	10^5
14	10^5
8	$>10^7$
2	$>10^7$
HG52	$>10^6$
HSV-1 (17 <u>syn</u>+)	10^5

3 week old BALB/c mice were used (6 mice/dose).

0.1ml of virus was inoculated.

Figure 3.3

One step growth curves of plaque stocks 14, 17, 2, 8 and

the elite stock of HG52 in BHK-21 Cl3 cells (50mm

monolayers 4x10^6 cells) at 37°C. The cells were

infected at a moi of 5 pfu/cell. After 45 min

absorption, cells were washed, overlaid with ETC5 and

incubated at 37°C. Cultures were harvested at the times

indicated and the virus titre measured by plaque assay

on BHK-21 Cl3 cells is given per ml (10^6 cells). O HG52

; □ plaque stock 14 ; ■ plaque stock 17 ; △ plaque stock
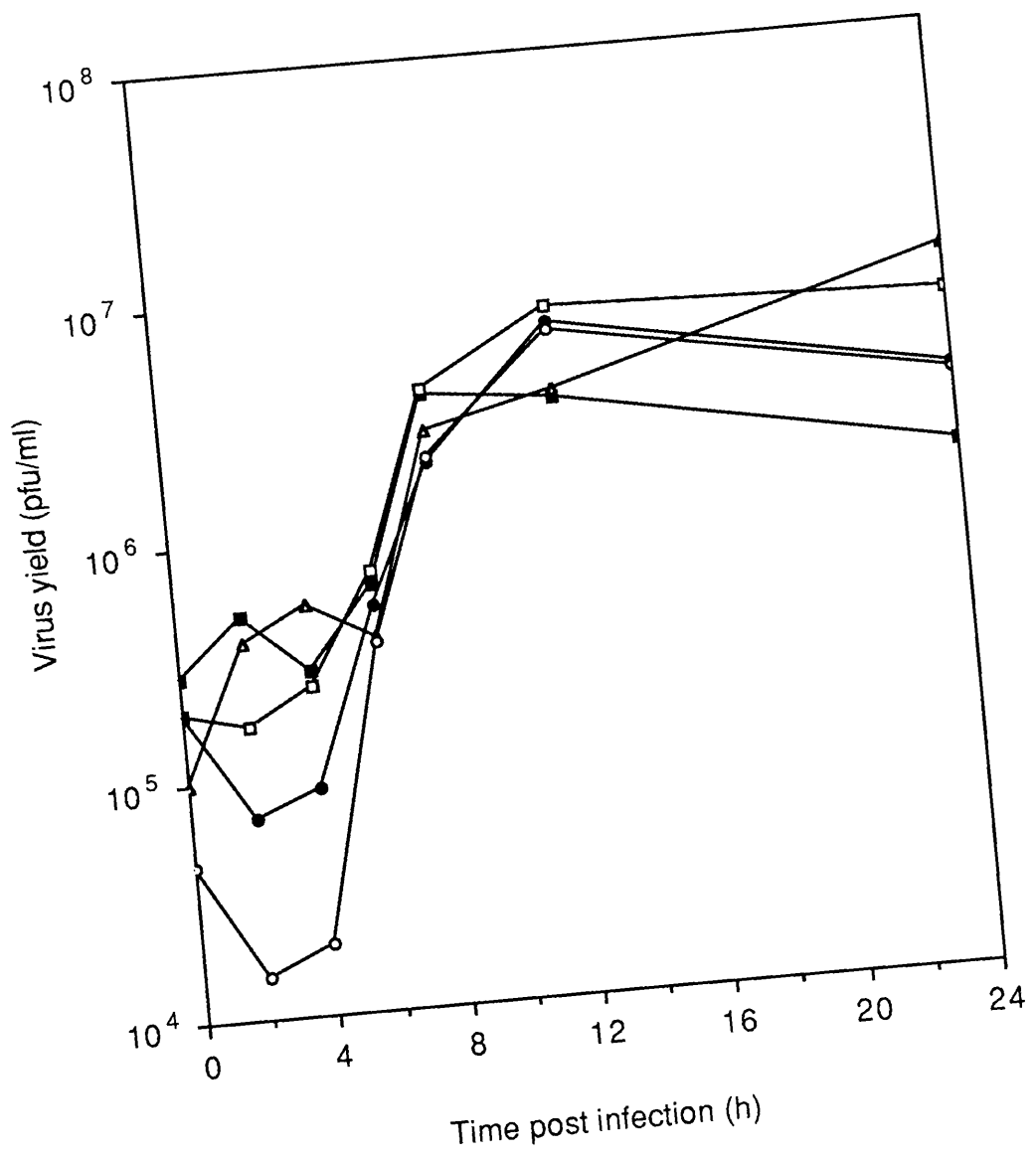
2 ; ● plaque stock 8.

(usually 2 mice/time point), their brains removed, homogenised and assayed for virus by titration on BHK-21 Cl3 cells at 37°C. The in vivo growth curves are presented in Figure 3.4. Plaque stocks 14, 17 and the elite stock of HG52 replicated while plaque stocks 2 and 8 replicated poorly. To determine whether the inefficient brain replication of plaque stocks 2 and 8 was specific to the brain cells or generalised to all mouse cells at the accepted body temperature of mice i.e 38.5°C, comparative one step growth experiments of plaque stocks (14, 17, 2 and 8) were carried out in BALB/c mouse embryo fibroblast (3T3) cells at 38.5°C, and the virus yield titrated on BHK-21 Cl3 cells at 37°C (see method section 2.9). The results are given in Figure 3.5. The plaque stocks (14, 17, 2 and 8) showed no replication and the virus titre diminished with time. The 3T3 cells appear to be non-permissive for HG52 at 38.5°C. The plaque stocks (14, 17, 2 and 8) were not temperature sensitive. Twenty four hour yield experiments (see method section 2.9) of plaque stocks 14, 17, 2 and 8 in BHK gave titres of $4x10^7$, $6x10^7$, $6.5x10^7$ and $5x10^7$ pfu/ml at 31°C and $3x10^7$, $4x10^7$, $3.5x10^7$ and $3x10^7$ pfu/ml respectively at 38.5°C.

3.1.6 Effect of passage on neurovirulence of individual stocks

To determine whether the plaque stocks (14, 17, 2 and 8) which had been categorised for virulence retained their virulence category following passage in BHK-21 Cl3 cells, they were passaged five times at low moi (see method section 2.8) and ten plaques were picked from each stock. Virus stocks were made from each of these ten plaques and their

Figure 3.4

Replication kinetics of plaque stocks 14, 17, 2, 8 and

the elite stock of HG52 in mouse brain in vivo. Three

week old BALB/c mice were inoculated intracranially into

the left cerebral hemisphere with 0.025ml containing 10^5

pfu of virus. At the time indicated post infection,

surviving mice were sacrificed (2 mice/time point),

their brains were removed, homogenised and the resulting

homogenate was titrated for virus on BHK-21 Cl3 cells at

37°C. O HG52 ; □ plaque stock 14 ; ■ plaque stock 17 ; △

plaque stock 2 ; ● plaque stock 8.
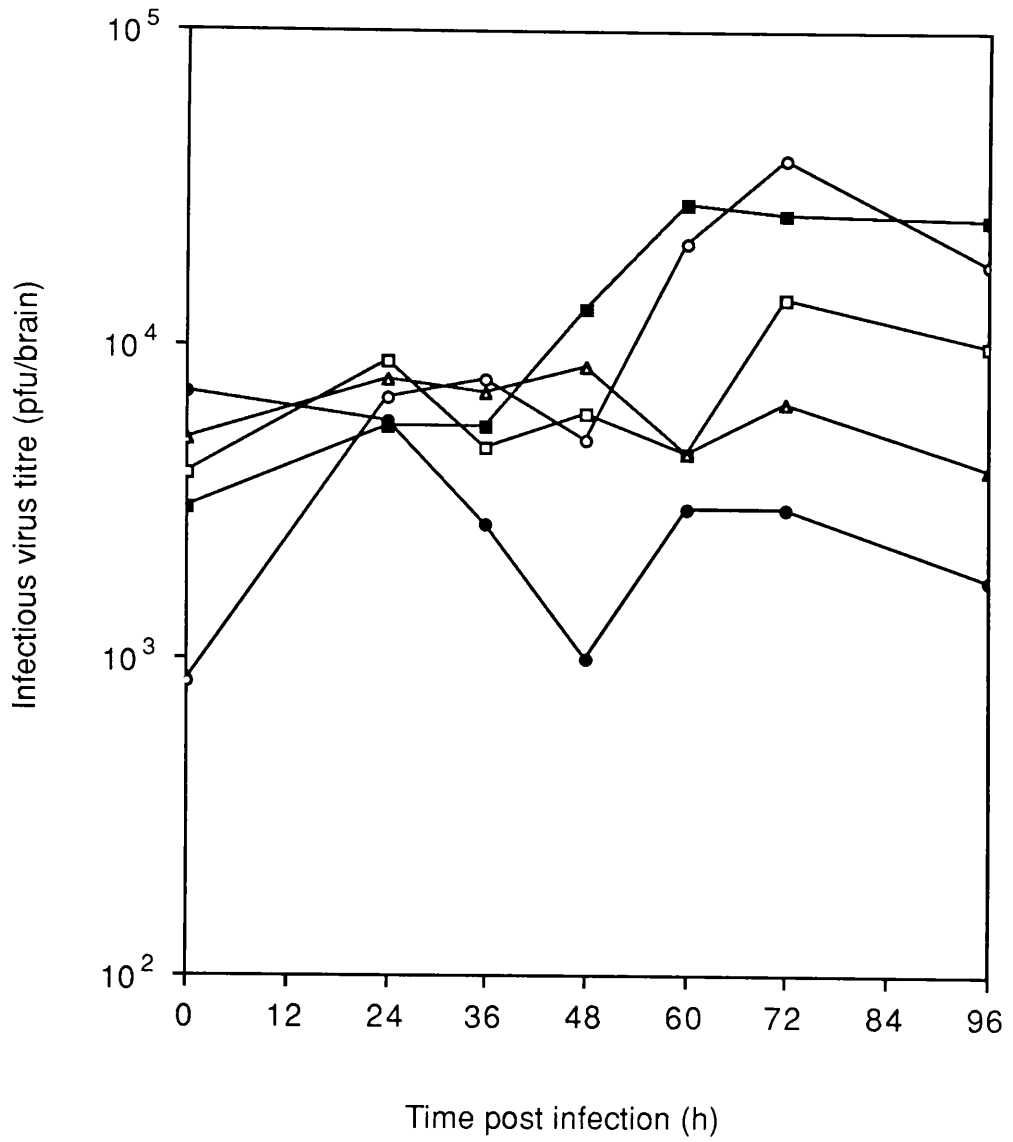
Figure 3.5

One step growth curves of the plaque stocks 14, 17, 2
and 8 in 3T3 cells at 38.5^{O}C. Cells were infected at a
moi of 5 pfu/cell. After absorption for 45 min at 37°C,
the monolayers were washed, overlaid with ETC5 and
incubated at 38.5^{O}C. Cultures were harvested at the
times indicated and the virus titre, measured by plaque
assay on BHK-21 Cl3 cells, is given per ml (10^6 cells). □
plaque stock 14 ; ■ plaque stock 17 ; △ plaque stock 2 ; ●
plaque stock 8.
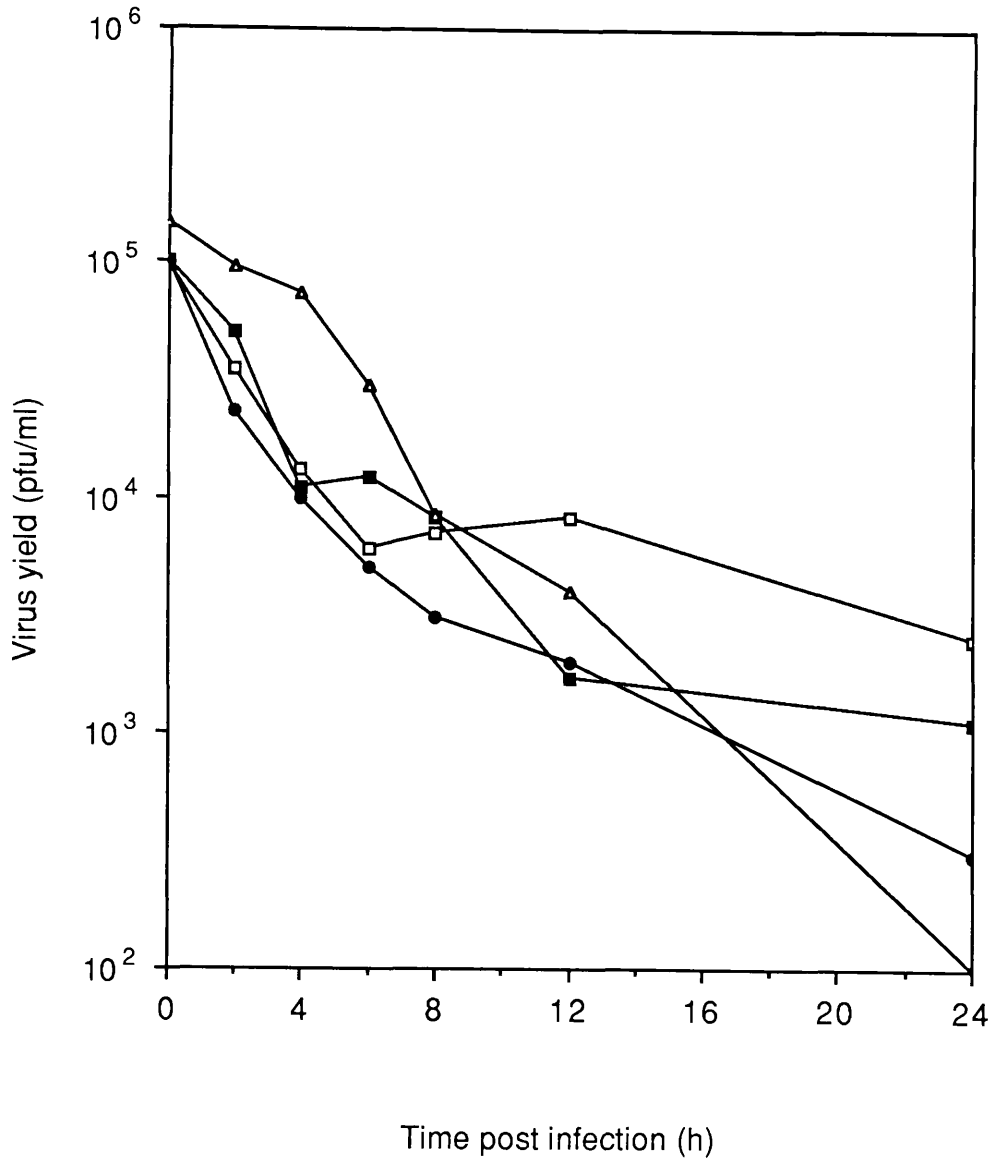
DNAs analysed using six different restriction enzymes. The results showed no apparent differences in the pattern of each DNA stock and the BamHI profiles of four independent plaque stocks and the elite stock of HG52 are shown as representative examples in Figure 3.6. Two stocks each from the plaque stocks 2, 8 and 14 and four from the plaque stock 17 at the 5th passage were chosen at random and inoculated intracranially into the left cerebral hemisphere of 3 week old BALB/c mice at doses of 10^2-10^5 pfu/mouse (0.025ml of virus) and the LD_{50} values were determined as before (see method section 2.11). The results are presented in Table 3.4. The virus stocks derived from the intermediate (plaque stock 2) and low (plaque stock 8) virulence stocks remained stably of intermediate or low virulence. The virus stocks derived from the high (plaque stocks 14 and 17) virulence stocks were more heterogeneous. Some demonstrated intermediate levels of virulenece (10^3-10^4 pfu/mouse) e.g virus stocks 14/3, 14/4 and 17/1 while others retained their levels of virulence. These results clearly demonstrated the virulence heterogeneity within the stock of HSV-2 strain HG52 and reemphesised the necessity of working with well characterised plaque purified stocks when assessing virulence. The high virulence plaque stock 17 was chosen as the baseline to study the effect of defined deletions in HG52 on virulence.

3.2 Virulence of deletion variants of HSV-2 strain HG52

3.2.1 Introduction

The identification of viral genes that control pathogenic properties _in vivo,_ and the elucidation of their functions are of fundamental importance to an understanding

Figure 3.6

Autoradiographs of BamHI digests of viral DNA
^{32}P-labelled <u>in vivo</u> (1.2% agarose) of the 5th passage
plaque purified stocks and the elite stock of HG52 (from
left to right), HG52, 8/10, 14/4, 17/1, and 17/3.
Letters refer to specific fragments.
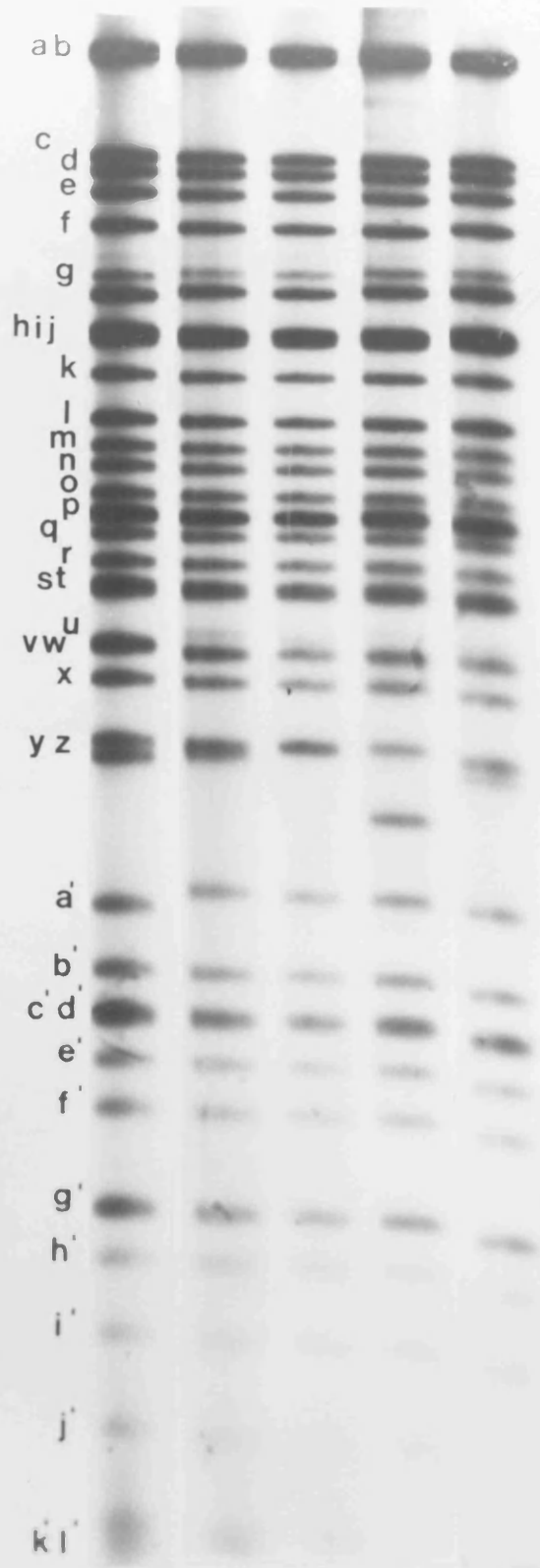
Table 3.4. LD_{50} values after intracranial inoculation of the 5th passage of the selected plaque purified stocks of HG52

Plaque number	LD_{50} (pfu/mouse)
2/7	$>10^5$
2/8	$>10^5$
8/4	$>10^5$
8/10	2.1×10^4
14/3	3.9×10^4
14/4	2×10^3
17/1	3×10^3
17/3	5.5×10^2
17/8	$<10^2$
17/9	$<10^2$

3 week old BALB/c mice were used (6 mice/dose).

0.025ml of virus was inoculated.

of the basic molecular mechanism of HSV pathogenesis. Over
the past several years, many laboratories have been involved
in studies of HSV genes and gene products specifically
related to neurovirulence. Most of these studies were
carried out on HSV-1 using deletion mutants, temperature
sensitive (ts) mutants, monoclonal antibody resistant (mar)
mutants, drug resistant mutants and intertypic recombinants.
Very little is known about HSV-2 genes or gene products
associated with neurovirulence.

A series of HG52 variants with deletions in the internal
long repeat (IR$_L$) or short unique/terminal short repeat
(U$_S$/TR$_S$) regions of the genomes were studied to define the
role of the deleted genes in HSV neurovirulence using the
mouse model system. The size and location of the deletions
and growth characteristics of the variants have been
reported by Harland and Brown (1985) and Brown and Harland
(1987). The fact that the variants were not impaired in
their lytic growth cycle demonstrated that the deleted genes
were not essential for growth in vitro. Figure 3.7 shows a
schematic map of the HG52 genome with the location of the
IR$_L$ deletions in the variants under study. The variants
used were as follows: their nomenclature when first
published is given in brackets.

JH2603 (HG52XD86): has a 3.75 kb deletion which includes the
BamHI p/f site at 0.775 m.u. Most or all of the deleted
sequences are within IR$_L$ and probably span the U$_L$/IR$_L$
junction. The deletion must remove at least part of the
coding region of the immediate early gene 1 (IE1)
(0.785-0.81 m.u.) which codes for the immediate early
polypeptide VmwIE118 (Preston et al., 1978; Easton and
Clements, 1980).

Figure 3.7

Structure of the HG52 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 an expansion of the XbaI $\underline{g}$ fragment (0.7-0.83 m.u.). The positions of the 'a' sequence and the transcripts for IE1 and IE2 genes are indicated. The third line shows the positions of the BamHI sites within XbaI $\underline{g}$. The location and the extent of the deletions in the variants is indicated. JH2604 is the only variant which contains a deletion in both copies of the R_L.
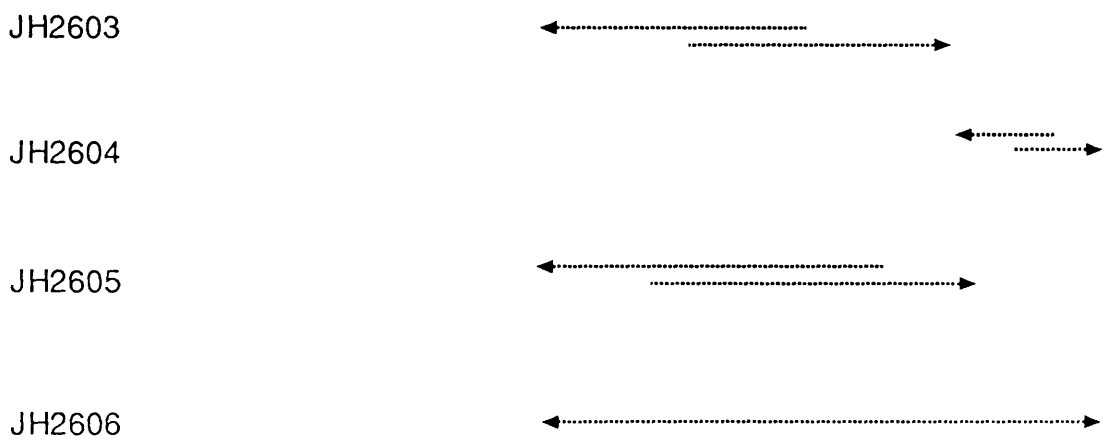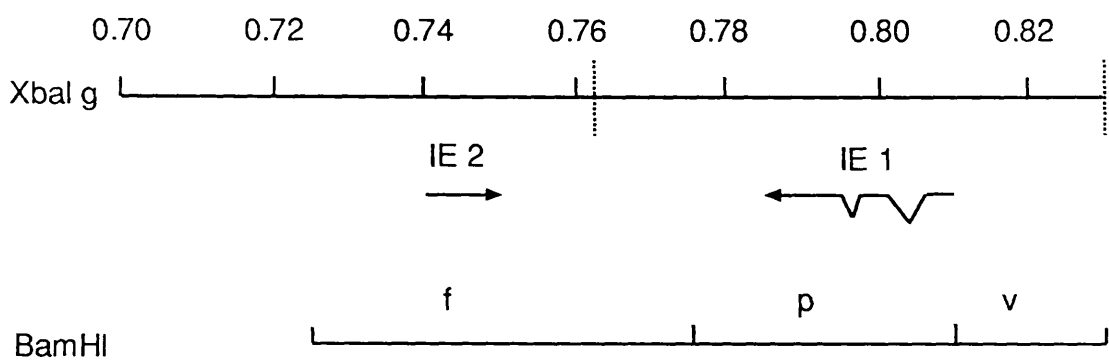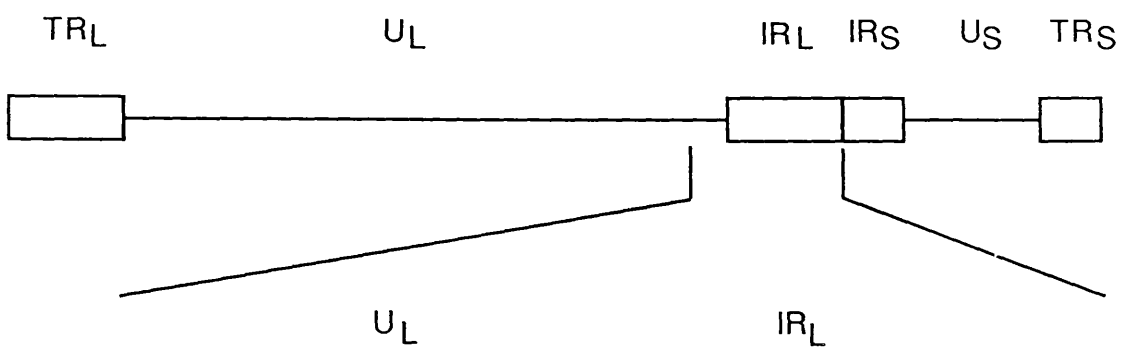
JH2604 (HG52XD192): has a 1.5 kb deletion in TR_L and IR_L within both copies of the BamHI v fragment (0-0.02 and 0.81-0.83 m.u.).

JH2605 (HG52XD85/5): has a deletion of 5.5 kb within IR_L including the BamHI p/f site and probably the U_L/IR_L junction.

JH2606 (HG52XD85/4): has 9 kb deletion encompassing the BamHI p/f site, and the BamHI p and v fragments excluding the 'a' sequences. The deletion extends from approximately 0.76 to 0.83 m.u. including the total coding region of the IE1 gene and the U_L/IR_L junction.

A diagrammatic representation of the location and extent of the deletions in the U_S and TR_S components of the genome in the altered variants is shown in Figure 3.8. All have lost the Xbal site at 0.7 m.u. The variants analysed are as follows: their nomenclature when first published is given in brackets.

JH2607 (HG52XD163X12): has a deletion of 7.75 kb extending from approximately 0.94-0.994 m.u. The genes deleted are US10, US11 and US12 coding for polypeptides of Mr 33K, 21K/22K, and VmwIE12 respectively (Watson et al., 1979; Marsden et al., 1982; Dalziel and Marsden, 1984; Lee et al., 1982), one copy of IE3 coding for VmwIE182 (Morse et al., 1978) and one copy of the short origin of replication (ori_S) (Stow and McMonagle, 1983).

JH2608 (HG52X163X14): has a deletion of 7.5 kb including the genes US10, US11 and US12. The deleted sequences have been replaced by inverted duplication of U_S/IR_S sequences between 0.83-0.91 and 0.94-1.2 m.u. such that the short repeat region is extended by 6 kb on either side. This means that this variant has two copies of the genes US1, US2, US3, US4

Figure 3.8

XbaI map for the DNA of HSV-2 strain HG52 (top line)
from Cortini and Wilkie (1978), showing the position of
XbaI sites. The location of the deletion in the
variants is indicated and the positions of the lost XbaI
sites (X) are also shown in each genome below the top
line.

The origin of the joint fragments is as follows; a =c+h,
b =c+i, e =g+h, f =g+i.
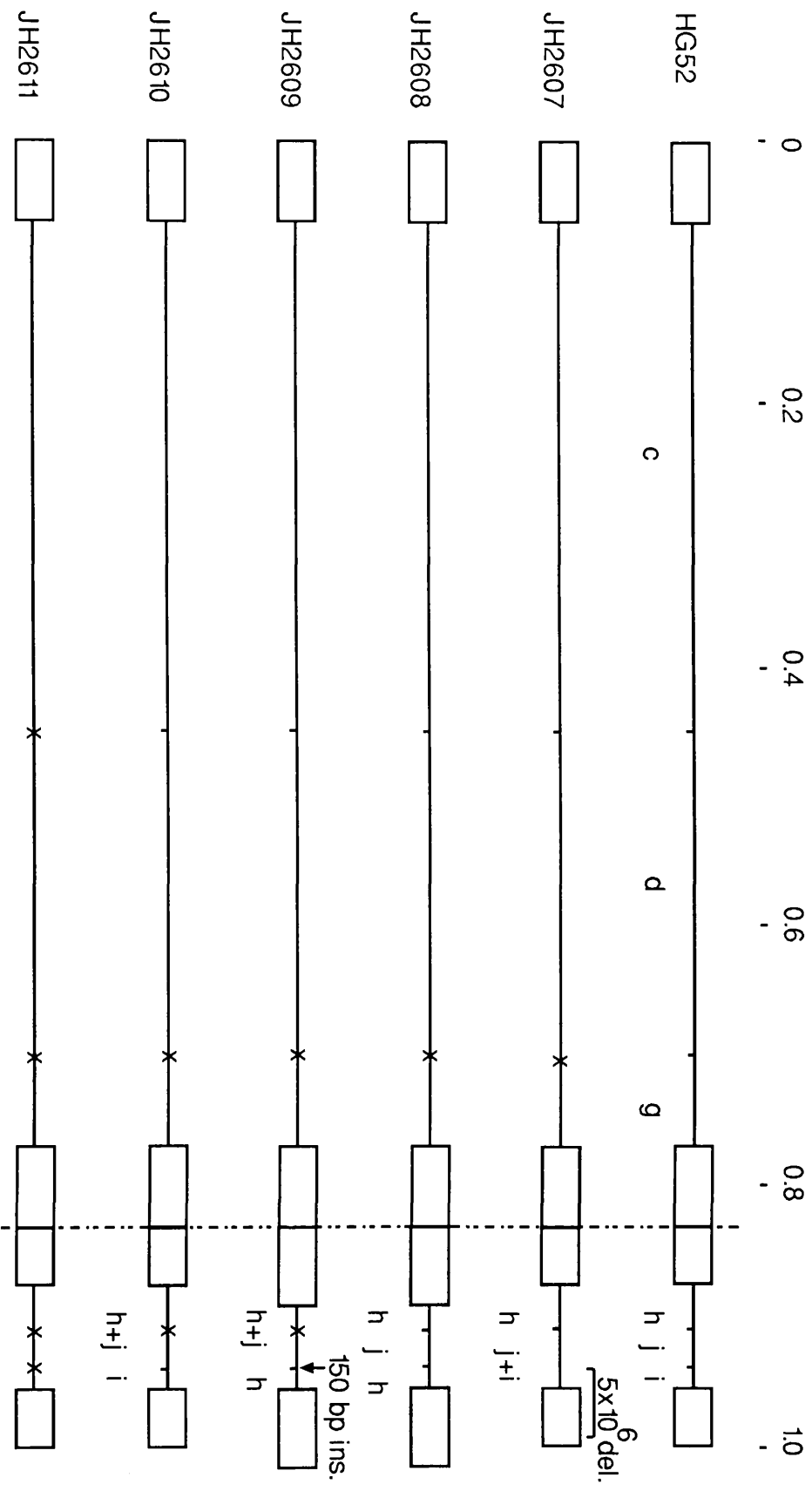
coding for VmwIE68, a protein of unknown function, protein

kinase and glycoprotein G respectively (McGeoch et al., 1985

and 1987; McGeoch and Davison, 1986; Frame et al., 1986b)

and the coding region of US5 (McGeoch et al., 1985).

JH2609 (HG52X163X21): is the same as JH2608 except that it

contains additional HindIII and EcoRI sites at 0.94 m.u. and

has lost the XbaI site at 0.91 m.u.

JH2610 (HG52X163X3): has lost the XbaI site at 0.91 m.u.

resulting in fusion of XbaI h and j.

JH2611 (HG52X163X53): has lost all the XbaI sites (0.45,

0.7, 0.91 and 0.94 m.u.).

3.2.2 Neurovirulence of the deletion variants

Neurovirulence of the HG52 deletion variants was assayed

by intracranial inoculation of 3 week old BALB/c mice at

doses ranging from 10^3-10^7 pfu/mouse (5-6 mice/dose). The

mice were monitored for 3 weeks following inoculation and

the LD_{50} values calculated (see method section 2.11). The

results are shown in Table 3.5. The LD_{50} of the wild type

elite stock of HG52 is shown for comparison. Most of the

deletion variants exhibited reduced neurovirulence and can

be segregated into three groups. The first group with an

LD_{50} value of $>10^7$ pfu/mouse, classified as avirulent

includes JH2604. The second group with an LD_{50} value of

10^5-10^6 pfu/mouse classified as of reduced virulence

includes JH2610, JH2608, JH2609, JH2605 and JH2606. The

third group with an LD_{50} value of 10^4 pfu/mouse classified

as attenuated includes JH2611, JH2607 and JH2603. These

results indicate that the capacity of all the variants to

kill mice following intracranial inoculation was reduced

with LD_{50} values ranging between 10^4->10^7 pfu/mouse compared

Table 3.5. LD$_{50}$ values after intracranial inoculation of the deletion variants of strain HG52

| Virus | Virus dose (pfu)/animal | | | | | | LD$_{50}$ pfu/mouse |
	10^2	10^3	10^4	10^5	10^6	10^7	
HG52	4/6[+]	5/6	6/6	ND[*]	ND	ND	$<10^2$
JH2603	ND	0/6	3/6	5/6	6/6	ND	10^4
JH2604	ND	ND	ND	0/6	0/6	1/6	$>10^7$
JH2605	ND	ND	0/6	3/6	5/5	ND	10^5
JH2606	ND	ND	0/6	0/6	4/5	5/5	5.5×10^5
JH2607	ND	0/6	3/6	6/6	ND	ND	10^4
JH2608	ND	0/6	0/6	2/6	6/6	ND	2.8×10^5
JH2609	ND	0/6	0/6	3/6	6/6	ND	10^5
JH2610	ND	ND	ND	0/5	1/5	5/5	4.2×10^6
JH2611	ND	ND	0/6	6/6	6/6	ND	5×10^4

+ Number of deaths/number of animals inoculated.

* ND not determined.

3 week old BALB/c mice were used.

0.025ml of virus was inoculated.

to $<10^2$ pfu/mouse for the HG52 elite stock. Comparing these
results with the range of neurovirulence results obtained
for individual plaque stocks of HG52 indicated that the LD_{50}
values of all the deletion variants except JH2604 and JH2610
fell within the range of the parental HG52.

It had previously been shown that although not
temperature sensitive the deletion variants in U_S/TR_S were
temperature restricted at $38.5^\circ C$ (Brown and Harland, 1987)
the ambient body temperature of mice. As any impairment in
neurovirulence may have been attributed to this restriction
it was decided to concentrate on the variants with deletions
in IR_L i.e JH2603, JH2604, JH2605 and JH2606. These
variants   displayed reduced neurovirulence.

 JH2603   was chosen as a model in cotransfection
experiments and to test whether the deletion in the variant
JH2603 has any effect on neurovirulence. The deletion in
the variant JH2603 was shown to be 3.75 kb in IR_L within the
Xbal g fragment. In order to introduce the deletion into
the genome of plaque 17 stock of HG52, purified DNA from the
the variant JH2603 was digested and the Xbal g fragment
(0.7-0.83 m.u.) containing the deletion was excised, eluted
and cotransfected with intact plaque 17 stock genome (see
method section 2.19). The fragment was used at a 5, 10 and
20 fold molar excess over the intact plaque 17 stock DNA.
Fifty plaques were picked, grown into plate stocks and the
DNAs of twenty five were digested with Xbal (see method
section 2.17) and their restriction profiles studied. One
designated R17XD86 showed a profile indicating that the
fragment containing the deletion had recombined into the
wild type genome. After three rounds of plaque
purification, Xbal digestion confirmed that recombination

had occurred and that the deletion in JH2603 had been inserted. The results are shown in Figure 3.9. The recombinant R17XD86 (lane 2) shows that the Xbal g and the Xbal g containing joint fragments (e and f) were now running at the positions of the equivalent bands in JH2603 (lane 3) and not in their normal wild type position (lane 1). The recombinant was inoculated intracranially into 3 week old BALB/c mice at doses ranging from 10^2 to 10^5 pfu/mouse (see method section 2.11). As controls, the highly neurovirulent plaque 17 stock of HG52 and JH2603 were used at doses ranging from 10^2-10^3 and from 10^2-10^5 pfu/mouse respectively. The results given in Table 3.6 showed that the recombinant had an LD_{50} value of 3.5×10^4 pfu/mouse which is the same as the variant JH2603 indicating that the sequences deleted in JH2603 had an effect on neurovirulence.

In conclusion, the results indicate that all the deletion variants of HG52 analysed had reduced neurovirulence following intracranial inoculation of 3 week old BALB/c mice with LD_{50} values ranging 10^4->10^7 pfu/mouse compared to $<10^2$ pfu/mouse for the wild type elite stock of HG52.

3.3 Analysis of the deletion variant JH2604

3.3.1 Neurovirulence of JH2604

The DNA structure of the deletion variant JH2604 has been described in detail by Harland and Brown (1985). Essentially the genome has a deletion of approximately 1.5 kb in both copies of BamHI v located between 0-0.02 and 0.81-0.83 m.u. in TR_L and IR_L respectively. The relevant restriction maps for HG52 are shown in Figure 3.10 (Cortini and Wilkie, 1978). To determine the neurovirulence of

Figure 3.9

Autoradiographs of XbaI digests of viral DNA
^{32}P-labelled <u>in vivo</u> (0.5% agarose) of HG52 (lane 1),

R17XD86 (lane 2) and JH2603 (lane 3). Letters refer to

specific fragments, arrowheads indicate the positions

where fragments are missing and stars indicate novel

fragments.

Table 3.6. LD_{50} values after intracranial inoculation of plaque 17 stock of HG52, JH2603 and the recombinant R17XD86

Virus	Virus dose (pfu)/animal				LD_{50} (pfu/mouse)
	10^2	10^3	10^4	10^5	
Plaque 17	2/6[+]	5/6	ND[*]	ND	3.5×10^2
JH2603	0/6	0/6	3/6	5/6	10^4
R17XD86	1/6	0/6	2/6	5/6	3.5×10^4

+ Number of deaths/number of animals inoculated.

* ND not determined.

3 week old BALB/c mice were used.

0.025ml of virus was inoculated.

Figure 3.10

Restriction endonuclease maps for the DNA of HSV-2
strain HG52 from Cortini and Wilkie (1978). The origin
of the joint fragments is as follows. Xbal : $\underline{a}$ =$\underline{c}$+$\underline{h}$, $\underline{b}$
=$\underline{c}$+$\underline{i}$, $\underline{e}$ =$\underline{g}$+$\underline{h}$, $\underline{f}$ =$\underline{g}$+$\underline{i}$. HindIII : $\underline{c}$ =$\underline{i}$+$\underline{k}$, $\underline{d}$ =$\underline{j}$+$\underline{k}$, $\underline{f}$ =$\underline{i}$+
$\underline{m}$, $\underline{g}$ =$\underline{j}$+$\underline{m}$. EcoRI : $\underline{b}$ =$\underline{f}$+$\underline{k}$, $\underline{c}$ =$\underline{h}$+$\underline{k}$, $\underline{d}$ =$\underline{f}$+$\underline{m}$, $\underline{e}$ =$\underline{h}$+$\underline{m}$.
BglII : $\underline{a}$ =$\underline{d}$+$\underline{k}$, $\underline{b}$ =$\underline{d}$+$\underline{m}$, $\underline{e}$ =$\underline{h}$+$\underline{k}$, $\underline{f}$ =$\underline{h}$+$\underline{m}$. HpaI : $\underline{b}$
=$\underline{f}$+($\underline{S}$), $\underline{e}$ =$\underline{g}$+($\underline{S}$). KpnI : $\underline{b}$ =$\underline{c}$+$\underline{r}$, $\underline{e}$ =$\underline{f}$+$\underline{r}$. BamHI : $\underline{g}$
=$\underline{v}$+$\underline{u}$.

0 · 0·2 · 0·4 · 0·6 · 0·8 · 1·0

XbaI c d g h j i

HindIII i b n h e a o j m l k

EcoRI f j i g e a p l h k

BglII d r p g j o c n i h m q l k

HpaI g a h d e f (S)

KpnI f k l p n h o q j m t g d i s c r a r

BamHI v p c x h q d i i r s n l' b h j' o y j m e t a w k f p v'u g' z e f' l c d' b a' g' u
m'
k'm'

JH2604 compared to the parental HG52 elite stock, experiments were carried out to estimate the LD_{50} values in mice. Twenty five ul aliquots of HG52 at doses of 10^2-10^5 pfu/mouse and JH2604 at doses of 10^5-10^7 pfu/mouse were inoculated into the left cerebral hemisphere of 3 week old BALB/c mice of each sex. Deaths were scored between days 3 and 21 post inoculation and the LD_{50} values calculated (see method section 2.11). The results are shown in Table 3.7.

The elite parental stock of HG52 showed an LD_{50} value of $<10^2$ pfu/mouse which was comparable to that determined previously and to the LD_{50} value of $<10^3$ pfu/mouse calculated for the plaque purified , plaque 17, stock of HG52 (see results section 3.1). The LD_{50} value of JH2604 was greater than 10^7 pfu/mouse; at a dose of 10^7 pfu/animal, only one animal out of nine died and no others showed signs of encephalitis or any other symptoms of illness or distress. Also, two mice each infected with 10^8 pfu/mouse of JH2604 remained healthy after inoculation. Thus the deletion variant JH2604 was at least 10^6 fold less neurovirulent than the parental stock of HG52. The particle : pfu ratios of 54 : 1 for HG52 and 71 : 1 for JH2604 are comparable and fall within the normal range of values for HSV-2. Following footpad inoculation (see method section 2.11) the variant JH2604 failed to kill any of the mice (15 mice) inoculated at doses of 10^6, 10^7 and 10^8 pfu/mouse giving an LD_{50} value of $>10^8$ pfu/mouse.

3.3.2 Growth of JH2604 in vivo

To determine whether the low neurovirulence of JH2604 was due to failure of replication in the mouse brain, the growth of the virus in vivo was tested. Samples of HG52 and

Table 3.7.   Neurovirulence of HG52 and JH2604 in BALB/c mice following intracranial inoculation

Virus	Virus dose (pfu)/animal					
	10^2	10^3	10^4	10^5	10^6	10^7
HG52[**]	7/12[+]	8/12	12/12	6/6	ND[*]	ND
JH2604[***]	ND	ND	ND	0/4	0/4	1/9

+ Number of deaths/number of animals inoculated.

* ND not determined.

** Particle : pfu 54 : 1.

*** Particle : pfu 71 : 1.

3 week old BALB/c mice were used.

0.025ml of virus was inoculated.

JH2604 (10^5 pfu/mouse) were inoculated into the left cerebral hemisphere of 3 week old BALB/c mice of both sexes. At 0, 24, 48, 72, 96, 120 and 144 h post infection, two mice were killed and their brains were removed, homogenised and the resulting suspension sonicated and assayed by plaque titration on BHK-21 Cl3 cells at 37°C (see method section 2.5). The results plotted in Figure 3.11 show that the parental HG52 grew well in the mouse brain with the titre continuing to rise at 120 h post inoculation; by 144 h all the mice had died from encephalitis. In contrast, the variant JH2604 failed to show any replication and the titre diminished progressively with time. These results indicated that the variant JH2604 was specifically restricted in the nervous system due to a replication deficiency in mouse brain cells.

3.3.3 Growth of JH2604 in vitro

The deletion variant JH2604 grows like wild type in a single cycle growth experiment in BHK-21 Cl3 cells at 37°C (Harland and Brown, 1985); the parental elite stock of HG52 is somewhat temperature restricted at 38.5°C in that, though displaying exponential growth, the highest titre of virus reached by 12 h post infection is not appreciably higher than the starting titre immediately post absorption (Brown and Harland, 1987). The variant JH2604 is not temperature sensitive but is impaired in single cycle growth experiments at 38.5°C to the same extent as the parental HG52. The titre of the JH2604 stock used for all experiments was 4×10^7 pfu/ml at 31°C and 3×10^7 pfu/ml at 38.5°C. HG52 stock generally gives a lower titre compared to HSV-1 strain 17 syn+, due to the fact that HG52 is mainly cell associated.

Figure 3.11

In vivo growth kinetics of HG52 and JH2604. Three week

old BALB/c mice were inoculated into the left cerebral

hemisphere with 25ul of virus containing 10^5 pfu/mouse.

At indicated times post infection, two surviving mice

from each time point were sacrificed. The brain tissue

was removed and homogenised; the resulting cell

suspension was sonicated and released infectious virus

titrated on BHK-21 C13 cells at 37°C. O HG52 ; ●

JH2604.

Y-axis: Infectious virus titre (pfu/brain)

X-axis values: 0, 24, 48, 72, 96, 120, 144

X-axis label: Time post infection (h)

Y-axis values: 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, $10^{0.5}$
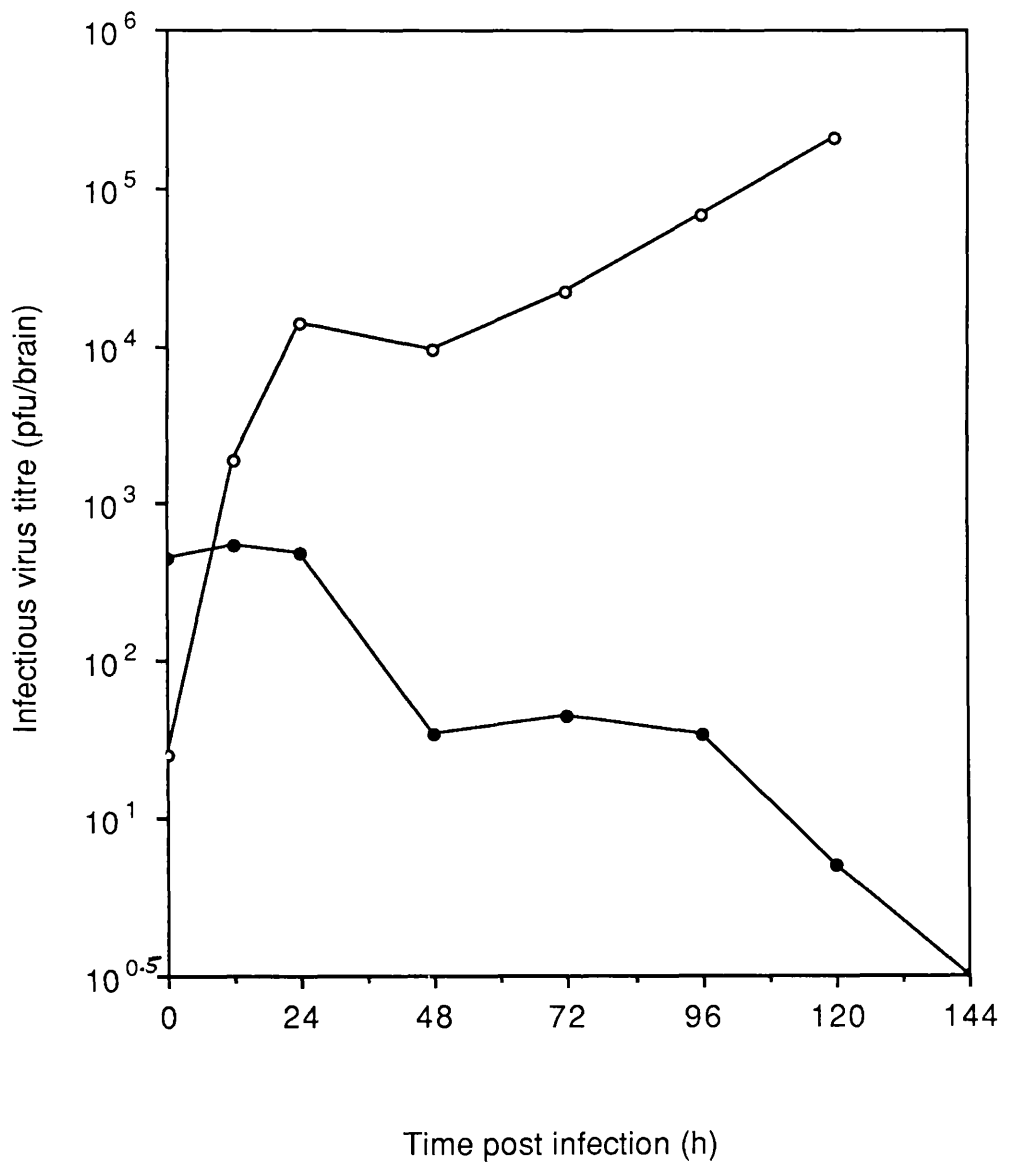
To determine whether JH2604 was temperature sensitive in mouse cells at the accepted ambient body temperature of mice i.e 38.5^{O}C, comparative one step growth experiments were carried out in BALB/c mouse embryo fibroblast (3T3) cells at 38.5^{O}C (see method section 2.9), and the virus yield was titrated on BHK-21 Cl3 cells at 37°C. Results are given in Figure 3.12 and show that the parental HG52 replicated poorly, reaching a titre comparable to the input titre by 8 h post infection and then diminished. The variant JH2604 showed little evidence of replication and the virus titre progressively fell by 2 logs with time. In one step growth experiments perfomed at 37°C in 3T3 cells (Figure 3.13), the parental HG52 demonstrated some replication by 24 h post infection while JH2604 failed to replicate. Since it was possible that HG52 grows more slowly in 3T3 cells, multistep growth experiments were performed at 37°C in 3T3 cells. The results are shown in Figure 3.14 and again the parental HG52 showed some replication and reached its highest titre by 24 h post infection. The variant JH2604 failed to replicate and the titre dropped by 4 logs with time. It is concluded that BALB/c 3T3 cells are poor hosts for replication of HSV-2 strain HG52 although some replication of wild type virus does occur; these cells are totally non-permissive for the deletion variant JH2604 both at 37°C and 38.5^{O}C.

3.3.4 Infected cell polypeptide synthesis by JH2604

It has been shown previously that JH2604 synthesises normal amounts of HSV-induced immediate early polypeptides under immediate early conditions (Harland and Brown, 1985). To determine whether the deletion in JH2604 affected any

Figure 3.12

Single cycle growth kinetics of HG52 (O) and JH2604 (●)
in 3T3 cells at 38.5^{O}C. Cells were infected at a moi of
5 pfu/cell. After absorption, cells were washed,
overlaid with ETC5 and incubated at 38.5^{O}C. At various
times post infection, cells were harvested, virus was
released by sonication and titrated on BHK-21 Cl3 cells
at 37°C. The titre is given per ml (10^6 cells).
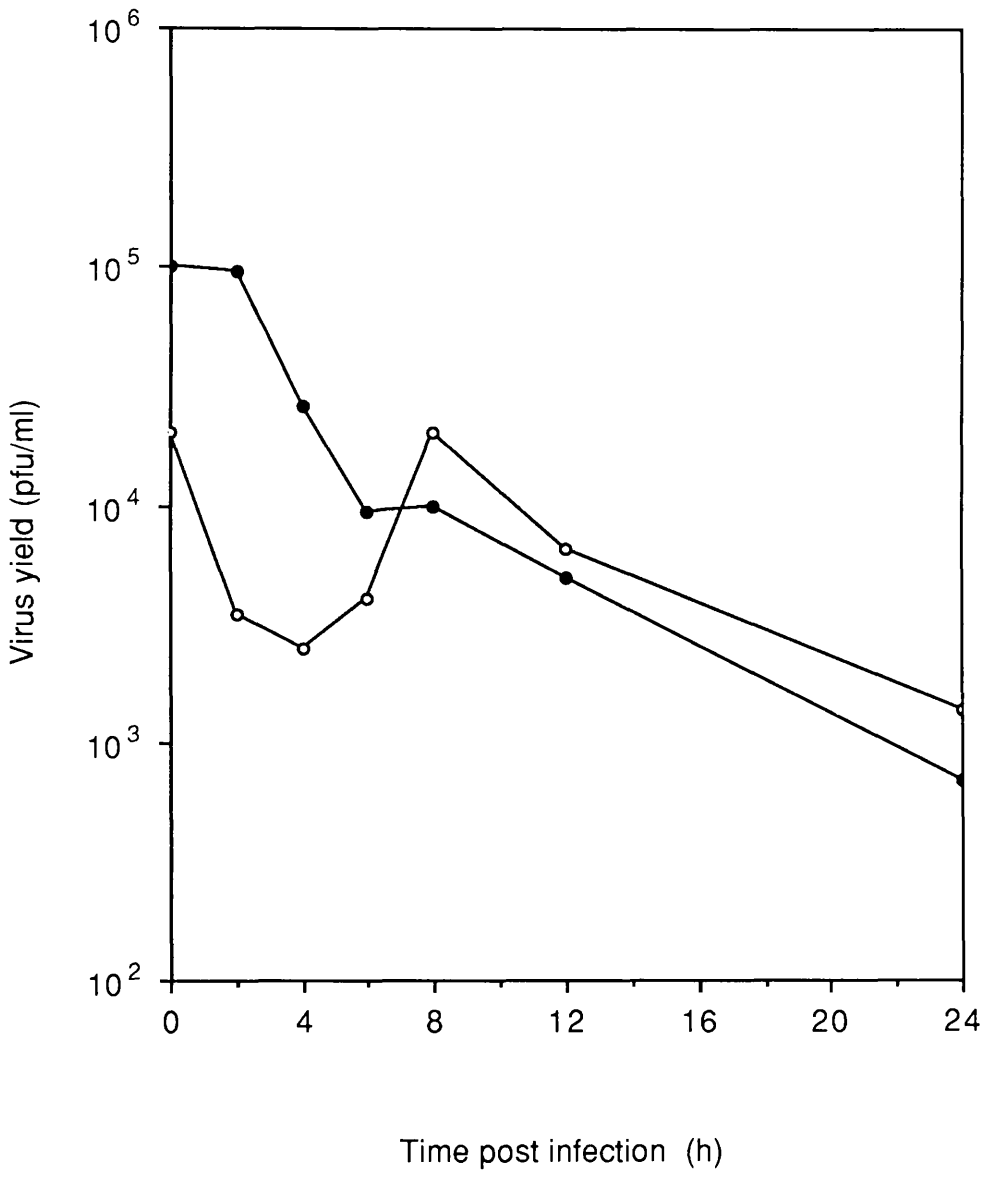
Figure 3.13

One step growth curves of HG52 and JH2604 in 3T3 cells
at 37°C. Cells were infected at a moi of 5 pfu/cell.
Following absorption, cells were washed, overlaid with
ETC5 and incubated at 37°C. Cells were harvested at
times indicated and the virus titre measured by plaque
assay on BHK-21 C13 cells at 37°C is given per ml (10^{6}
cells). O HG52 ; ● JH2604.
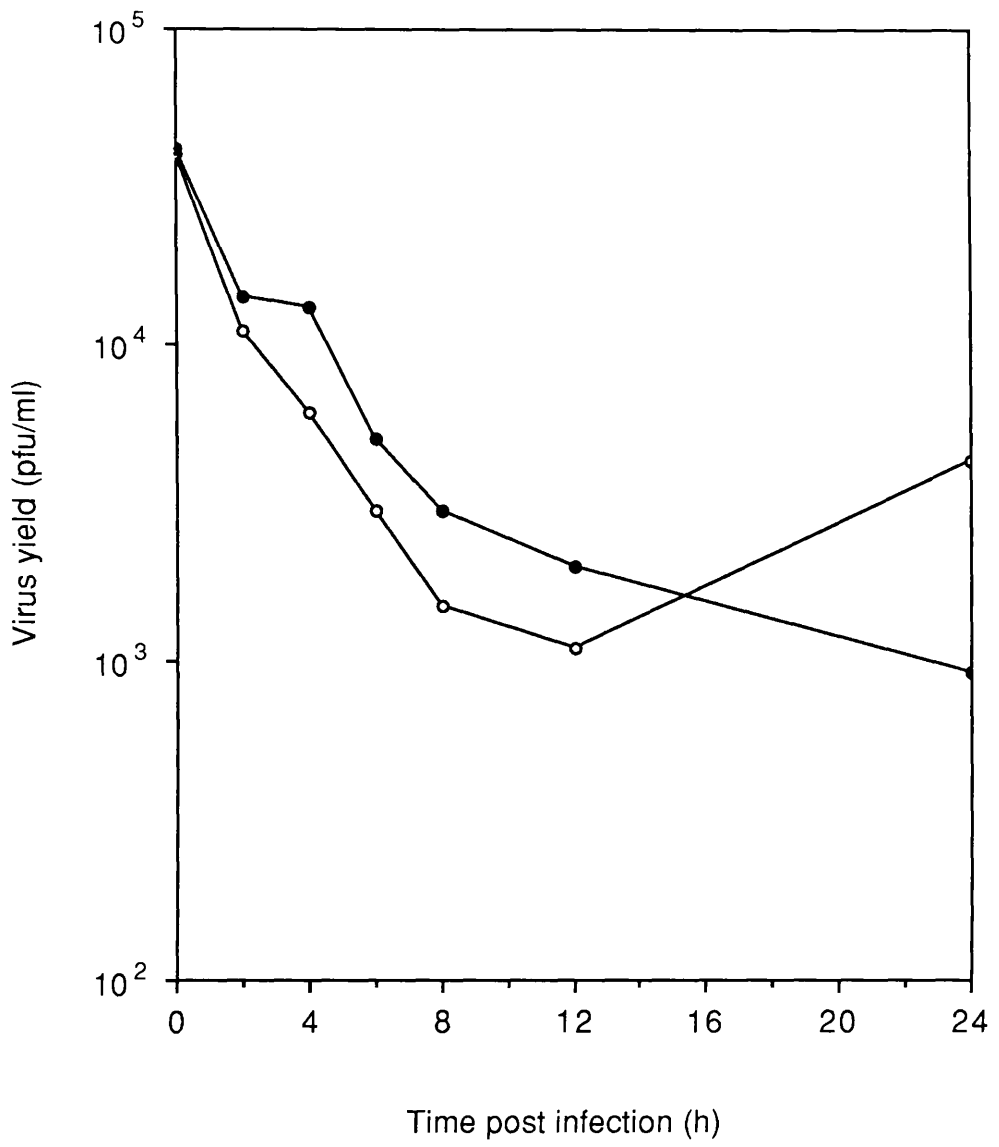
Figure 3.14

Multistep growth kinetics of HG52 and JH2604 in 3T3
cells at 37°C. Cells were infected at a moi of 0.002
pfu/cell. After absorption, cells were washed, overlaid
with ETC5 and incubated at 37°C. At various times post
infection, cells were harvested, virus released by
sonication, titrated on BHK-21 Cl3 cells at 37°C and the
titre is given per ml (10^6 cells). O HG52 ; ● JH2604.
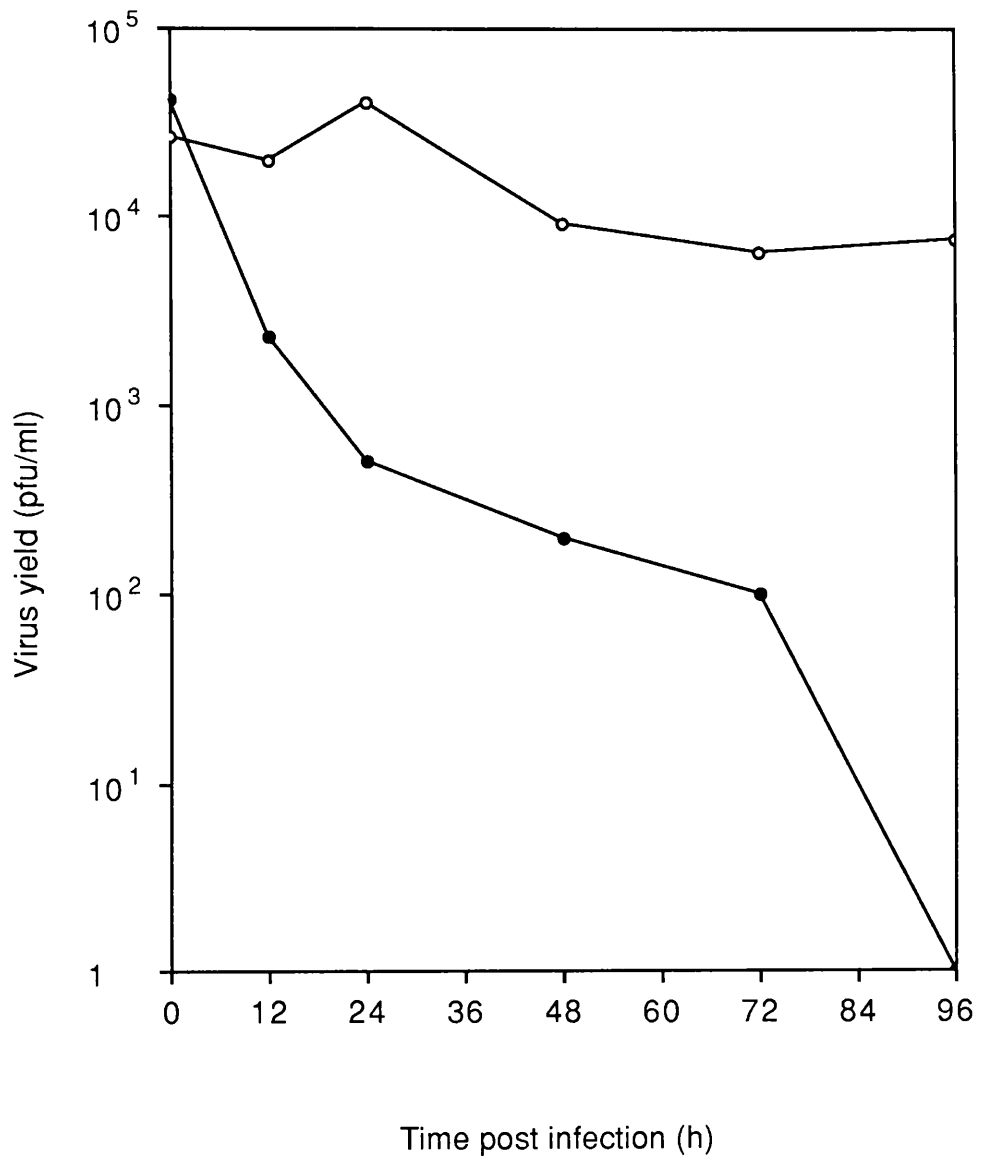
other detectable polypeptide synthesis, the infected cell

polypeptides of HG52 and JH2604 were labelled with

^{35}S-methionine and analysed by SDS-PAGE (see method section

2.15). The results are shown in Figure 3.15. There were no

detectable differences between the infected cell

polypeptides synthesised by HG52 (lanes 1 and 6) and by

JH2604 (lanes 2 and 5) compared to the mock infected cells

(lanes 3 and 4) indicating that the deletion in JH2604 does

not affect detectable general polypeptide synthesis.

3.3.5 Thymidine kinase synthesis by JH2604

It is widely accepted that the expression of

virus-encoded thymidine kinase (TK) enzyme activity is

necessary for the maximum demonstration of virulence

phenotype by HSV (Field and Wildy, 1978). To determine

whether the lack of neurovirulence of JH2604 was due to a

mutation in TK synthesis, TK assays were performed on HG52,

JH2604 and a known TK-negative mutant (TK^{-7}) using the

method described in section 2.13. The results of two

separate experiments are shown in Table 3.8. With the

TK-negative mutant (TK^{-7}) the ^{3}H counts per minute (cpm)

were marginally below the background (mock infected) levels

in both experiments, while the wild type HG52 gave counts of

3.4 and 2.4 times the background levels and the variant

JH2604 gave counts of 3.6 and 3.0 times the mock infected

levels. Thus JH2604 is at least as efficient as HG52 in

synthesizing the TK enzyme and thereforethe avirulent

phenotype of JH2604 was not due to lack of expression of TK

enzyme activity.

3.3.6 Correction of the deletion in JH2604

Figure 3.15

Autoradiographs 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 single
10% (lanes 1 and 3) and gradient 5-12.5% (lanes 4 and 6)
concentrations of acrylamide gels. HG52 (lanes 1 and 6)
; JH2604 (lanes 2 and 5) and mock infected (lanes 3 and
4). Numbers refer to the apparent molecular weight
$(x10^{-3})$ of HSV-2 infected cell polypeptides.
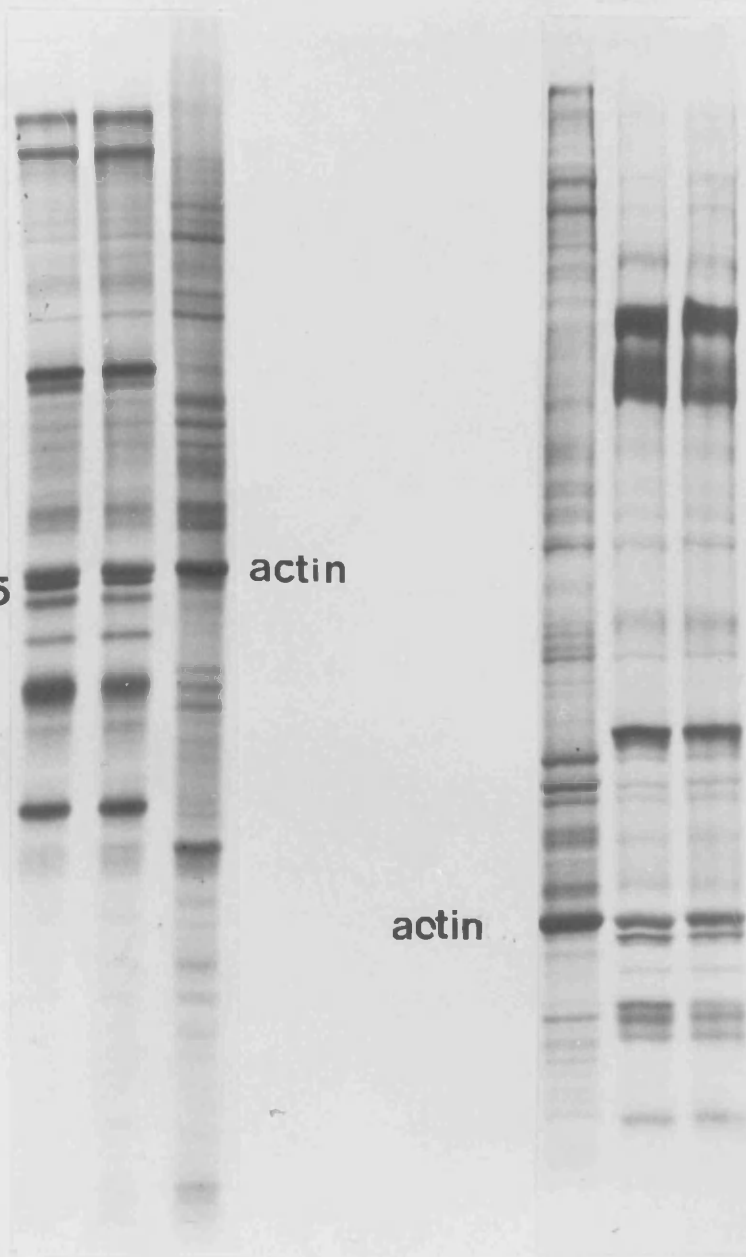
Table 3.8. TK assays on HG52, JH2604 and TK^{-7} viruses

Virus	Radioactivity (cpm)/ug protein	
	Experiment 1	Experiment 2
Mock infected	2573	3460
TK^{-7} (17)	2216	2812
HG52	8713	8424
JH2604	9265	10645

The strategy employed to correct the deletion in the genome of JH2604 and subsequent isolation of recombinants is shown in Figure 3.16. Essentially unit length JH2604 genomes and restriction endonuclease fragments spanning the deletion from the HG52 genome were cotransfected onto BHK-21 Cl3 cells. The progeny viruses were allowed to amplify until the cpe was complete. Cultures were harvested independently, titrated out and plaques were picked, grown into plate stocks and their DNAs were analysed by restriction endonucleases using the method of Lonsdale (1979). Recombinant viruses were isolated, plaque purified, their DNA profiles and LD_{50} values determined (see method section 2.11). In this study purified DNA from the highly neurovirulent plaque 17 stock of HG52 (LD_{50} <10^3 pfu/mouse) was digested with XbaI and the XbaI g and g containing joint fragments (e and f) were excised and after purification by electroelution (see method section 2.18) were used independently in cotransfection experiments with the intact JH2604 DNA. The deletion in JH2604 was shown to be within XbaI g and the g joint fragments (e and f). The Mr of fragment XbaI g (0.7-0.83 m.u.) is $13x10^6$; that of XbaI e ($g+h$ 0.83-0.91 m.u.) is $21x10^6$ and of f ($g+i$ 0.94-1.0 m.u.) is $19x10^6$. In cotransfection experiments the fragments were used at a 5, 10 and 20 fold molar excess to the intact JH2604 DNA. Although the deletion in JH2604 is only $1x10^6$ Mr (1.5 kb) in both copies of BamHI v, these large XbaI fragments were used to facilitate recombination between the fragment and the intact genome (the larger the fragment, the greater the chance of recombination occurring) as no selection system was available. Two hundred plaques were picked, and the DNA of 46 of these was digested with XbaI;

Figure 3.16

Strategy employed to correct the deletion in the variant

JH2604 <u>in vitro</u>.

Intact JH2604 genomes   XbaI RE fragments from HG52

Amplification in culture (BHK-21 C13 cells)

Pick plaques

RE analysis

Isolation of the recombinants

Plaque purification

RE analysis

Neurovirulence of the recombinants

(LD_{50} values)
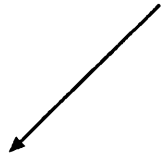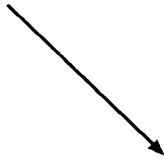
four of these digests showed profiles indicating that the deletion in XbaI g had been corrected, as the g, e and f bands ran in the normal wild type position. Three were from cotransfections using a 20 fold molar excess of XbaI e and the fourth from a plate in which XbaI f had been cotransfected at a 20 fold molar excess with JH2604 DNA. After three rounds of plaque purification, XbaI digestion confirmed that recombination had occurred and that the deletion in JH2604 had been corrected. The recombinants were designated R17(192)E pl. 8, R17(192)E pl. 14, R17(192)E pl. 15 and R17(192)F pl. 10. Figure 3.17 shows XbaI profiles of the four recombinants (lanes 2-5) compared to HG52 (lane 1) and JH2604 (lane 6). XbaI digestion demonstrated that the deletion had been corrected in IR_L i.e within XbaI g. The size of the other XbaI fragment containing BamHI v (i.e c, 45×10^6 Mr) is such that an alteration in size of Mr 1×10^6 would not be detectable. Therefore HindIII and HpaI digestion was carried out. The results are shown in Figure 3.18. BamHI v in TR_L is located within HpaI g, and BamHI v in IR_L is located within HpaI f. The variant JH2604 shows f and g deleted by approximately 1×10^6 Mr (lane 6) compared to f and g of HG52 (lane 1). The size of the HpaI g and f containing joint fragments (b and c) are so large that a deletion of 1×10^6 Mr would not be detectable. In the recombinants HpaI f and g have returned to their wild type positions (lanes 2-5). BamHI v in TR_L is located within HindIII i, and in IR_L within HindIII j. Therefore with the variant JH2604, HindIII i and j and the i and j containing joint fragments i.e c, d, f and g (lane 12) are deleted compared to the corresponding HG52 fragments (lane 7). In the recombinants, i, j, c, d, f and g have all

Figure 3.17

Autoradiographs of restriction digests of viral DNA
^{32}P-labelled in vivo. XbaI digestion (0.5% agarose) of

HG52 (lane 1), R17(192)E pl. 8 (lane 2), R17(192)E pl.

14 (lane 3), R17(192)E pl. 15 (lane 4), R17(192)F pl. 10

(lane 5) and JH2604 (lane 6). Letters refer to specific

fragments, arrowheads indicate the positions where

fragments are missing and e', f', g' indicate novel

fragments.
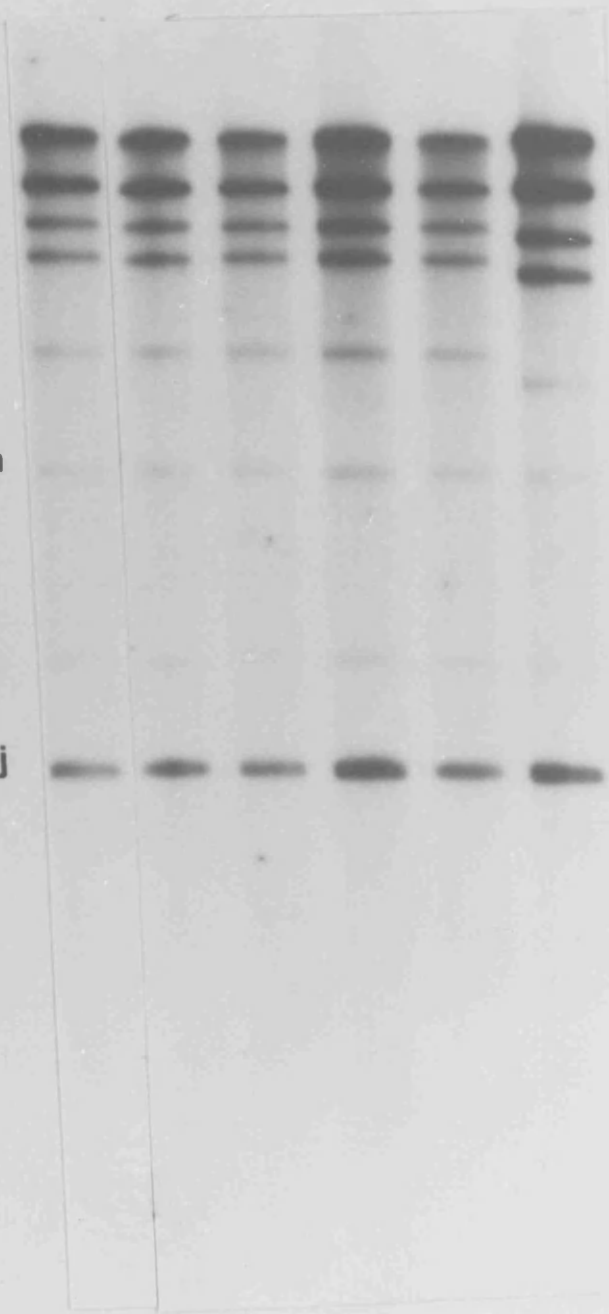
Figure 3.18

Autoradiographs of restriction digests of viral DNA
^{32}P-labelled in vivo. HpaI (lanes 1 to 6) and HindIII
(lanes 7 to 12) digestion of HG52 (lanes 1 and 7),
JH2604 (lanes 6 and 12), R17(192)E pl. 8 (lanes 2 and
8), R17(192)E pl. 14 (lanes 3 and 9), R17(192)E pl. 15
(lanes 4 and 10) and R17(192)F pl. 10 (lanes 5 and 11).
Letters refer to specific fragments, arrowheads indicate
the positions where fragments are missing and f', g'
etc. indicate novel fragments.

HpaI

1 2 3 4 5 6

abcd
e

f
g

h

g
f
▲▲

HindIII

7 8 9 10 11 12

a
b
efgcd
h

ijk
l
i
m

n

o

cd'
fg'
▲▲ ▲▲
i'
j'
▲▲
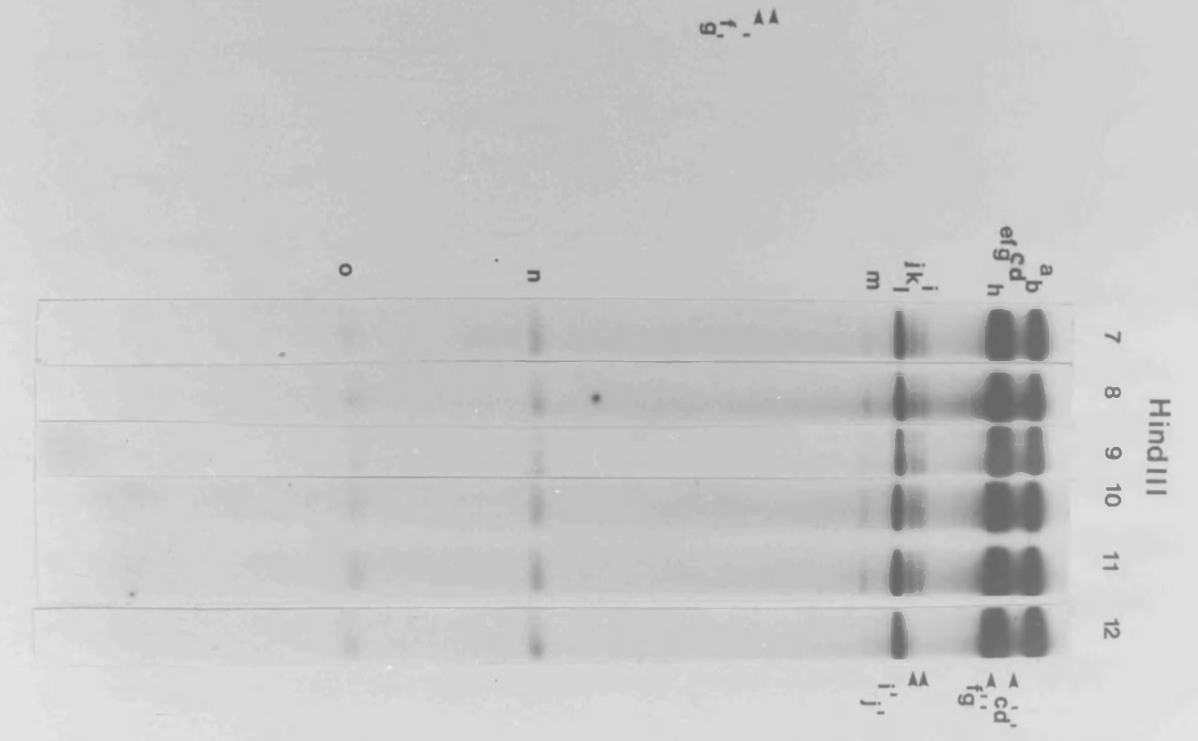
returned to their wild type positions (lanes 8-11). The

HpaI and HindIII digests demonstrate that the deletion has

been corrected in both copies of BamHI $\underline{v}$ i.e in TR_L and IR_L.

The TK phenotype of the recombinants was tested by TK assays

(see method section 2.13) to ensure that no secondary

mutation$_\wedge$affecting virulence had occured and the results are
in TK

shown in Table 3.9. The recombinants were as efficient as

the wild type in synthesizing the TK enzyme.

3.3.7 Neurovirulence of the R17(192) recombinants

To determine their neurovirulence, the three times

plaque purified recombinants were inoculated intracranially

into the left cerebral hemisphere of 3 week old BALB/c mice

at doses ranging from 10^2 to 10^6 pfu/mouse. As controls the

highly neurovirulent plaque 17 stock of HG52 and JH2604 were

used at doses ranging from 10^2-10^5 and 10^5-10^7 pfu/mouse

respectively. The results are given in Table 3.10 show that

all four recombinants have regained neurovirulence with LD_{50}

values ranging from 10^3 to $3x10^5$ pfu/mouse. These LD_{50}

values are within the range of the values of the individual

plaque stocks of HG52 ($<10^2-3x10^5$ pfu/mouse) (see section

3.1). The avirulent phenotype of JH2604 has been confirmed

with an LD_{50} value of $>10^7$ pfu/mouse. The particle : pfu

ratios of the recombinants are shown in Table 3.10 and are

within the acceptable range for HSV-2. The avirulent

phenotype of JH2604 has therefore been corrected by

insertion of wild type sequences replacing the deletion in

both copies of the BamHI $\underline{v}$ fragment.

The range of the LD_{50} values obtained with the

recombinants in which the deletion has been corrected

suggest either (i) there are sequences apart from those

Table 3.9. TK assys on the R17(192) recombinants, R192(17) recombinants, HG52 and TK^{-7}

Virus	Radioactivity (cpm)/ug protein
Mock infected	2352
TK^{-7}	2115
HG52	8220
R17(192)E pl. 8	7924
R17(192)E pl. 14	8140
R17(192)E pl. 15	8080
R17(192)F pl. 10	7982
R192(17)E pl. 2	9630
R192(17)E pl. 5	8356

Table 3.10. LD$_{50}$ values after intracranial inoculation of the R17(192) recombinants

Virus	Particle : pfu	Virus dose (pfu)/animal						LD$_{50}$ pfu/mouse
		10^2	10^3	10^4	10^5	10^6	10^7	
JH2604	71 : 1	ND*	ND	ND	0/12+	0/12	2/18	>10^7
HG52 pl. 17	6 : 1	1/6	9/12	11/12	6/6	ND	ND	2x10^2
R17(192)E pl. 8	78 : 1	ND	0/6	0/6	4/11	4/5	ND	2x10^5
R17(192)E pl. 14	63 : 1	0/14	6/14	12/12	ND	ND	ND	1x10^3
R17(192)E pl. 15	150 : 1	ND	0/6	0/6	3/11	3/5	ND	3x10^5
R17(192)F pl. 10	115 : 1	1/6	0/12	0/12	3/11	3/5	ND	3x10^5

* ND not determined.

+ Number of deaths/ number of animals inoculated.

3 week old BALB/c mice were used.

0.025ml of virus was inoculated.

deleted in JH2604 which act either in conjunction with or independently of those between 0-0.02 and 0.81-0.83 m.u. to determine neurovirulence or (ii) the DNA stock of HG52 plaque 17 is heterogeneous, in that viruses arising from individual genomes have different virulence potential. The latter would mean that the virus population of HG52 plaque 17 despite its recent purification contains a genome mixture with virulence LD_{50} values similar to those of the individual plaque stocks of the elite HG52 laboratory stock ($<10^3-3x10^5$ pfu/mouse). To determine whether this was the case, a DNA stock of HG52 plaque 17 was transfected and five well separated progeny plaques were isolated. Virus stocks from the five plaques were inoculated into 3 week old BALB/c mice at doses ranging from 10^2-10^4 pfu/mouse and the LD_{50} values were determined (see method section 2.11). The results in Table 3.11 show that the LD_{50} values of these plaque stocks were $<10^2$ to $>10^4$ pfu/mouse confirming the virulence heterogeneity within the stock of HG52 plaque 17.

3.3.8 Introduction of the JH2604 deletion into the HG52 genome

Having confirmed that the avirulent phenotype of JH2604 could be corrected by insertion of wild type fragments to correct the deletion, reciprocal experiments were carried out to determine whether removal of the relevant sequences from HG52 resulted in an avirulent phenotype. In order to insert the deletion in JH2604 into the wild type genome, purified DNA from JH2604 was digested with XbaI and the three fragments g, e and f were excised and purified from the gel (see method section 2.18). The three fragments were independently used in cotransfection experiments with intact

Table 3.11. LD_{50} values after intracranial inoculation of the plaque stocks picked from transfected HG52 plaque 17 DNA

Plaque number	Virus dose(pfu)/animal			LD_{50} (pfu/mouse)
	10^2	10^3	10^4	
17/1	0/5[+]	3/5	5/5	8.5×10^2
17/2	3/5	5/5	5/5	$<10^2$
17/3	0/5	0/5	2/5	$>10^4$
17/4	0/5	0/5	1/5	$>10^4$
17/5	1/5	3/5	5/5	7×10^2

+ Number of deaths/number of animals inoculated.

3 week old BALB/c mice were used.

0.025ml of virus was inoculated.

DNA from HG52 plaque 17 stock (high neurovirulence) at a 5, 10 and 20 fold molar ratio. No selection system was used to isolate recombinants when 200 plaques were picked. Of the DNA of 40 of these digested with XbaI, two showed XbaI profiles indicating that the fragment containing the deletion had recombined into the wild type genome (Figure 3.19 lanes 2 and 3) in that the $\underline{g}$, $\underline{e}$ and $\underline{f}$ bands were now running at the positions of the equivalent bands in JH2604 (lane 4) and not in their normal wild type positions (lane 1). HpaI and HindIII digestion (Figure 3.20) confirmed that the deletion was in both copies of R_L in that HpaI $\underline{g}$ and $\underline{f}$ (lanes 2 and 3) and HindIII $\underline{i}$,$\underline{j}$, $\underline{c}$, $\underline{d}$, $\underline{f}$ and $\underline{g}$ (lanes 6 and 7) were running below their normal wild type positions (lanes 1 and 5). The recombinants were plaque purified three times, their restriction profiles confirmed and virus stocks were grown. The recombinants were as efficient as wild type in synthesizing TK enzyme (Table 3.9). The recombinants were designated R192(17)E pl. 2 and R192(17)E pl. 5.

3.3.9 Neurovirulence of the R192(17) recombinants

To determine their neurovirulence, the two recombinants R192(17) were independently inoculated intracranially into the left cerebral hemisphere of 3 week old BALB/c mice at doses of 10^5-10^7 pfu/mouse. Control infections with the HG52 plaque 17 stock and JH2604 were performed at the same time. The results are shown in Table 3.12. It can be seen that each of the recombinants had an LD_{50} value of $>10^7$ pfu/mouse i.e the same as that of the non-neurovirulent deletion variant JH2604. The particle : pfu ratios of the recombinants are shown in Table 3.12 and were comparable to

Figure 3.19

Autoradiographs of restriction digests of viral DNA
^{32}P-labelled in vivo. XbaI digestion (0.5% agarose) of
HG52 (lane 1), R192(17)E pl. 2 (lane 2), R192(17)E pl. 5
(lane 3) and JH2604 (lane 4). Letters refer to specific
fragments, arrowheads indicate the positions where the
fragments are missing and e', f' etc. indicate novel
fragments.
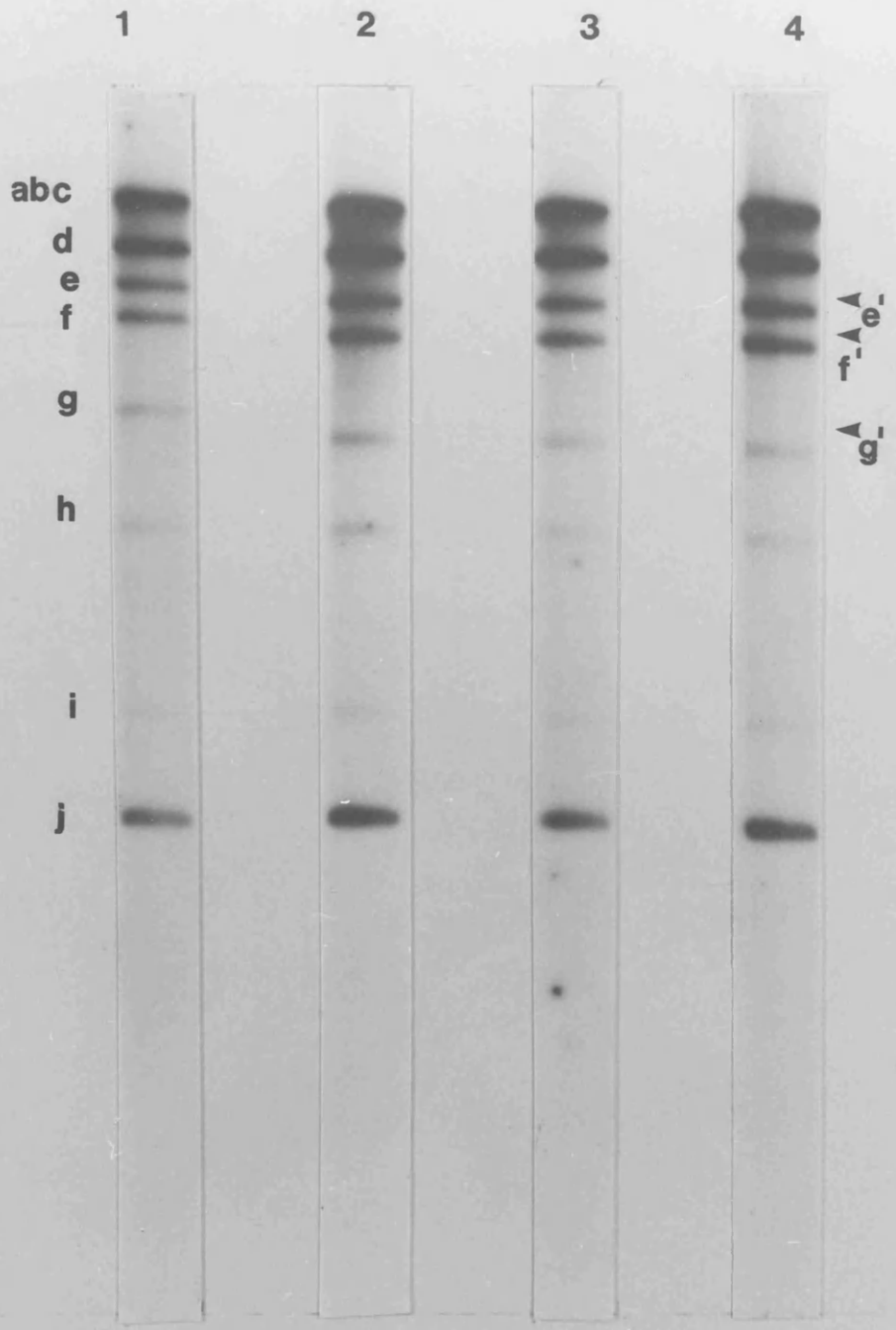
Figure 3.20

Autoradiographs of restriction digests of viral DNA ^{32}P-labelled in vivo. HpaI (lanes 1 to 4) and HindIII (lanes 5 to 8) digestion of HG52 (lanes 1 and 5), R192(17)E pl. 2 (lanes 2 and 6), R192(17)E pl. 5 (lanes 3 and 7) and JH2604 (lanes 4 and 8). Letters refer to specific fragments, arrowheads indicate the positions where fragments are missing and c', d' etc. indicate novel fragments.
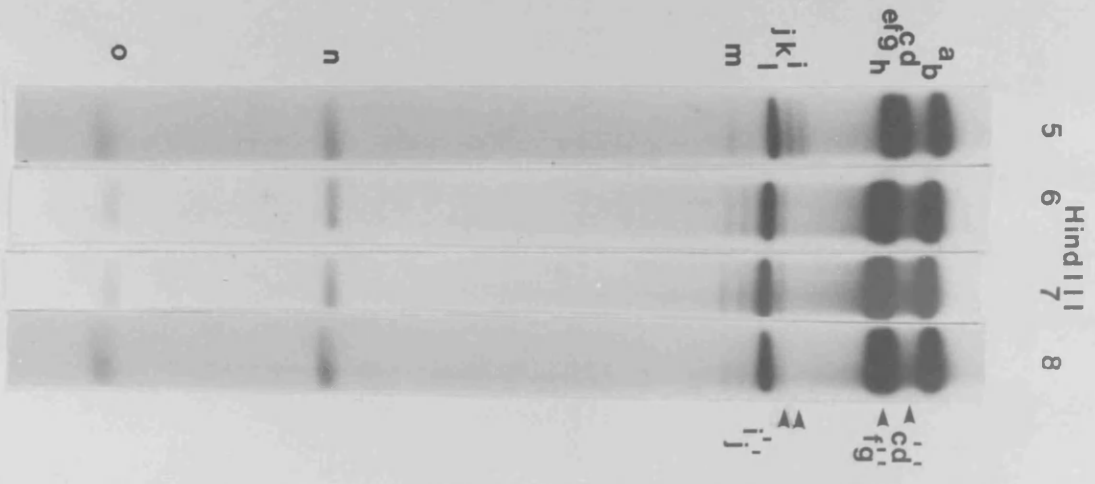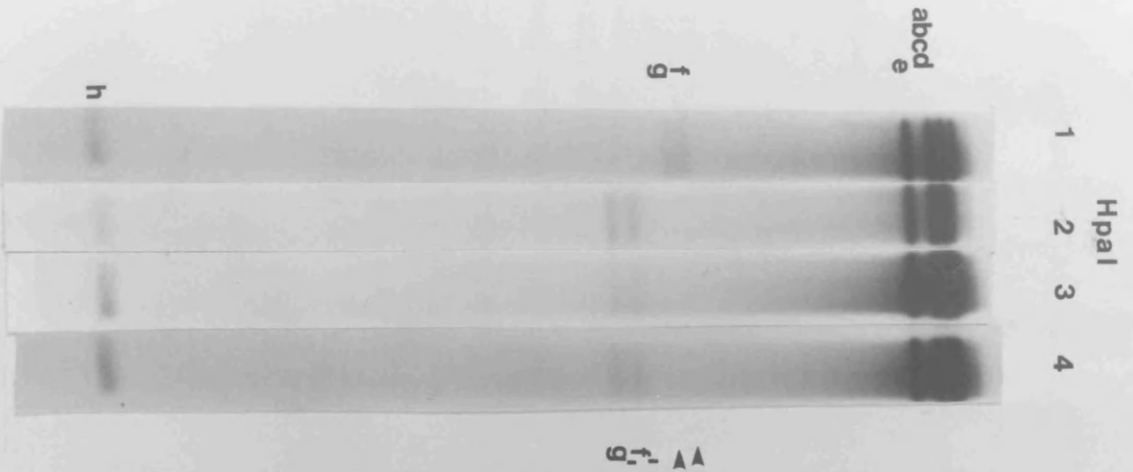
Table 3.12. LD_{50} values after intracranial inoculation of the R192(17) recombinants

Virus	Particle : pfu	Virus dose (pfu)/animal						LD_{50} pfu/mouse
		10^2	10^3	10^4	10^5	10^6	10^7	
JH2604	71 : 1	ND*	ND	ND	0/12+	0/12	2/18	$>10^7$
HG52 pl. 17	6 : 1	1/6	9/12	11/12	6/6	ND	ND	2×10^2
R192(17)E pl. 2	96 : 1	ND	ND	ND	0/6	0/6	2/6	$>10^7$
R192(17)E pl. 5	37 : 1	ND	ND	ND	0/6	0/6	2/6	$>10^7$

* ND not determined.

+ Number of deaths/number of animals inoculated.

3 week old BALB/c mice were used.

0.025ml of virus was inoculated.

that of wild type. These results imply that the sequences within the 3 kb terminal portion of R_L probably within BamHI $\underline{v}$ are required for neurovirulence of HSV-2 strain HG52.

3.3.10 In vivo selection

The JH2604 deletion had been shown to be within the BamHI $\underline{v}$ fragment (0-0.02 and 0.81-0.83 m.u.) (Harland and Brown, 1985) but we chose to correct or insert the deletion using large XbaI fragments because of the small size of the relevant BamHI fragment and the lack of a selection system. The position of the relevant BamHI, XbaI, HindIII and HpaI fragments is seen in Figure 3.21. Using the large XbaI fragments is open to the criticism that as well as replacing the deleted sequences, the recombinants could also have contained other sequences from the cotransfecting XbaI $\underline{e}$ or $\underline{f}$ and that the observed effect on neurovirulence was not due to the deletion but to a secondary mutation. To counter this argument, correction of the deletion by a small fragment BamHI $\underline{g}$ i.e $\underline{v}$ (0.81-0.83 m.u.) + $\underline{u}$ (0.83-0.85 m.u.) using an in vivo selection system in BALB/c mice was carried out. The strategy employed to correct the deletion using an in vivo selection system and isolation of recombinants was a modification of that described by Thompson et al. (1983) and is summerised in Figure 3.22. Since the variant JH2604 does not kill mice due to its lack of a biologically selectable marker i.e failure of replication in mouse brain, any deaths will be due to recombination of the JH2604 genome with a restriction endonuclease fragment of HG52 which displays the desired phenotype (neurovirulence) and the genotype of the recombinants can then be defined by restriction endonuclease analysis. In this study intact JH2604 genomes were

Figure 3.21

Structure of the HSV-2 strain HG52 genome (top line) showing U_L and U_S flanked by TR_L/IR_L and IR_S/TR_S respectively. The positions of the relevant BamHI, Xbal, HpaI and HindIII fragments are indicated.

	0	0.1	0.2	0.7	0.8	1.0

TR$_L$ U$_L$ IR$_L$ IR$_S$ U$_S$ TR$_S$

BamHI v

Xbal c

Hpal g

Hindlll i

g v

f

j
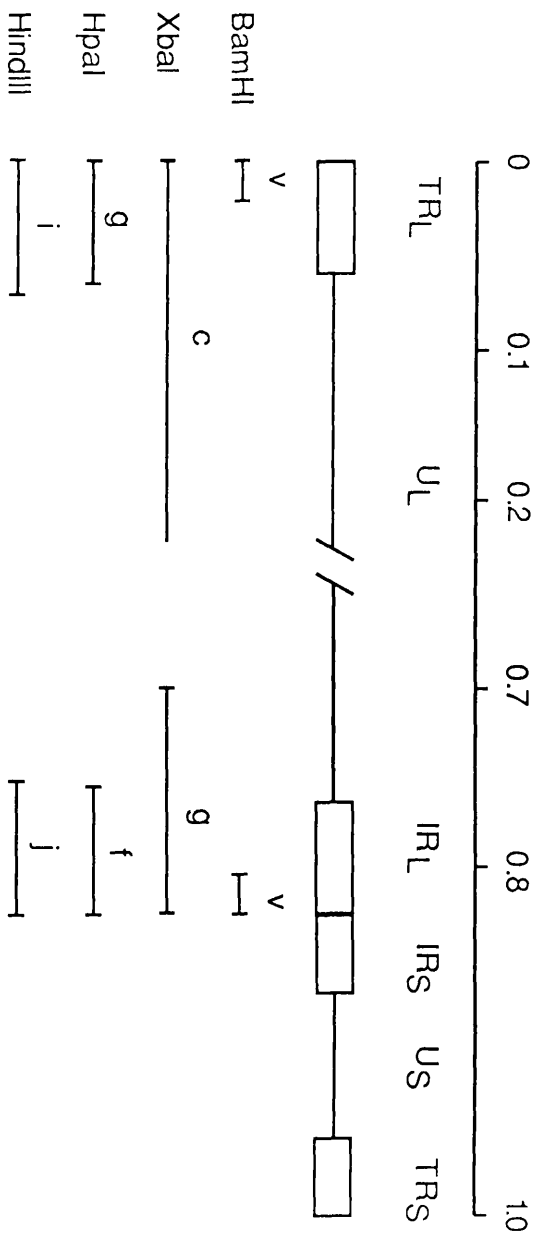
Figure 3.22

Strategy employed for correction of the deletion in the variant JH2604 using _in vivo_ selection in mice.

Intact JH2604
genomes

BamHI RE fragments
from HG52

Amplification in culture (BHK-21 C13 cells)

Intracranial inoculation of mice

Isolation of virus from brain tissue

Reinfect mice (LD_{50} values)

RE analysis

Plaque purification

RE analysis

Neurovirulence of the recombinants
(LD_{50} values)
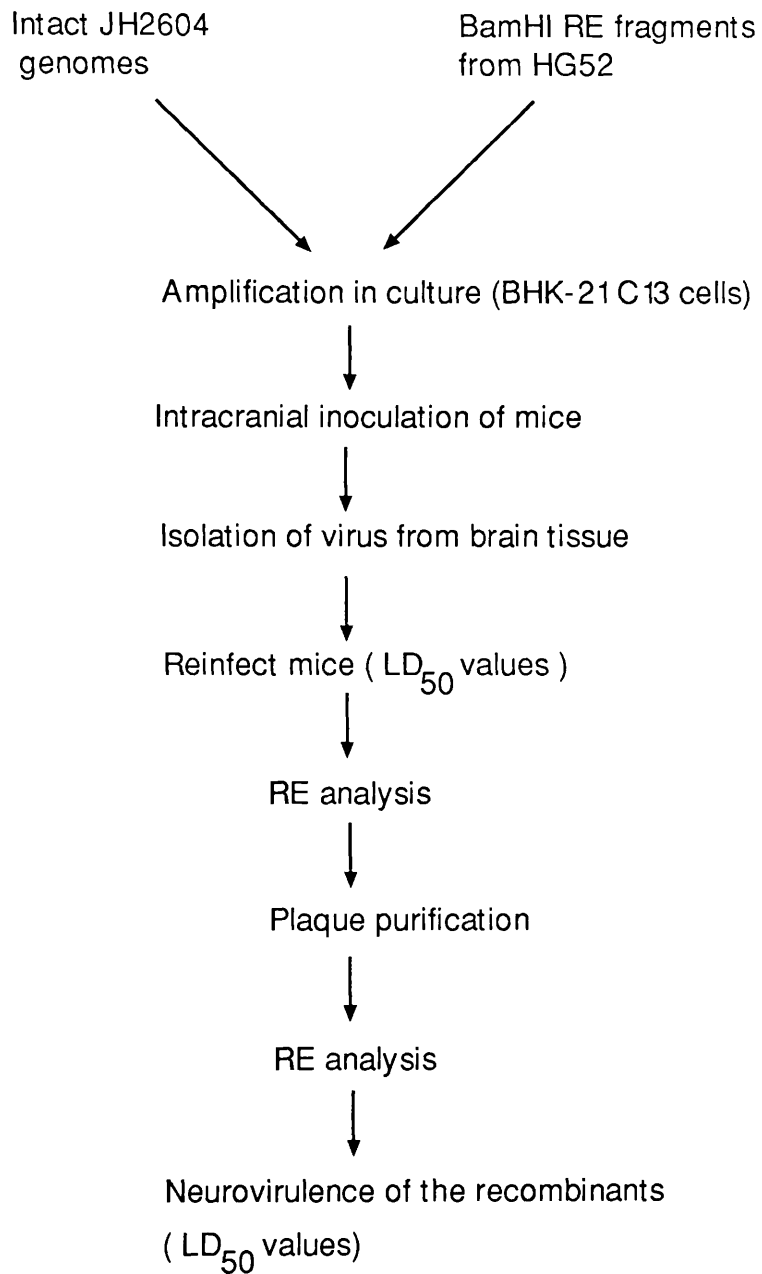
cotransfected in BHK-21 C13 cells with different molar

ratios (1, 5 and 10 fold) of the BamHI g fragment of the

highly neurovirulent plaque 17 stock of HG52 (see section

3.1). When extensive cpe was apparent the cells were

harvested, sonicated and the resulting virus suspension

serially diluted. Twenty five ul was inoculated

intracranially into the left cerebral hemisphere of 3 week

old BALB/c mice (2 mice/dilution). By three days post

infection, two mice had died, one inoculated with the virus

from a transfection plate infected with a 10 fold molar

excess of BamHI g and the other from a transfection with a 5

fold molar excess of BamHI g. The brain of the mouse

inoculated with the products of the 10 fold BamHI g

transfection plate was necrotic and could not be used. The

brain of the other dead mouse was removed, homogenised and

0.2ml of the resulting suspension inoculated onto BHK-21 C13

cells in a 50mm petri-dish. The infected plate was

harvested, the virus released by sonication and used to

reinfect mice intracranially to determine its LD_{50} value.

This stock gave a LD_{50} value of $<10^3$ pfu/mouse. The DNA of

the plate stock was subjected to restriction enzyme analysis

and the HindIII and HpaI DNA profiles are shown in Figure

3.23. The analysis showed that the virus recovered from the

brain (lanes 3 and 6) was a mixture of JH2604 (lanes 2 and

5) and HG52 (lanes 1 and 4). Therefore the plate stock of

the virus recovered from the brain was titrated out on

BHK-21 C13, separate plaques were picked, plate stocks from

each were made and their DNA analysed by HindIII and HpaI

digestion. Two plaque stocks that showed a HG52 profile

were subjected to three rounds of stringent plaque

purification and a stock of virus was grown from each after

Figure 3.23

Autoradiographs of restriction digests of viral DNA
^{32}P-labelled <u>in vivo</u>. HpaI (lanes 1 to 3) and HindIII

(lanes 4 to 6) digestion of HG52 (lanes 1 and 4), JH2604

(lanes 2 and 5) and virus recovered from the brain

(lanes 3 and 6). Letters refer to specific fragments

and stars indicate novel fragments.
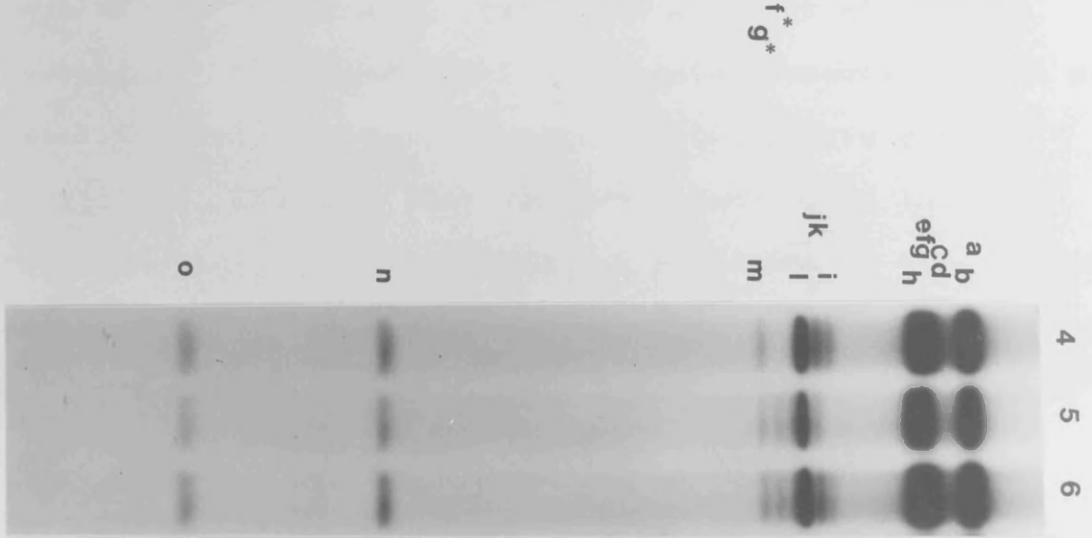
the third round. The recombinants were designated

recombinant 1 and 2. HindIII and HpaI DNA profiles of the

two virus stocks are shown in Figure 3.24 and it can be seen

that the two recombinant viruses (lanes 1, 2, 5 and 6) have

DNA structures equivalent to the wild type HG52 (lanes 4 and

8) and not the deletion variant JH2604 (lanes 3 and 7). The

two recombinant viruses were each inoculated intracranially

into the left cerebral hemisphere of 3 week old BALB/mice at

doses of 10^2, 10^3 and 10^4 pfu/mouse (5 mice/dose) and their

LD_{50s} determined. The results are shown in Table 3.13. It

can be seen that for both recombinants, all infected mice

died at a dose of 10^2 pfu/mouse giving an LD_{50} value for

each of $<10^2$ pfu/mouse. Using the approach of _in vivo_

recombinant selection HSV-2 function(s) associated with at a

least 10^6 fold increase in neurovirulence have been

localised. The fact that the two recombinants isolated

contained the DNA region (BamHI g fragment) in both copies

of the R_L from the highly neurovirulent plaque 17 stock of

HG52 demonstrate unequivocally that the sequences within

BamHI g are required for JH2604 to restore its

neurovirulence to the level of wild type virus.

3.4 Sequence analysis of the fragment containing the
deletion in the variant JH2604
3.4.1 Size variability of the BamHI g (v + u) joint fragment
of the variant JH2604

JH2604 had been shown to contain a deletion of

approximately 1.5 kb in each copy of BamHI _v_ fragment

between 0-0.02 and 0.81-0.83 m.u. (Harland and Brown, 1985).

It was initially thought that the deletion in both copies of

BamHI _v_ may not have been identical but that the variant had

Figure 3.24

Autoradiographs of restriction digests of viral DNA
^{32}P-labelled in vivo. HpaI (lanes 1 to 4) and HindIII
(lanes 5 to 8) digestion of recombinant viruses (lanes
1, 2, 5 and 6), JH2604 (lanes 3 and 7) and HG52 (lanes 4
and 8). Letters refer to specific fragments and stars
indicate novel fragments.
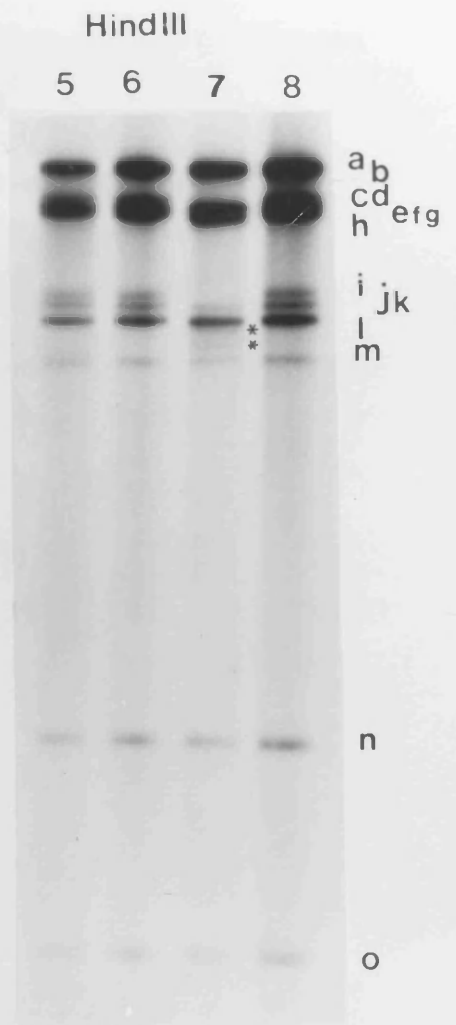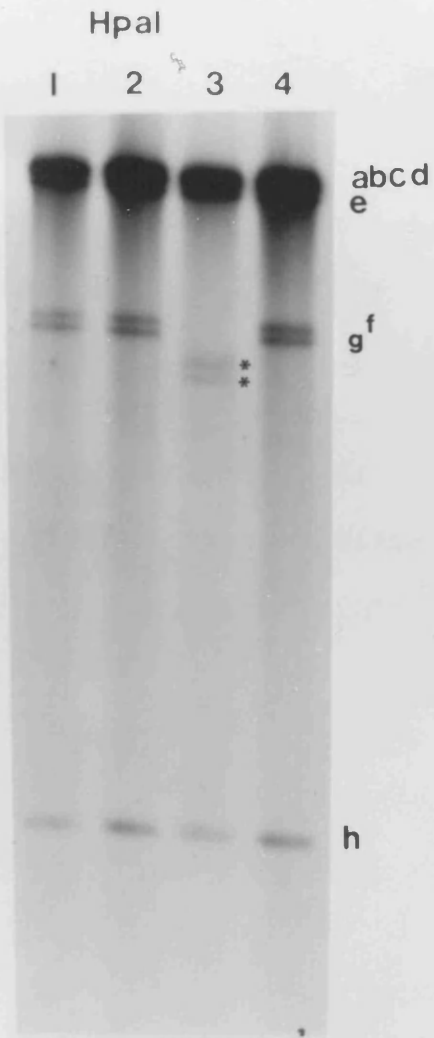
Table 3.13. LD_{50} values after intracranial inoculation of the _in vivo_ recombinants

Virus	Virus dose (pfu)/animal			LD_{50} (pfu/mouse)
	10^2	10^3	10^4	
Recombinant 1	5/5[+]	5/5	5/5	$<10^2$
Recombinant 2	5/5	5/5	5/5	$<10^2$

+ Number of deaths/number of animals inoculated.

3 week old BALB/c mice were used.

0.025ml of virus was inoculated.

lost at least 150 bp of equivalent sequences from both

copies of R_L. Therefore it was important to delimit the

deletion and sequence the fragment spanning the deletion to

know its exact location. BamHI digestion of JH2604 DNA

shows that the BamHI g fragment ($\underline{v}$ + $\underline{u}$) is missing from its

normal position and appears to run in two positions (1) just

above the $\underline{l}$ fragment and (2) just above the $\underline{m}$ fragment

(Figure 3.25). These two fragments were excised and

purified from a 1.2% agarose gel (see method section 2.18),

and cloned into the BamHI site of pAT153 plasmid (see method

section 2.22). Two positive clones designated 22 and 28

were isolated. Southern blot analysis of a BamHI digest

using nick translated intact HG52 genomes as the probe (see

method section 2.28) showed hybridization to one band in

clone 22 running just above the BamHI $\underline{l}$ fragment and one

band in clone 28 running just above the BamHI $\underline{m}$ fragment

(Figure 3.26). When clone 22   DNA was nick translated

and used as a probe in Southern blots with BamHI digested

DNA of HG52, JH2604, clone 22 and clone 28, it can be seen

that; with HG52 there is positive hybridization to BamHI $\underline{v}$,

$\underline{u}$ and $\underline{g}$; with JH2604 there is positive hybridization to $\underline{u}$

and to the two bands running coincidentally with BamHI $\underline{l}$ and

$\underline{m}$. It is assumed that the small size of the deleted $\underline{v}$

fragment precludes its identification on this gel. With

clones 22 and 28 there is hybridization to two bands, the

lower one representing the pAT153 DNA and the upper one in

each case the deleted BamHI $\underline{g}$ fragment running

coincidentally with BamHI $\underline{l}$ and $\underline{m}$ respectively. The

variation in the mobility of the fragments in clones 22 and

28 could be due to either (a) the deletion in each case

being a different size or (b) variability in the copy number

Figure 3.25

Autoradiographs of restriction digests of viral DNA
^{32}P-labelled in vivo. BamHI digestion (1.2% agarose) of
JH2604 (lane 1) and HG52 (lane 2). Letters refer to
specific fragments, arrowheads indicate the positions
where the fragments are missing and stars indicate novel
fragments.
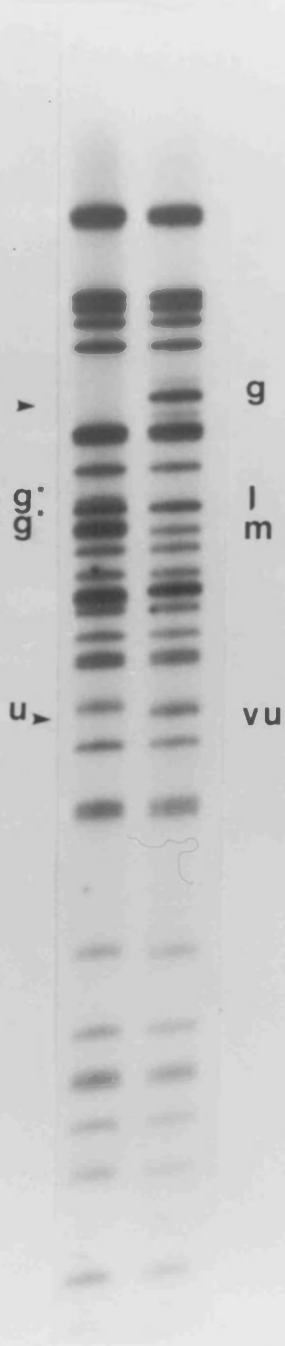
Figure 3.26

Autoradiographs of Southern blots containing BamHI

restriction fragments of clone 28 (lanes 1 and 5), clone

22 (lanes 2 and 6), JH2604 (lanes 3 and 7) and HG52

(lanes 4 and 8) to which nick translated probes have

been hybridised. The probes were the recombinant

plasmid clone 22 (lanes 1 to 4) and HG52 DNA (lanes 5 to

8). Letters refer to specific fragments.

Clone 22 probe

1 2 3 4

Wt probe

5 6 7 8

g

vu

g

l

m

vu
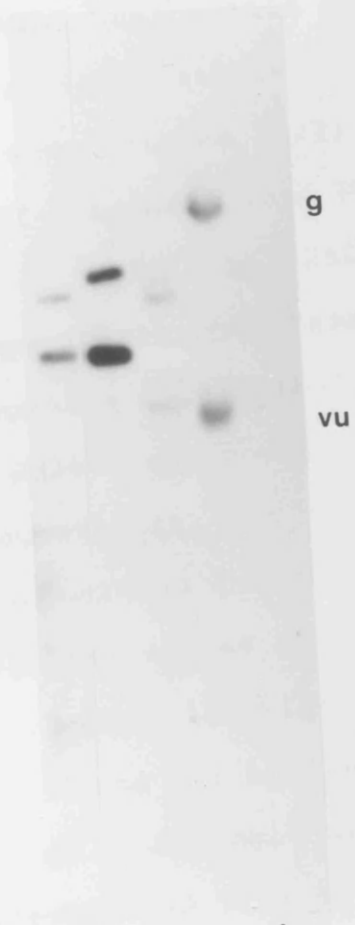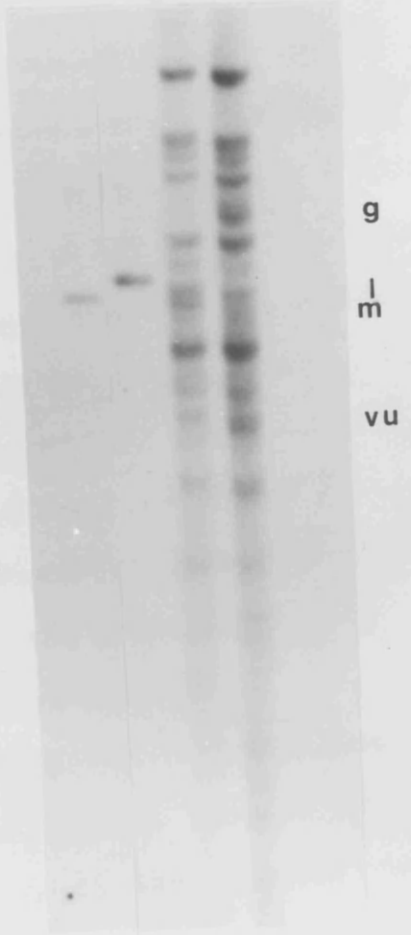
of the reiterated sets of sequences known to exist in this region of the genome (Davison and Wilkie, 1981).

3.4.2 Subcloning of BamHI g of the variant JH2604 into M13 bacteriophage and nucleotide sequencing

The wild type HG52 BamHI g fragment has been shown to be 5815 bp in length extending from IR$_S$ to IR$_L$ (D. J. McGeoch, unpublished results). There is a HincII site at nucleotide position 3053 within the 'a' sequence and a XhoI site at nucleotide position 5069 just upstream of the 5' end of the IE1 gene (Figure 3.27). Plasmids containing the JH2604 BamHI g inserts were cut with XhoI-HincII giving a fragment of 516 bp in size (2016 bp in the case of wild type BamHI g fragment) in both cases and the remainder of the BamHI g fragments were normal. This indicated that (1) the deletion in JH2604 was contained completely within the XhoI-HincII fragment, (2) the deletion as predicted from restriction endonuclease analysis was approximately 1.5 kb in size and (3) both copies of BamHI v contained a deletion with apparently identical end points and size.

These 516 bp fragments were ligated into the SmaI-SalI sites of M13 mp18 and mp19 phage and the ligated DNA was transfected into E. coli strain JM101 (see method section 2.30). The nucleotide sequence of the M13 clones was determined by the dideoxynucleotide chain termination method (see method section 2.32). Sequencing of clones 22 and 28 showed homology to the wild type sequence, starting at the HincII site (3053) and continuing for 30 bp to nucleotide position 3083 (Figure 3.28). There is then a deletion of 1488 bp and homology starts again at nucleotide position 4571 and continues with no alteration to the XhoI site at

Figure 3.27

Structure of the HG52 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 an expansion of the BamHI g fragment (0.81-0.85 m.u.) which is 5815 bp. The position of the 'a' sequence and the transcript of the IE1 gene is indicated. The bottom line shows the positions of HincII and XhoI sites within BamHI g.

TR$_L$ U$_L$ IR$_L$ IR$_S$ U$_S$ TR$_S$

IR$_L$ IR$_S$

5815   0

BamHI g
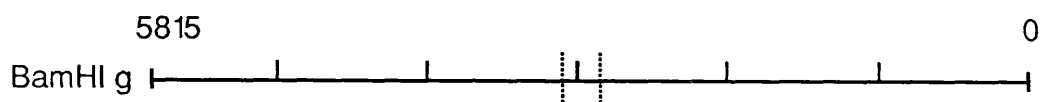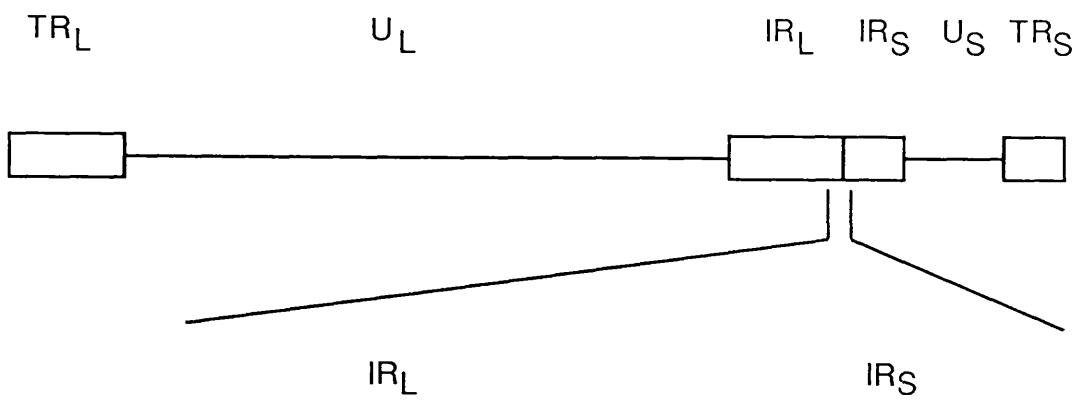
IE 1   'a'seq

Xhol - HincII

5069   3053   1769   1237

Figure 3.28

Portion of an autoradiograph of a 6% sequencing gel
showing the cloning site (3053) within the 'a' sequence
and the start (3083) and end (4571) of the deletion in
JH2604. Sequencing products were separated on denatured
polyacrylamide gels (Sanger et al., 1980) containing 9M
urea (McGeoch et al., 1986).
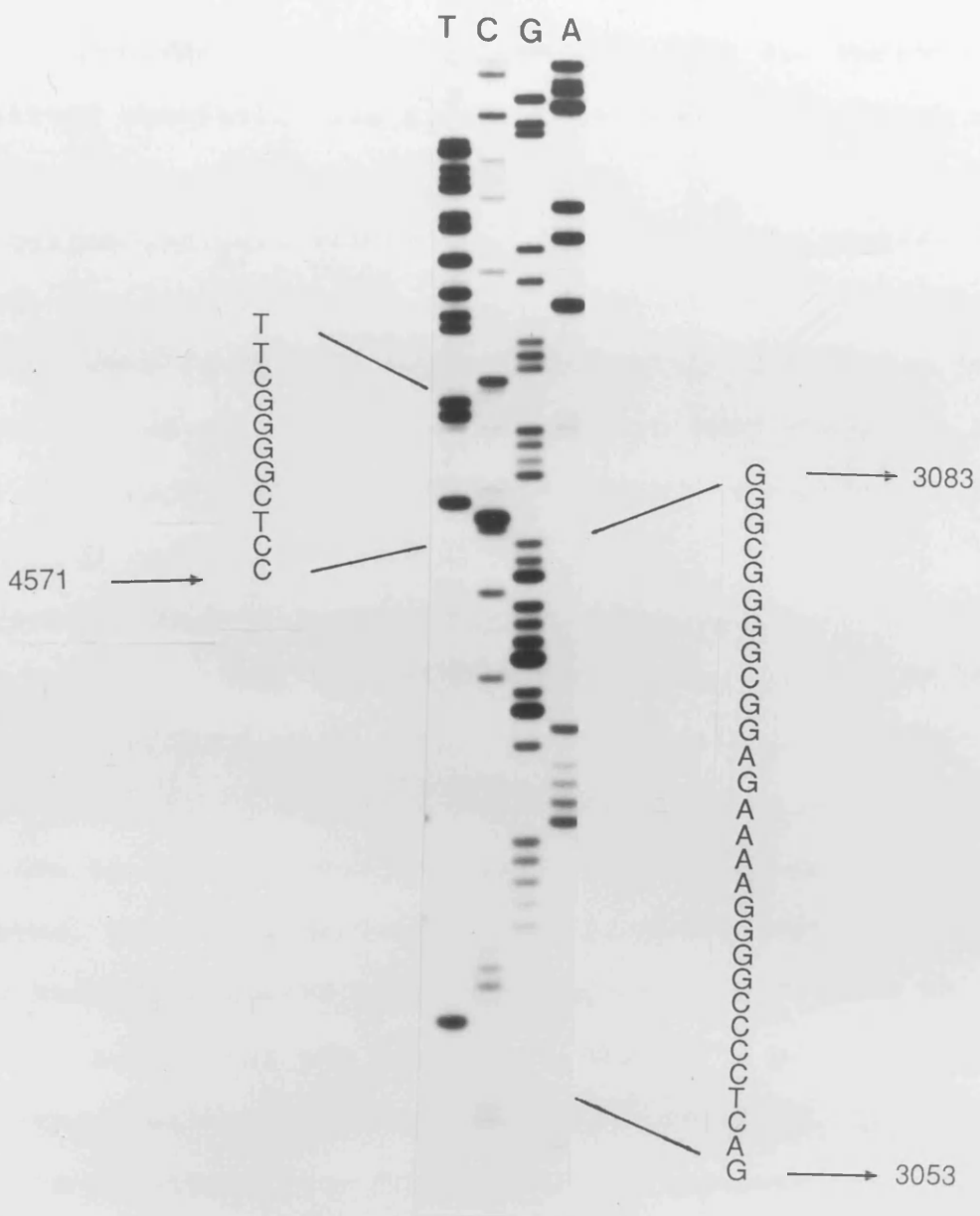
5069 (Figure 3.27). The deletion includes one copy of the 17 bp direct repeat (DR1) element of the 'a' sequence, AGTCCCCGTCCTGCCGC and four copies of a 19 bp sequence, CCCCTCCGACCCCCTGACG starting at position 3771 (D. McGeoch, unpublished results). The deletion terminates 522 bases upstream of the 5' end of the IE1 gene.

Sequence analysis showed that the nucleotide sequences deleted, and hence the size of the deletion in both clones 22 and 28 were identical indicating that the difference in the mobility of the remnant of BamHI g in each clone was due to variable copy numbers of the reiterated sequences outwith the deleted region.

HincII also cuts BamHI g at nucleotide position 1237 within BamHI u. XhoI in addition to cutting BamHI g at 5069 cuts within BamHI u at nucleotide position 1769 (Figure 3.27). This 532 bp sequence within BamHI u was sequenced and shown to be identical to that of HG52 with no point mutations, deletions or insertion. In total 2548 bp of the JH2604 BamHI g fragment have been sequenced and shown to be identical except for the deletion. However a point mutation within the remaining 3267 bp can not be excluded.

It is concluded from the nucleotide sequence analysis and the in vivo studies that the sequences within the 1488 bp fragment located between 0-0.02 in TR_L and 0.81-0.83 m.u. in IR_L of HSV-2 strain HG52 confer neurovirulence for BALB/c mice. The analysis clearly demonstrates a function in vivo for the deleted sequences which may suggest that the region is protein coding.

3.5 Neuropathology

In order to study the morphological changes produced by

the elite stock of HG52 and the variant JH2604 and the
distribution of the viral antigens in mice brains,
experiments were carried out in 3 week old BALB/c mice.
Mice were inoculated intracranially into the left cerebral
hemisphere (see method section 2.11) with virus stocks (10^5
pfu/mouse) and at 0, 24, 48, 72, 96, 120, 144, and 168 h
post infection, two surviving mice from each time point were
sacrificed and their brains were removed and processed for
histopathology and immunohistochemistry (see method sections
2.35 and 2.36). Two mice were inoculated intracranially
with PBS/calf serum as a control.

3.5.1. Macroscopic examination of brains infected with JH2604 and HG52

In 20% of the brains it was possible to identify the
site of intracranial inoculation over the lateral aspect of
the left cerebral hemisphere. There were no intracranial
complications and in particular there was no evidence that
the intracranial pressure had been high during life.

3.5.2 Microscopic examination of paraffin sections of brains infected with JH2604 and HG52

For the controls, they were normal and there was no
evidence of inflammation, hydrocephalus or intracranial
herniation (Figure 3.29). The nature and severity of
neurohistological changes in the brains inoculated with
either HG52 or JH2604 are shown in Table 3.14. At 24 h post
infection, all the mice brains inoculated with either the
elite stock of HG52 or JH2604, showed a mild degree of
lymphocytic infiltration of the meninges. This infiltration
was most conspicuous at the base of the brain. In all other

Figure 3.29

Paraffin embedded section of normal mouse brain. Figure

shows normal white matter H & E X430.

Table 3.14 Nature and severity of neurohistological changes in brains inoculated with the elite stock of HG52 and JH2604.

Virus	Survival (h)	Lymphocytic meningitis	Encephalitis	Ependymitis	Hydrocephalus
HG52	0	-	-	-	-
	24	+	-	-	-
	48	+	-	-	-
	72	+	+ (FN)	-	-
	96	++	++ (FN)	-	-
	120	+	++ (FN)	-	-
	144	+	++ (FN + PC)	+ (PC)	+/-
JH2604	0	-	-	-	-
	24	+	-	-	-
	48	+	-	+ (PC)	-
	72	+	+ (PC)	+ (PC)	+
	96	+	+ (PC)	+ (PC)	+
	120	++	++ (PC)	+ (PC)	+
	144	++	++ (PC)	+ (PC)	+
	168	+	+ (PC)	++ (PC)	+

Grade: + = mild; ++ = moderate.

FN = focal necrosis.

PC = perivascular cuffing by lymphocytes.

aspects the brains looked normal and there was no evidence

of hydrocephalus. At 48 h post infection, the brains

inoculated with HG52 showed abnormality limited to a mild

lymphocytic infiltration of the basal meninges. In

contrast, the brains inoculated with the variant JH2604

showed in addition to the basal meninges, focal infiltration

of the walls of the third ventricle by lymphocytes and an

associated mild enlargement of the ventricular system. By

72 h post infection, the brains inoculated with HG52 showed

small foci of necrotising encephalitis in the internal

capsule and thalamus of the left cerebral hemisphere. Even

though these lesions appeared to be associated with the site

of inoculation, their absence in the earlier time points

clearly indicated a response by the brain to the

inoculation. By 96 h post infection, however, the lesions

were larger and bilateral (Figure 3.30), affecting both grey

and white matter. Within these areas there was focal

necrosis of all neurons and glia, vacuolation of the white

matter, swelling of the related astrocytes and a mild

infiltration by neutrophil polymorphs. Some of the related

blood vessels appeared to be highly cuffed by macrophages.

There was also a moderate amount of lymphocytic infiltration

of the basal meninges.

In contrast, the changes in the cerebral hemisphere of

the brains infected with JH2604 at 72 h and 96 h post

infection were different in that there was no evidence of

necrotising encephalitis, even at the site of inoculation.

There was, however, an encephalitis with mild cuffing of

small vessels, largely by lymphocytes, but also with

occasional macrophages (Figure 3.31). This perivascular

cuffing was limited to the site of inoculation and was

Figure 3.30

Paraffin embedded section of mouse brain inoculated
intracranially with HSV-2 strain HG52 96 h post
infection. Figure shows focus of acute necrotising
encephalitis in which there is a vacuolation of the
neuropile, necrosis and fragmentation of glia with mild
infiltration by lymphocytes and neutrophil polymorphs H
& E X430.
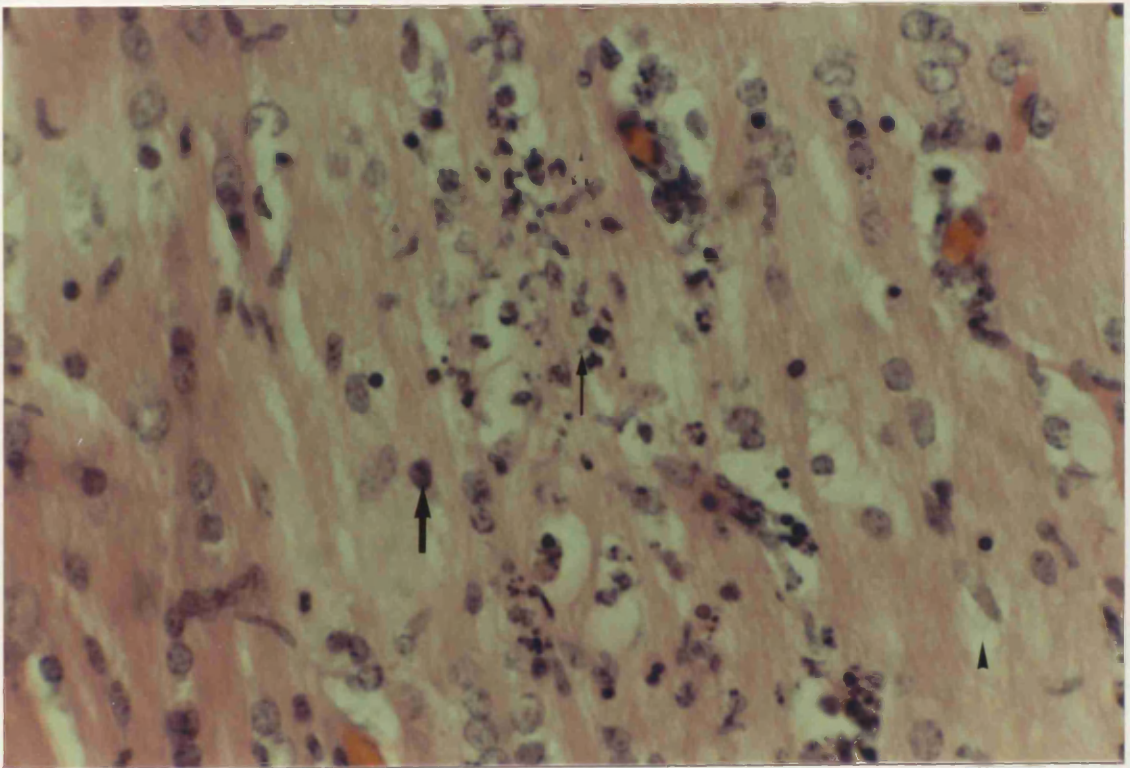
Figure 3.31

Paraffin embedded section of mouse brain inoculated

intracranially with the deletion variant JH2604 96 h

post infection. The figure shows localised infiltration

of lymphocytes and macrophages,and perivascular cuffing /

of blood vessel. In contrast to Figure 3.30 notice the

complete absence of necrosis H & E X430.

associated with a mild basal lymphocytic meningitis and a marked focal infiltration of the periventricular walls by lymphocytes. There was evidence of mild hydrocephalus. At 120 h and 144 post infection, the nature of histological changes in both H657 and Br124 infected brains remained unchanged though the extent increased over time. For example, in most infected brains there were multiple foci of necrotising encephalitis widely scattered throughout the

seen in the brain stem and cortex in all experiments.

The changes in the brains infected with H657 at 120 h and 144 h post infection were different from those above, similar to those noted at earlier time periods of this study. These changes consisted of widely distributed foci of perivascular cuffing by lymphocytes, in addition, there was a variable but moderate amount of lymphocytic infiltration of the basal meninges and an extension of this inflammatory process from the meninges along the intracerebral vessels. In this latter site there was also mild and occasional response, but no evidence of neuronal necrosis. There was

associated with a mild basal lymphocytic meningitis and a marked focal infiltration of the ventricular walls by lymphocytes. There was evidence of mild hydrocephalus. At 120 h and 144 post infection, the nature of histological changes in both HG52 and JH2604 infected brains remained unchanged though the amount increased over time. For example, in HG52 infected brains there were multiple foci of necrotising encephalitis widely scattered throughout the grey and white matter of both the cerebral hemispheres and hindbrains. By 122 h post infection, the changes were more easily seen with considerable proliferation of microglia, macrophage formation and a sprinking of neutrophil polymorphs. There was also both hypertrophy and hyperplasia of astrocytes. Oligodendroglia were difficult to recognise and there was vacuolation of myelinated fibers. There was a small amount of perivascular cuffing by lymphocytes, changes that were also seen in relation to the walls of the ventricular system. There was a mild degree of hydrocephalus and small numbers of lymphocytes were present in the basal meninges. Similar foci of necrosis were also seen in the brain stem and cortex of the cerebellum.

The changes in the brains infected with JH2604 at 120 h and 144 h post infection were different from HG52 though similar to those noted at earlier time points in this study. These changes consisted of widely distributed foci of perivascular cuffing by lymphocytes. In addition, there was a variable but moderate amount of lymphocytic infiltration of the basal meninges and an extension of this inflammatory process from the meninges along the intracortical vessels. In this latter site there was also glial and astrocytic response, but no evidence of neuronal necrosis. There was

also perivascular infiltration by lymphocytes and all brains
had developed a moderate degree of hydrocephalus. By 168 h
post infection, only the brains inoculated with JH2604 were
available since no mice had survived at that time point with
HG52. The changes were similar to those described earlier.
The only possible quantitative change was an increased
amount of perivascular infiltration by lymphocytes which
resulted in a moderate degree of ventricular enlargement.

3.5.3 Immunohistochemistry of paraffin sections of brains infected with JH2604 and HG52

In control brains there was no evidence of staining in
various cell types of the brain, its meningeal covering or
the ependymal lining of the ventricles (Figure 3.32). In
the brains infected with HG52 at 24 h and 48 h post
infection, positive staining was seen in some astrocytes
along the line of inoculation as well as in some of the
ependymal cells of the third ventricle. Similar changes
were seen in the JH2604 infected brains in which positive
staining was limited to the immediate vicinity of the site
of inoculation and one or two ependymal cells in the wall of
the third ventricle. By 96 h post infection the HG52
infected brains showed a positive staining of astrocytes not
only to the site of inoculation, but also in and around the
foci of necrotising encephalitis (Figure 3.33). Some of the
staining appeared to be intranuclear and intracytoplasmic
but where the cells had undergone necrosis, chromagen
appeared to be extracellular. Prior treatment with trypsin
removed much of the staining from the foci of necrosis but
left positive staining in what appeared to be both
astrocytes and nerve cells. Apart from staining of a few

Figure 3.32

Paraffin embedded section of normal mouse brain
immunostained with avidin biotinylated horseradish
peroxidase rabbit anti-HSV 1:100. Notice the complete
absence of staining in the white matter X430.
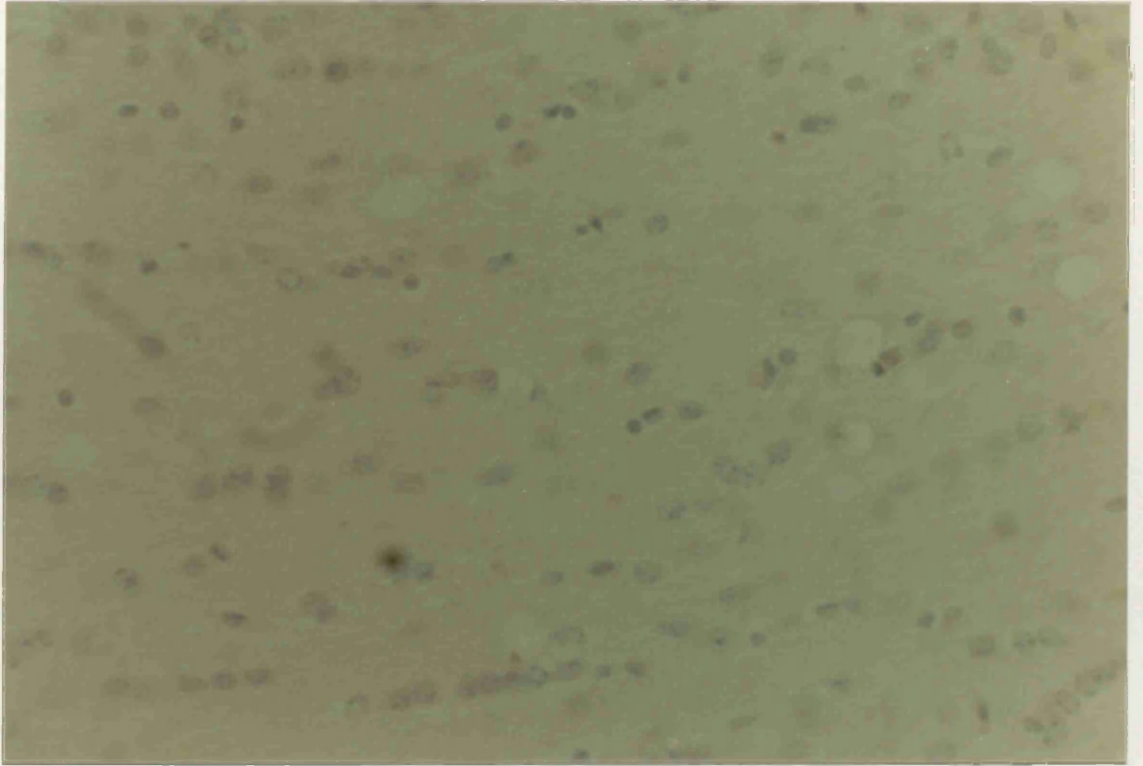
Figure 3.33

Paraffin embedded section of mouse brain inoculated
intracranially with HSV-2 strain HG52 96 h post
infection and immunostained with avidin biotinylated
horseradish peroxidase rabbit anti-HSV 1:100. Positive
staining (brown) is seen in both viable/ and necrotic/
nuclei and extracellularly in the neuropile X430.

ependymal cells, there was no positive staining elsewhere in the forebrain. There was, however, positive staining of both Purkinji cells and related reactive Bergmann glia in foci of early necrosis within the cortex of the cerebellum.

The changes in JH2604 infected brains at 72 h and 96 h post infection were different, positive staining being limited to astrocytes in the immediate vicinity of the site of inoculation (Figure 3.34), a few ependymal cells in the wall of the third ventricle and related subependymal astrocytes. There was no evidence of staining of neuronal cells, either within the forebrain or hindbrain. The remainder of the brain appeared normal. The patterns of positive staining in the brains infected either with HG52 or JH2604 at 120 h and 144 h post infection were different but similar to those identified in the earlier time points. In the HG52 infected brains, it was possible to see positive staining in the astrocytes and nerve cells in the immediate vicinity of the foci of necrotising encephalitis in both forebrain and cerebellum.

These results clearly demonstrated that the variant JH2604 is restricted in its growth in brain cells especially in neuronal cells.

Figure 3.34

Paraffin embedded section of mouse brain inoculated with
the deletion variant JH2604 96 h post infection and
immunostained with avidin biotinylated horseradish
peroxidase rabbit anti-HSV 1:100. Positive staining
(brown) is limited to astrocytes along the line of
inoculation X430.
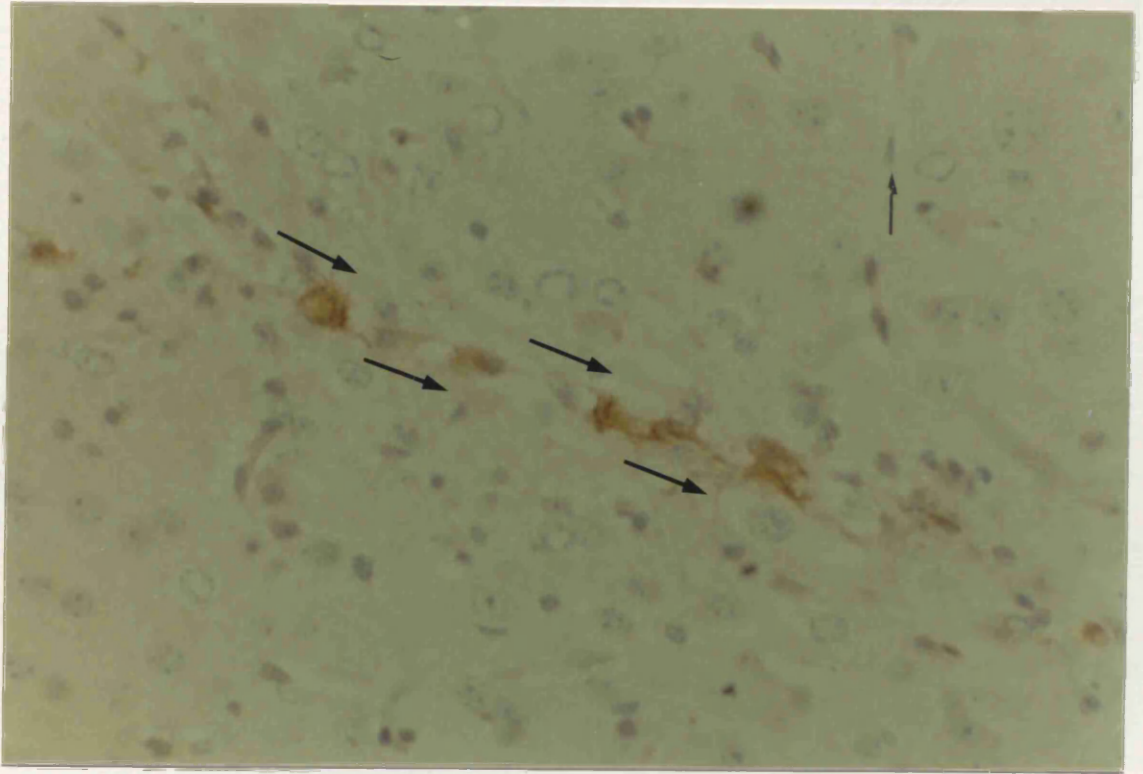
CHAPTER 4

DISCUSSION

The aim of this thesis was to identify gene(s) involved in neurovirulence of HSV-2 strain HG52 in the mouse model system by analysis of the effect in vivo of deletion variants of the virus. Before studying biological properties such as latency and virulence of defined deletion variants in an animal model system, it is essential to determine the phenotype of the parental wild type virus in the system under study. Therefore we have evaluated the neurovirulence of individual plaque stocks isolated from the elite laboratory stock of HG52 to determine the baseline from which to evaluate the neurovirulence of the HG52 deletion variants.

The ten individual stocks isolated from the elite stock of HG52 showed differences in their LD_{50} values following intracranial inoculation of 3 week old BALB/c mice. On the basis of the LD_{50} values compared with that of the elite stock of HG52, the plaque stocks were subdivided into three classes of neurovirulence; low with a LD_{50} value $>10^5$ pfu/mouse, intermediate with a LD_{50} value 10^3-10^4 pfu/mouse and high with a LD_{50} value of $<10^3$ pfu/mouse. None were designated as avirulent. The difference between low and high virulence stocks is over 3 logs. The same differences in the LD_{50} values between high and low virulence stocks were seen using the intraperitoneal route of inoculation. Although HG52 showed a normal dose response curve following intracranial inoculation, the inversion of the dose response curve following intraperitoneal inoculation was unexpected. This inversion of the HG52 dose response curve is an observation which is specific for HSV-2 strain HG52, since

HSV-1 strain 17 showed a normal dose response curve. The reason for the inversion is unclear and is possibly due to the intraperitoneal route of inoculation. It has been shown that intraperitoneal infection with HSV-2 is an excellent inducer of macrophage phagocytosis activity in mice (Armerding et al., 1981). The activated macrophages, possibly induced by the local IFN generated or by the virus infection alone, might be required for resistance to an intraperitoneal infection. Injecting a high dose of virus intraperitoneally could result in activation of more macrophages and production of high levels of IFN which may result in resistance to infection. Although this explanation might be correct for a high infectious dose, it does not fit with the lower dose of virus.

Passaging of the single plaque stocks in vitro at $37^{\circ}C$ showed in some cases a shift from high to intermediate virulence whereas the phenotype of low virulence was generally retained after repeated passage at $37^{\circ}C$. The shift from high to intermediate virulence could result from the presence of a mixture of the three classes of virulence in the high virulence stocks, despite their plaque purification. Therefore, in an in vivo experiment, the score would be for high virulence. When the high virulence stocks were passaged in vitro i.e in the absence of in vivo selection pressure they segregated into the three virulence classes. This explanation has been supported by the virulence heterogeneity of high virulence stocks during cotransfection experiments which will be discussed later.

Thompson and Stevens (1983a), using 4-6 week old Swiss Webster mice, found the LD_{50} value of the elite stock of HG52 to be 10^4 pfu/mouse following intracranial inoculation.

Five plaques were isolated and stocks from these passaged

four times at 31°C or 37.5°C. Those passaged at 31°C

retained their low virulence, however, after passage at

37.5°C, virulence increased in four cases and in one case

remained low. Similar findings have also been observed in

HSV-2 strain Curtis (Thompson and Stevens, 1983a). It is

clear from these findings that there is virulence

heterogeneity within HSV-2 stocks, a finding we have now

clearly confirmed, quantitated and extended. As Thompson

and Stevens (1983a), showed the effect of temperature on

selection of virulent HSV-2 stocks, Goodman and Stevens

(1986) have demonstrated that passage of HSV-1 strain 17 in

vitro in chick embryo fibroblast cells enhanced the

virulence of the virus for chick embryos in vivo suggesting

that in vitro passage of HSV potentiated, rather than

attenuated virulence. In our hands, there was no such

effect of temperature and passage in vitro on virulence of

HSV-2 strain HG52. Our findings are similar to those

reported in PRV by Lomniczi et al. (1984).

Restriction endonuclease analysis of the DNAs of the

isolated plaque stocks was carried out at different passage

numbers using a range of restriction enzymes and no

differences were seen in the size and distribution of

fragments. In addition, the DNAs of virus stocks reisolated

from mice brains retained their original restriction

endonuclease profiles. Restriction endonuclease analysis

however can not detect point mutations or small changes

(deletions/insertions <150 bp) in the viral genome which

could be possible sources of variation in the biological

behaviour of the virus in vivo. The particle : pfu ratios

of the plaque stocks can not be evoked to explain the

differences in biological behaviour. Using the two criteria
of restriction endonuclease profile and particle : pfu
ratio, all of the ten plaque stocks used to infect animals
were classified as wild type.

The replication kinetics of the individual plaque stocks
have shown that the observed difference in neurovirulence
was due to inefficient replication in mouse brain in vivo as
the plaque stocks 17 and 14 (high virulence) replicated
with kinetics similar to the elite stock of HG52, while
plaque stock 8 (low virulence) replicated poorly (see Figure
3.4). The replication capacities of plaque stocks 17 and 14
in mouse brain were specific for brain tissue as they did
not show differential replication kinetics in vitro in
BHK-21 C13 cells. Therefore, the restriction of the growth
of the low virulence stock appeared to be specific and not
generalised. The results obtained with individual plaque
stocks clearly demonstrate that the laboratory strain HG52
is heterogeneous with respect to neurovirulence in mice.
HG52 is not unique in this respect as it has been shown that
both virulent and avirulent stocks of HSV-1 strain KOS exist
(Dix et al., 1983). Although the elite stock of HG52 was
initially derived by rigorous plaque purification of a
patient isolate, it is heterogeneous not only in terms of
neurovirulence, but also contains a proportion of viable
virus with aberrant genomic structures comprising at least
24% of the population (Harland and Brown, 1985; 1988; Brown
and Harland, 1987).

The basis for the variation in an important biological
property is unclear but one possible reason is the presence
of point mutations, deletions and/or insertions in the
genome which can not be detected by restriction endonuclease

analysis. Such changes in the viral genome might affect any of the stages in the viral replication. Our results emphasise the importance of working with well characterised parental virus when assessing the biological behaviour of mutants.

Having confirmed the heterogeneity within HG52, the plaque stock 17 has been used as the prototype virus and its LD_{50} value has been taken as the baseline from which to evaluate the neurovirulence of the deletion variants.

The nervous system is a critical target for HSV infection. The relationship between HSV and the peripheral nervous system is usually benign in that the virus remains latent in the neurons of dorsal root ganglia from which it reactivates intermittently. However, when HSV invades the central nervous system the likely outcome is a fatal encephalitis. It is therefore important to determine those genes of HSV which predispose it to invade and destroy nervous tissue. Viral genes controlling neurovirulence have only been identified recently and various laboratories have predominantly implicated the viral sequences between 0.7-0.83 m.u. (Thompson and Stevens, 1983b; Thompson et al., 1985; Rosen et al., 1986; Javier et al., 1987). This region contains the whole of IR_L and part of U_L and the genes encoded therein are one copy of IE1 and UL50 to UL56 (Clements et al., 1979; Whitton et al., 1983; Perry et al., 1986; McGeoch et al., 1988). The mapping of virulence genes has come from the analysis of intertypic HSV recombinants constructed between low an high virulence strains of HSV using cloned viral fragments (Thompson et al., 1983; 1985). Deletion variants more readily allow defined regions of the genome to be studied for their neurovirulence properties.

The deletion variants analysed in this study have deletions ranging from 1.5 kb to 9 kb in the TR_L/IR_L and U_S/TR_S regions of the genome. All the deletion variants had reduced pathogenicity for mice following intracranial inoculation and depending on their LD_{50} values they fell into three classes of neurovirulence with LD_{50} values ranging from $10^4 -> 10^7$ pfu/mouse. The variants deleted in the U_S/TR_S regions are JH2607, JH2608, JH2609, JH2610 and JH2611. The size and extent of the deletion in these variants relied mainly on restriction endonuclease and Southern blot analysis (Brown and Harland, 1987). As no sequence data was available at the time of their isolation, the exact sequences deleted in each variant remains unresolved at present making the interpretation of the neurovirulence results slightly difficult.

The variants JH2607, JH2608 and JH2609 had LD_{50} values of 10^4, 2.5×10^5 and 10^5 pfu/mouse respectively and the feature they have in common is the loss of the US10, US11 and US12 genes. These LD_{50} values are at least 3 logs higher than the HG52 plaque 17 LD_{50} value ($<10^2$ pfu/mouse) implying a role for the US10, US11 and US12 genes either individually or cummulatively in neurovirulence. In addition the variant JH2607 has lost one copy of the IE3 gene and one copy of ori_S and comparing its LD50 value (10^4 pfu/mouse) with those of JH2608 (2.5×10^5 pfu/mouse) and JH2609 (10^5 pfu/mouse) indicated that the deletion of one copy of the IE3 gene and one copy of ori_S had no effect on neurovirulence. The LD_{50} values of the variants JH2608 and JH2609 are comparable (2.5×10^5 and 10^5 pfu/mouse respectively). The additional changes in the genomes of JH2608 and JH2609 compared to JH2607 are extension of the

short repeat regions by 6 kb on either side as well as loss
of the XbaI site at 0.91 m.u. in the variant JH2609 (Brown
and Harland, 1987). The common features of the variants
JH2609, JH2610 and JH2611 are the loss of XbaI sites at 0.7
and 0.91 m.u. (Brown and Harland, 1987). In addition the
variant JH2611 has lost the XbaI sites at 0.45 and 0.94 m.u.
(Brown and Harland, 1987). The DNA sequences around the
XbaI sites at 0.45, 0.7 and 0.94 m.u. are not known for
HSV-2, but in HSV-1 these sites lie within UL33, between
UL49 and UL50 and in the intergenic region of US9 and US10
respectively (McGeoch et al., 1985: 1988). The DNA
sequences around 0.91 m.u. in HSV-1 lie within the US4 gene,
encoding gG (McGeoch et al., 1985). The glycoprotein gG in
HSV-2 strain HG52 is encoded by the US4 gene. The XbaI site
at 0.91 m.u. lies within the coding region of the gene
(McGeoch et al., 1987). It has been shown that in the
variants JH2610 and JH2611 neither gG-2 nor its precursor
were synthesised, but two polypeptides migrating above the
normal positions of gG-2 and its precursor were made, while
the variant JH2609 failed to synthesise gG-2 or its
precursor showing that gG-2 is not essential for virus
growth in vitro (Harland and Brown, 1988). The variants
JH2609, JH2610 and JH2611 had reduced pathogenicity for mice
following intracranial inoculation having LD_{50} values of
10^5, 4.2×10^6 and 5×10^4 pfu/mouse respectively. These LD_{50}
values are much higher than the LD_{50} value of the elite
stock of HG52 ($<10^2$ pfu/mouse), indicating the importance of
gG-2 in neurovirulence. These results are consistent with
the findings of Meignier et al. (1988) which showed that a
gG^- mutant of HSV-1 strain F had reduced pathogenicity for
mice.

Generally, the variants deleted in the U_S/TR_S regions were shown to be temperature restricted at 38.5°C (Brown and Harland, 1987), so we can not rule out this growth disadvantage from contributing to reduced neurovirulence although Javier et al. (1988a) have shown that neurovirulence functions are independent of high titre virus replication in mouse brain.

The variants with deletion in IR_L/TR_L were shown to grow normally in tissue culture in BHK-21 Cl3 cells (Harland and Brown, 1985). It has been shown that the IR_L region of the HSV-1 strain 17 genome (Figure 4.1) contains the IE1 gene between 0.785-0.81 m.u. (Perry et al 1986) and the LAT transcripts between 0.782-0.796 m.u. running in the opposite orientation to the IE1 gene (Wagner et al., 1988; Perry and McGeoch, 1988). In HSV-1 strain F a gene has been proposed which codes for a polypeptide designated ICP34.5 in the same orientation as the IE1 gene (Chou and Roizman, 1986). Again, restriction endonuclease analysis and until recently the lack of sequence data for this region in the HG52 genome has not allowed precise determination of the extent of the deletions. The IE polypeptide analysis of the variant JH2603, whose genome is deleted by 3.75 kb in IR_L, showed a reduction in the amount of VmwIE118 synthesised in infected cells, supporting the conclusion that only one copy of the coding region of VmwIE118 had been deleted (Harland and Brown, 1985). Although the LAT transcripts have recently been identified in HSV-1 (see section 1.9), their existence in HG52 is under analysis. If they exist in the same positions as those identified in HSV-1, theoretically the deletion in JH2603 would remove one copy of the LAT transcripts in IR_L. The deletion in JH2605 is 5.5 kb, is

Figure 4.1

Gene organization of IR_L in HSV-1 strain 17. The top line shows the U_L (dotted line), IR_L (open box), 'a' sequence (solid box) and IR_S (dotted open box). Locations of the coding regions of genes (open boxes) and orientations of transcripts (arrows) for IE1 (Perry et al., 1986), LAT (Wagner et al., 1988) and proposed ICP34.5 in strain F (Chou and Roizman, 1986) are indicated.

The minnor LAT (Mitchell et al., 1990) is indicated by dotted arrow.

U_L

LAT

IE1

minor LAT

ICP 34.5

IR_L

'a'   IR_S

confined to IR_L and removes the BamHI $\underline{f}/\underline{p}$ site at 0.775 m.u.
(Harland and Brown, 1985). Whether the deletion affects
VmwIE118 synthesis is unresolved. The deletions in the
variants JH2603 and JH2605 may involve the U_L/IR_L junction.
The U_L gene adjacent to the U_L/IR_L junction in HSV-1 strain
17 is UL56 (McGeoch et al., 1988) and it has already been
implicated in intraperitoneal pathogenicity in tree shrews
(Rosen et al., 1986) and mice (Becker et al., 1986). The
deletion in the variant JH2606 is 9 kb in length and removes
the U_L/IR_L junction, the BamHI $\underline{f}/\underline{p}$ site at 0.775 m.u., the
total coding region for VmwIE118 (Harland and Brown, 1985)
and possibly the LAT transcripts. As in JH2603 and JH2605,
there is also a possibility that UL56 is removed from the
variant JH2606. The variant JH2604 which is deleted by 1.5
kb in both the TR_L and IR_L copies of BamHI $\underline{v}$ between 0-0.02
and 0.81-0.83 m.u. is the only variant in which IE
polypeptide synthesis is not affected (Harland and Brown,
1985).

All the deletion variants in TR_L/IR_L had reduced
pathogenicity for mice following intracranial inoculation
with LD_{50} values ranging from 10^4 to $>10^7$ pfu/mouse compared
to $<10^2$ pfu/mouse for HG52. Therefore the variants were at
least 3 logs and at most 6 logs less neurovirulent than the
elite stock of HG52. These results imply that the genes in
the IR_L region of HG52 genome are important in the control
of neurovirulence. Although the variants JH2603 and JH2606
had lost one copy of the IE1 gene, they still produced
disease in mice. Recently Clements and Stow (1989) have
shown that the mutant dl1403, which contains a 2 kb deletion
within both the TR_L and IR_L copies of the IE1 gene (Stow and
Stow, 1986), had reduced virulence for mice following

intracranial and footpad inoculation.

The precise role of the LAT transcripts in latency is unresolved but their deletion results in impairment of virus reactivation from explanted latently infected ganglia (see section 1.9). Their role in neurovirulence is not yet known. However, preliminary latency experiments of the IR_L/TR_L deletion variants showed that all the variants were capable of establishing latent infection in dorsal root ganglia of mice, but they were impaired to a different extent in reactivation from latent infection (G.B. Clements, personal communication).

The variant JH2604, whose genome is deleted by 1.5 kb in both copies of the BamHI v fragment, was avirulent for mice with a LD_{50} value $>10^7$ pfu/mouse compared to $<10^2$ pfu/mouse for HG52 implicating the sequences between 0-0.02 and 0.81-0.83 m.u. in neurovirulence. As the DNA sequences between 0-0.02 and 0.81-0.83 m.u. deleted in JH2604 appeared to be important in neurovirulence and since no potential ORF has been clearly identified in this region in HSV-1 strain 17 or in HSV-2 strain HG52, it was important to characterise this variant in detail. It was calculated from restriction endonuclease analysis and Southern blot data that the variant JH2604 had a 1.5 kb deletion in both copies of the BamHI v fragment between 0-0.02 and 0.81-0.83 m.u. in TR_L and IR_L respectively (Harland and Brown, 1985). As no sequence data for this region of HG52 was available at the time of its isolation, the size and location of the deletion relied on restriction endonuclease and Southern blot analysis. It was thought that the same sequences were deleted from each copy of the BamHI v fragment but the size of the deletion (1.5 kb) and the size of the BamHI v

fragment (3 kb) did not preclude the deletion in each copy

being at least in part contiguous (Harland and Brown, 1985).

We have demonstrated that the variant JH2604 is

avirulent for 3 week old BALB/c mice on intracranial

inoculation with a LD_{50} value $>10^7$ pfu/mouse compared to

$<10^2$ pfu/mouse for plaque stock 17. It also failed to kill

any mice following footpad inoculation at doses of 10^6, 10^7

and 10^8 pfu/mouse giving a LD_{50} value $>10^8$ pfu/mouse. In

protection experiments, JH2604 failed to produce zosteriform

spread of disease in mice following flank inoculation and

those mice were protected following the inoculation of a

lethal challenge of the HG52 elite stock by the same route

of inoculation (B. Rouse, personal communication to S.M.

Brown). The variant JH2604 and its parental HG52 grew

equally well in BHK-21 C13 cells at 37°C and is no more

temperature restricted at 38.5^{o}C than HG52 (J. Harland,

personal communication). However, JH2604 failed to grow in

mouse 3T3 cells at both 37°C and 38.5^{o}C, but as the elite

stock of HG52 also showed poor replication in these cells,

the apparent lack of growth of JH2604 is not significant.

It has recently been shown that using mouse 3T6 cells,

JH2604 grew as well as the HG52 elite stock (A.R. MacLean,

personal communication) and that the restriction in 3T3

cells was specific for this particular clone of cells and

not for mouse cells per se.

Of significance is the finding that JH2604 does not

replicate in vivo in mouse brain. The elite stock of HG52

grows efficiently within the brain at the accepted ambient

mouse temperature of 38.5^{o}C, reaching peak titres by 120 h

post infection with all the infected animals dying by 144 h

post infection. JH2604 showed some replication by 12 h and

24 h post infection, but by 48 h post infection the amount of infectious virus recovered from brains was over 1 log unit lower than the input virus measurable immediately post absorption. The growth kinetics of JH2604 in vivo in mouse brain and in vitro in BHK-21 Cl3 and 3T6 cells imply that JH2604 is specifically restricted in neural tissue and not generaly restricted in murine or other cell types. These findings have been supported by neuropathological and immunohistochemical studies of brains infected with JH2604 and the elite stock of HG52.

The results of neuropathological studies showed a fundamental difference in the histopathological appearence of the two types of lesion produced by JH2604 and the wild type virus. The wild type virus being characterised by disseminated widely distributed foci of necrosis throughout the brain with minimum associated inflammatory cell response. In contrast to these changes, the microscopy of the brains infected with JH2604 was strikingly different with complete absence of necrotising encephalitis even at the site of inoculation. However, the lesion was characterised by perivascular cuffing of small vessels by a combination of lymphocytes and macrophages, these changes being limited to the site of inoculation or the ependymal lining of the ventricles. A probable consequence of the ependymitis that characterised the variant JH2604 was the development of hydrocephalus, a feature that was more often and readily seen in the JH2604 infected brain compared with the parental HG52. The hydrocephalus was probably due to a narrowing of cerebrospinal fluid pathways due to a combination of desquamating debris and the marked inflammatory cell infiltration, an appearance described

previously in HSV-1 infected mice (Hayashi et al., 1986).
The distribution of the virus in the brain was determined by
immunohistochemical studies which reflected the histological
identification of the pathological process in the brains of
infected animals. In JH2604 infected brains there was a
positive staining (viral antigens) limited to astrocytes in
the immediate vicinity of the site of inoculation with a few
ependymal cells in the wall of the ventricular system. In
contrast, HG52 infected brain showed a wide distribution of
positive staining in neurons and astrocytes. These results
confirmed that JH2604 has a blockage in its replication in
neural cells. In the absence of JH2604 replication, the
infiltrating lymphocytes and neutrophil polymorphs are
hypothesised to clear the remaining input virus from the
brain.

The mechanism by which a deletion of 1.5 kb in the
JH2604 genome resulted in failure of replication in the
central nervous system is not clear. The function of the
deleted sequences is unlikely to be involved in attachment,
penetration or uncoating because JH2604 is capable of
establishing and reactivating from a latent infection in
mice (G.B. Clements, personal communication), therefore
JH2604 must be capable of entering neurons and expressing
some of its genes despite the lack of growth in neural
tissue. In this respect, Steiner et al. (1990) have
recently shown that HSV-1 mutant in Vmw65 TIF (Ace et al.,
1989) can establish latent infection in mice following eye
infection in the absence of viral replication. Similar
findings have also been observed in the avirulent RE6
(Thompson et al., 1989). The possible functions of deleted
sequences might affect gene expression at one or more of

several stages; transcription, translation, DNA synthesis, packaging or encapsidation. Evidence of lower efficiency of translation in an attenuated poliovirus, which contains a mutation in the 5' non-coding region of its RNA that resulted in attenuated virus which failed to replicate in the mouse brain (LaMonica et al., 1987), has been reported (Svitkin et al., 1985).

It has been shown that viral encoded TK modulates virulence, since HSV without this enzyme is relatively non-neurovirulent. The TK phenotype of the variant JH2604 was indistinguishible from that of the parental HG52. The non-neurovirulence and lack of growth in mouse brain is therefore not a result of the virus lacking TK.

In order to determine the role of genomic information in the regions between 0-0.02 and 0.81-0.83 m.u. in neurovirulence, cotransfection experiments were carried out. The intact JH2604 DNA was cotransfected with the XbaI fragments of HG52 plaque stock 17 DNA containing sequences deleted from JH2604. The isolation of the recombinants, in which the deleted sequences had been replaced by equivalent sequences from the XbaI fragments, enabled the determination of whether the replaced sequences had a direct effect on neurovirulence. From 28 mice infected with R17(192)E pl. 8, the LD_{50} value of the recombinant was calculated to be 2×10^5 pfu/mouse; from 40 mice infected with R17(192)E pl. 14, 1×10^3 pfu/mouse; from 28 infected with R17(192)E pl. 15, 3×10^5 pfu/mouse and from 46 infected with R17(192)F pl. 10, 3×10^5 pfu/mouse. These values are within the range of LD_{50} values for individual plaque stocks of HG52 (see Table 3.1). There are two possibilities to explain the range of LD_{50} values obtained with the recombinants, in which the deletion

has been corrected, either (i) there is another mutation outside the regions between 0-0.02 and 0.81-0.83 m.u. which precludes successful rescue of neurovirulence functions or (ii) the DNA stock of plaque 17 used for cotransfection produced virus which was heterogeneous with respect to neurovirulence. The latter was shown to be the case when five plaques picked from transfected plaque stock 17 DNA, showed LD_{50} values ranging from $<10^2$ to $>10^4$ pfu/mouse (see Table 3.11). However, the deaths of 10/33 animals infected with R17(192) recombinants at a dose of 10^5 pfu/mouse and 10/15 with 10^6 pfu/mouse compared to 0/12 with 10^5 pfu/mouse, 0/12 with 10^6 pfu/mouse and 2/18 with 10^7 pfu/mouse of JH2604 point to the avirulent phenotype having been corrected by replacement of the deleted sequences within both copies of the BamHI v fragment or the exchange of sequences within the remainder of the XbaI e fragment (this will be discussed later). It obviously would have been more critical to correct the deletion in JH2604 by cotransfection with the BamHI g joint fragment (v + u) of HG52 plaque 17 DNA, but because this fragment is so small (4×10^6 Mr) and no selection system was available, we initially chose, to facilitate recombination by using large XbaI fragments.

The isolation of two recombinants in which the deletion in JH2604 has been introduced back into the HG52 plaque stock 17 genomes allowed the determination of how the reciprocal recombinants behaved following inoculation of mice. Intracranial inoculation of 18 mice with each recombinant produced an LD_{50} value of $>10^7$ pfu/mouse in each case. This is identical to the LD_{50} value determined from 42 animals infected at the same time with JH2604. These

results provided substantive evidence that the sequences deleted in JH2604 are involved in the determination of neurovirulence of HSV-2 strain HG52.

The possibility that R17(192) recombinants might contain other sequences from the contransfecting XbaI fragment of the plaque 17 stock of HG52 and the implication that besides the deleted sequences, the sequences within XbaI e and f were not equivalent in HG52 plaque 17 and JH2604 and that the observed effect on neurovirulence was not due to the deletion but to some other mutation, was tested. The deletion in the variant JH2604 has been corrected by the small BamHI g (v + u) joint fragment from plaque 17 DNA using in vivo selection in mice. The isolation of two recombinants that displayed plaque 17 neurovirulence with LD_{50} values of $<10^2$ pfu/mouse, demonstrated that the sequences within the BamHI g fragment and probably within the BamHI v fragment are required to restore the neurovirulence of JH2604 to the level of HG52 plaque 17.

The BamHI v fragment of HG52 co-maps with the BamHI s fragment of HSV-1. The nucleotide sequence of the BamHI v fragment was not known at the time of animal experiments, but in HSV-1 strain 17 no complete genes had been identified in BamHI s (Perry and McGeoch, 1988). The 5' end and 500 bp from the end of the IE1 gene are located within the BamHI s fragment. It has been shown previously that JH2604 makes VmwIE118 (equivalent to VmwIE110 in HSV-1) of normal size and in normal amounts compared to the parental HG52 (Harland and Brown, 1985), indicating that the deletion within the BamHI v fragment in both copies of R_L does not extend into the reading frame for the IE1 gene. It has also been shown that the general infected cell poypeptides synthesised by

JH2604 are not identifiably different from those of HG52.
In HSV-1 strain F, Chou and Roizman (1986) have reported a
gene in R_L coding for a polypeptide ICP34.5 whose promoter
is in the terminal 'a' sequence. The same putative gene has
not been identified in the sequences of HSV-1 strain 17
(Perry and McGeoch, 1988) and it is now known that its
equivalent is not present in HG52 (D.J. McGeoch, personal
communication). The in vivo analysis of the HG52 deletion
variant JH2604 has demonstrated that at least 500 bp of the
BamHI v fragment within or spanning 1500 bp of the terminal
sequences of R_L are required to confer neurovirulence for
JH2604.

Towards the end of the experimental work described in
this thesis, the BamHI g joint fragment of HG52 was
sequenced and the sequence made available by Dr. D.J.
McGeoch. It therefore became important to delimit the
deletion in JH2604 by sequencing the fragment in which the
deletion was located and to clarify the possibility of the
presence of other mutations.

The isolation of two positive pAT153 clones containing
the deleted JH2604 fragment and their restriction
endonuclease analysis and subcloning, demonstrated that the
deletion in each clone was approximately 1.5 kb and was
contained within the HincII-XhoI (between nucleotide
position 3053 to 5069) subfragment and had apparently
identical end points and size. Sequence analysis confirmed
that the sequences deleted in both clones were identical and
were 1488 bp in length. The deletion included a complete
copy of the 17 bp DR1 element (AGTCCCCGTCCTGCCGC) of the 'a'
sequence, four copies of a 19 bp reiterated sequence
(CCCCTCCGACCCCCTGACG) and stopped 522 bp upstream from the

5' end of IEl gene. The variability in the mobility of the joint fragment spanning the deletion in both clones was confirmed as not being due to variation in the size of the deletion in each copy of the BamHI $\underline{v}$ fragment but possibly to variation in copy numbers of reiterated sets of sequences located in the repeats (see section 1.3.1). Such variation has been shown previously in HSV-2 strain HG52 (Davison and Wilkie, 1981) and in HSV-1 strain KOS (Wagner and Summers, 1978).

Whether the deleted sequences contain an ORF is under analysis. Chou and Roizman (1986) presented sequencing data and proposed that the region between the IEl gene and the 'a' sequence in HSV-1 strain F contains a gene in the same orientation as the IEl gene (see Figure 4.1). An antipeptide serum based on this predicted ORF identified a protein ICP34.5, in extracts of infected cells (Ackermann et al., 1986). When comparisons were made between the published sequence for this region in strain F and the equivalent region in HSV-1 strain 17, Perry and McGeoch (1988) found a number of differences including a difference in the copy number of a 9 bp tandem repeat family (10 complete copies in strain F and five in strain 17) and 20 points in HSV-1 strain 17 at which an addition or deletion breaks the proposed coding ORF. This led to the conclusion that the entire ORF proposed by Chou and Roizman (1986) was not genuinely protein coding. The data presented by Perry and McGeoch (1988) were thought to be consistent with their being protein coding sequences in this locality but they did not consider the analysis satisfactory enough to propose any gene layout.

The BamHI $\underline{g}$ joint fragment of HSV-2 strain HG52 has been

found to be 5815 bp in length (D.J. McGeoch, personal communication). The sequences deleted in the variant JH2604 are shown in Figure 4.2. When comparisons were made between these sequences and the published sequences for this region in HSV-1 strain 17, there is a degree of homology between the two sequences (D.J. McGeoch, personal communication). The sequence arrangement in this region of HG52 is at present under critical analysis and it is thought that there is a potential ORF, but its precise end points have not been identified (D.J. McGeoch, personal communication).

The data presented in this thesis on correction and introduction of the deletion by a variety of methods suggests that the region deleted in the HSV-2 variant JH2604 must be protein coding. Support for the work in this thesis has recently come from Thompson et al. (1989) who showed that an HSV intertypic recombinant (RE6) was avirulent for mice and cotransfection followed by in vivo selection in mouse brain of RE6 DNA with a 1.6 kb cloned fragment mapping between 0.82-0.832 m.u. from the pathogenic HSV-1 strain 17 restored the neurovirulence phenotype of RE6.

FUTURE PROSPECTS

The information gained from this thesis is important both for further study of the molecular events controlling pathogenicity of HSV as well as the potential of the variant JH2604 for a prototype live vaccine.

As all the deletion variants analysed in this study had reduced pathogenicity for mice, it would be important to delimit the deletions by sequence analysis. The exact sequences deleted would be determined and marker rescue experiments correcting the deletions and analysis of

Figure 4.2

The sequences deleted in the variant JH2604. These
sequences are a part of the BamHI g joint fragment of
HG52 running from IR_S to IR_L (D.J. McGeoch, personal
communication). The start (3083) and end (4571) of the
deletion are indicated by stars. The 17 bp DR1 element
is also indicated by the dotted box. The four copies of
a 19 bp reiterated sequence are underlined.

```
2801  GTGTGGTGGT CTGCGGCACA GCCTGCTAGT CCCCGTCCTG CCGCGCGGGG

2851  GCGGGCGCGG GAAAAAAGCC GCGCGGGGGC GCCCGCGGGA AGGCCAGCCC

2901  CGCGGCGCGC GGGGGGAGGG GCGGCGCCCG CGGGGGAGCG GCCGGCTCCG

2951  GGGGAGGGAC GGGGAAGGGG GCGCGCGGGG CTGCCCTGCC GCCCGCCCGC

3001  CCGCCGCCGC CCGCCTTCGC GCCCCCCCCC AAAAAACACC CCCCCCGGGG

                                          *
3051  GTTGACTCCC CGGGGGAAAA GAGGCGGGGC GGGAGTCCCC GTCCTGCCGC

3101  CGCCCCCTTA AGAGGGCCCG CAACACGGCC CGGGCTGCGC ACGCCAGCCG

3151  GGACGGGTGA GTTCGCTAGG CAAGCACGGA CTGGCGGTTA CACGTGCATG

3201  CGTGCCGAGT GAACTCTCCC GCCCCGACGC GCTCCGGCTC CGGGCCTACG

3251  CCGAGCCCCA GCCGCCCGCC ATGTCCCGCC GCCGGGGTCC CCGCCGCCGG

3301  GGTCCCCGGC GCCGGCCGCG CCCCGGCGCT CCAGCCGTGC CGCGCCCGGC

3351  GCTCCAGCCG TGCCGCGCCC CGGCGCGCTC CCAACCGCAG ACTCCCAAAT

3401  GGTCCCTGCG TACGACTCGG GAACCGCGGT CGAGAGCGCG CCGGCCGCGT

3451  CCTCGCTCCT GCGGCGCTGG CTGCTGGTGC CCCAGGCGGA CGACAGCGAC

3501  GACGCGGACT ACGCCGGCAA CGACGACGCA GAGTGGGCGA ACAGCCCCCC

3551  GAGCGAGGGC GGGGGGAAGG CGCCGGAGGC CCCGCACGCC GCGCCTGCCG

3601  CCGCCTGCCC CCCGCCGCCG CCGCGCAAGG ACGCGGGCCG CAGCGCCCCC

3651  TTCCGCCCCA CCTGGCGCTA CGGCTGCGCA CCACGACGGA GTACCTGGCG

3701  CGCCTGAGCT GCGCCGGCGG CGGCCCCCCG CGTCCCCGCC CGGGAACGCG

3751  CCGCGCGGGA AGGTACGCCT CCCCTCCGAC CCCCTGACGC CCCTCCGACC

3801  CCCTGACGCC CCTCCGACCC CCTGACGCCC CTCCGACCCC CTGACGCCCC

3851  TCCGACCCCC GTGTCTCCCC GCCCGCAGTG TGCTTCTCGC CGCGCGTGCA

3901  GGTGCGCCAT CTGGTGGCCT GGGAGACGGC CGCGCGCCTG GCCCGACGGG

3951  GGTCCTGGGC GCGCGAGGGG CCGACCGGGA CCGGTTCCGG CGCCGCGTGG

4001  CGGCGGCCGA GGCGGTCATC GGACCGGTGC CTGGAGCCCG AGGCCCGAGC

4051  TCGGGCCCGA GCCCGGAGCC CGGGCCCACG ACAGACGGCG GACCCGCGGA

4101  GGAGGAGGAG GCGGCGGCGG CGGCGCGCGG GTCCTCCGCC GCCGCGGGCC

4151  CGGGCCGTCG GGCGGTCTAG GGTTGAACCG GCGAGGGCGG CCTCGGCCGG

4201  CGGAGCCCCG GAGCTCCGAA GTCTGCGCGA GGCCGCTCTC CGAAGAGACG

4251  ATGGGAGCCC CGCGTATATA TCCGCGAGGG CCCGGCGCCG CCCCGCCGCT

4301  CCGCCCGCCC CAGGGGGCGG CGCCGGCCAA CCGCGCGCCG CCGCGCGGGC

4351  CCGGACTCCG CCCCGGCGAC CGCCCCGCGC CGGCTTCCCG GTATGGTAAT

4401  TAGAAACTTT TAATAGGCGG TCCCGGCCGC CATCCCCGCG CATGGTAATT

4451  AGCAACTTTT AATGGGCCGG CGTTCCCGCT CGCGGTAATT AGCAGCTTTT

4501  AACGGGCCGC CATTCCCGCT TATGGTAATT AAAAACGTTC GGACGGCCCC
                                      *
4551  TCGCTCCCCG CGTAATTACT CCCTCGGGGT TCCGGGTTAT GCTGATTACT
```

resulting recombinants _in vivo_ would give a better understanding of the precise role of the deleted sequences in neurovirulence.

The variant JH2604 which is avirulent for mice and failed to replicate in mouse brain should be further investigated to determine the stage of blockage in viral replication in neural tissue. A combined immunohisto-chemical and transcriptional analysis of infected neuronal tissue should help in understanding where the blockage in viral replication occurs. In this respect it would be of great interest to determine the _in vivo_ functions of the deleted sequences in latent and acute ganglionic infections and examine the gene expression, if any, in neural cells.

Finally the identification of an ORF in the region deleted in JH2604 which has homology to equivalent sequences in HSV-1 identifies the avirulent phenotype of JH2604 probably being due to a functional protein. In this respect designing a variant in HSV-1 with a similar deletion as that in JH2604 as well as heterologous marker rescue would help in understanding the role of the region between 0-0.02 and 0.81-0.83 m.u. in neurovirulence of both HSV-1 and HSV-2.

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The JH2604 deletion variant of HSV2 (HG52) fails to produce

necrotising encephalitis following intracranial

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