HERPES SIMPLEX VIRUS LATENCY : ANALYSIS OF VIRAL GENES CONTROLLING REACTIVATION

by

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SUMMARY

Ocular infections with herpes simplex virus(HSV) in humans vary in clinical manifestation ranging from self limiting benign epithelial disease to nectrotizing stromal keratitis and uveitis. A more serious clinical problem is the recurrence of ocular lesions due to reactivation of latent virus from dorsal root ganglia. The rabbit eye has provided an efficient experimental model system for study of the mechanisms underlying herpes virus keratitis and the various factors affecting the establishment, maintenance and reactivation of latent HSV. The work described in this thesis stems from the observations of Gerdes and Smith(1983) who showed that in the rabbit eye model, spontaneous shedding of HSV following ocular infection and establishment of a latent infection in the trigeminal ganglia, is virus strain specific and the virus genes controlling reactivation are different from those needed for the establishment and maintenance of HSV latency.

Using the rabbit eye model of latency, HSV-1 strain McKrae invariably reactivates upon induction with epinephrine iontophoresis, whereas HSV-2 strain HG52 fails to reactivate. Both strains establish a latent infection of the dorsal root ganglia(trigeminal ganglia) with the same frequency. The work described in this thesis was aimed at identifying the viral gene(s) involved in the reactivation differential between HSV-1 strain McKrae and HSV-2 strain HG52 and has addressed itself to (i) the construction of intertypic recombinants between McKrae and HG52,(ii) analysis of the genome structure of the recombinant viruses by restriction endonuclease digestion ,(iii) the

reactivation and latency potential of the recombinants compared to the parental viruses following rabbit ocular infection,(iv) the virulence differential of the recombinants compared with the parental viruses following rabbit ocular infection,(v) the ability of the HSV to go latent in the cells of the rabbit cornea and (vi)the analysis of the genome structures of the recombinants in terms of the positions of heterologous inserts in relation to recombination between sequences containig an origin of replication.

In order to map the viral gene(s) controlling reactivation of HSV from latency, recombinants were constructed using intact McKrae DNA and total fragments from HG52 DNA digested with either Xbal or Hpal endonucleases. Eleven recombinants having a range of HG52 DNA inserts were isolated. The structure of the recombinants was deduced from analysis of restriction endonuclease digests with a number of enzymes and the HG52 inserts were mapped between 0.35 and 0.576 map units(m.u.) and/or between 0.82 and 1.00 m.u. of the viral genome. Four of these recombinants with HG52 inserts spanning the above regions were selected to study their reactivation and latency potential in the rabbit eye model. On induction with epinephrine iontophoresis, the 4 recombinants reactivated with the same frequency as the parental McKrae virus indicating that the genes between 0.35 and 0.576 and/or 0.82 to 1.00 m.u. are not solely or in combination involved in determining reactivation differential.

Rabbits could tolerate 3-fold to 40-fold excess doses of recombinant virus relative to the pathogenic McKrae parent indicating moderation in virulence of the McKrae

genome on insertion of HG52 sequence. The reduction in virulence of the McKrae genome was independent of the location of heterologous inserts and indicated a multigenic control of virulence upon ocular inoculation of HSV in rabbits.

During the course of this study virus was isolated from some of the corneal explants from the rabbits latently infected with McKrae and/or recombinant viruses. The delay in shedding of virus from corneal explants was similar to that from trigeminal ganglia indicating that cells of the cornea are capable of harboring a latent as compared to a persistent infection. The low frequency of isolation of HSV from corneas together with the isolation from a minority proportion of the cornea lends support to the findings from the human corneas and suggests that the cornea may be a site additional to the preferred dorsal root ganglia for the establishment of a latent infection.

It was observed that in cotransfection experiments involving intact McKrae genomes and Hpal digested HG52 DNA, only recombinants containing type II inserts from Hpal d(0.35 to 0.576 m.u.) and/or containing an intact type II [S] region(0.82 to 1.00 m.u.) were isolated. Similarly with Xbal cleaved HG52 DNA only recombinants containing type II sequences from the right hand end of Xbal c(0.0 to 0.45 m.u.) were isolated. In effect, the type II insert always contained one or both origins of replication(Ori_L /Ori_S). In the reciprocal experiments isolation of two recombinants from cotransfection of Hpal cleaved McKrae DNA with intact HG52 DNA confirmed this finding; one contained both copies of Ori_S and the intervening short region sequences of McKrae, the other contained approximately 3 Kb of McKrae in

which Ori_L is located. These results indicate that either (a) the presence of an origin of replication in a restriction endonuclease fragment amplifies the fragment thereby increasing its concentration and hence recombination potential with intact genomes; and/or (b) recombination and replication may occur simultaneously. The implications of these findings are discussed. In either case isolation of the recombinants containing Ori_L and Ori_S from the restricted DNA parent strongly suggests that both origins of DNA replication are functional <u>in vitro.</u>

ABBREVIATIONS

ACG	acycloguanosine
APS	ammoniumpersulphate
BHK	baby hamster kidney cells
bp	base pairs
BSA	bovine serum albumin
BUDR	5-bromo-2-deoxyuridine
Ci	Curies
cm	centimetre
cpe	cytopathic effects
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double-stranded
E.coli	Escherichia coli
EDTA	sodium ethylene diamine tetra-acetic acid
g	grams
hr	hour
HFL	human foetal lung
HSV	herpes simplex virus
ICP	infected cell polypeptide
IE	immediate early
IgG	immunoglobulin G
K	kilo
kd	kilodalton
kb	kilobase
M	molar
min	minute
ml	millilitre
mM	millimolar

mm	millimetre
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
m.u.	map units
MW	molecular weight
NP40	nonidet p40
NPT	non-permissive temperature
ori	origin of viral DNA replication
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming units
pi	post infection
PRV	pseudorabies virus
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
sec	seconds
syn	syncytial
$\underline{syn}^+$	non-syncytial
TEMED	N,N,N-N-tetramethylethylenediamine
tk	thymidine kinase
$\underline{ts}$	temperature-sensitive
$\underline{ts}^+$	wild-type for temperature-sensitivity
UV	ultra-violet
v/v	volume per volume
Vmw	molecular weight of virus-induced polypeptide
w/v	weight per volume

wt	wild-type
uCi	microcuries
ug	microgram
ul	microlitre
%	percentage

CHAPTER 1

INTRODUCTION

INTRODUCTION

The aim of the introduction is to provide background information on the general biology of herpes simplex virus (HSV). A brief review of current HSV genetics is provided. Particular reference is made to advances at the molecular level in understanding the viral factors controlling reactivation of HSV from latency and virulence of HSV in experimental animals.

1.1. FAMILY HERPETOVIRIDAE AND ITS CLASSIFICATION

More than 80 distinct members of the family Herpetoviridae have been isolated from hosts as diverse as fish, fungus and man (reviewed by Roizman, 1982). Membership of the family is based upon the presence of four basic morphological characteristics (Roizman and Furlong, 1974). (i) An electron dense core containing a double stranded virus genome of 80 to 150 x 10^6 m w; (ii) An icosahedral capsid consisting of 162 capsomeres of 100 nm diameter; (iii) A largely ill-defined layer of proteinaceous tegument located between the capsid and the envelope; and (iv) A lipid envelope (150 to 200 nm in diameter) enclosing the nucleocapsid. The envelope is usually acquired by budding through the inner nuclear membrane and contains glycoprotein spikes which are virus encoded.

1.1.a.I. Sub-classification on the basis of biological properties

Because of their morphological similarities, herpes viruses, have been classified into 3 sub-families on the basis of their biological properties such as host range, duration of the reproductive cycle, cytopathology and location of the virus genome during a latent infection in vivo (Roizman et al., 1981). The 3 sub-families are:

(i) Alphaherpesvirinae: Members of this sub-family have a variable host range both in vivo and in vitro and a rapid life cycle of less than 24 hr, which results in destruction of the infected cells. The latent infection in vivo is usually established in sensory neurones of the ganglia. Herpes simplex types-I, -II (HSV-1, HSV-2), varicella-zoster virus (VZV) and pseudo-rabies virus (PRV) are included in the alphaherpesvirinae.

(ii) Betaherpesvirinae; Members of this sub-family typified by human and murine cytomegaloviruses (HCMV, MCMV) are characterized by a

relatively long (more than 24 hr) reproductive cycle and a narrow host range frequently restricted to the species or genus to which the host belongs. Enlarged cells (cytomegalia) production is the characteristic pathology of Betaherpesvirinae. Secretory glands and lymphoreticular cells are the main sites of latent infections.

(iii) Gammaherpesvirinae: This group of viruses have a narrow host range in vivo and in vitro and are either B or T cell lymphotropic. Arrest of the virus reproductive cycle either at the prelytic or lytic stage results in persistence or cell death without production of complete virus. Lymphoid tissues are the site of latent infection. The prototype examples of the group include Epstein-Barr virus (EBV) in humans, Marek's disease virus (MDV) and herpes virus of turkeys (HVT).

1.1.a.II. Herpes viruses of humans

At least five of the herpes viruses belonging to 3 different sub-families are known to parasitise man. These include HSV-1, HSV-2, VZV, EBV and HCMV. Besides these, a novel herpesvirus has recently been isolated from immunocompromised patients (Salahuddin et al., 1986), but its genuine role in human pathology awaits confirmation.

Infections with HSV is ubiquitous and is commonly associated with lesions of mucous membranes of oral and genital organs. The vesicular lesions of lips and mouth commonly known as 'cold sores' are frequently caused by HSV-1 whereas HSV-2 is frequently encountered in genital tract infections. Spread of infection usually occurs either by close contact (HSV-1) or by the venereal route (HSV-2). Other important infections caused by HSV include herpes eye disease (see section 1.15), herpes neonatorum and herpes encephalitis. The latter diseases though rare in occurrence are usually life threatening. The clinical manifestations of HSV-1 and HSV-2 have recently been reviewed

by Whitley (1985). Establishment of latency in neurones following primary infection at the peripheral site is one of the characteristic biological properties of herpes viruses (reviewed by Hill, 1985). Recurrent lesions in HSV may occur at the same or at other peripheral sites (neurodermatome). Recurrence may result in sytemic illness, encephalitis or recurrent keratitis in the eye which may lead to blindness. Recurrent infections are usually more severe in immunocompromised individuals.

Primary lesions of varicella or chicken pox caused by VZV are similar but distinguishable from those of HSV and are commonly seen in childhood. Reactivation of VZV from latency in adults results in a more severe form of the disease i,e, 'shingles' or herpes zoster (reviewed by Gleb, 1985).

Infections due to CMV though widespread in the human population are usually sub-clinical. Both primary and recurrent disease due to CMV is a severe problem in immunocompromised hosts; particularly in transplant therapy and in patients with acquired immuno-deficiency syndrome (AIDS) (Hamilton, 1982). CMV has also been implicated in the development of cervical cancer (Alford and Britt, 1984).

EBV causes infections of human B lymphocytes and is a causative agent of infectious mononucleosis. A strong association of EBV with Burkitt's lymphoma and nasopharyngeal carcinoma has been observed (Epstein et al., 1964).

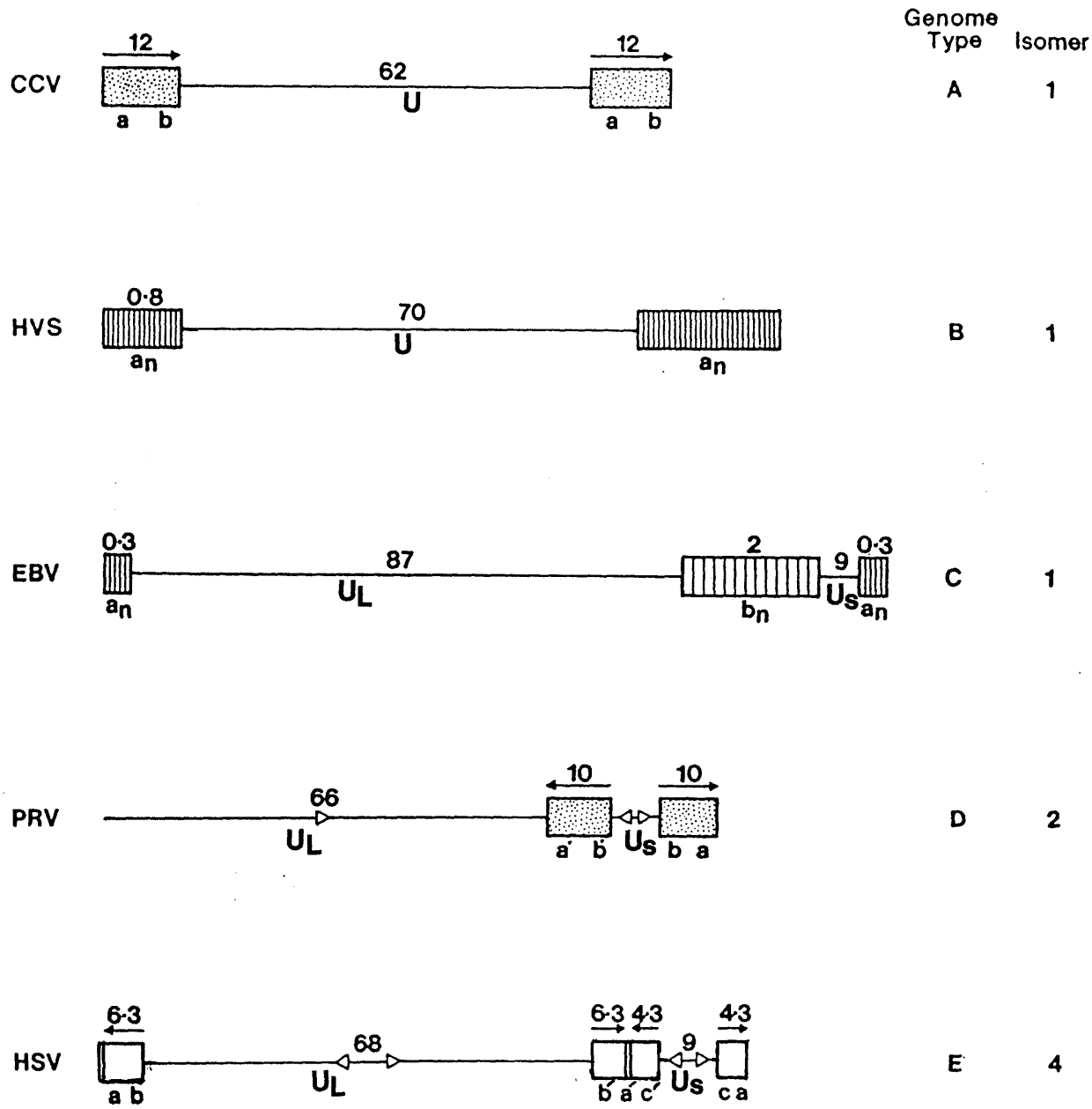
1.1.b. Sub classification of herpetoviridae on the basis of genome structure:

Despite similar biological properties, a wide variation has been observed in the size (80 to 150 x 10^6 mw), base composition (32 to 75% G+C moles%) and arrangement of DNA sequences within the genomes of member viruses of a sub family. Depending upon the arrangement within

FIGURE 1

Schematic diagram of the genome structures of channel catfish virus (CCV), herpes virus saimiri (HVS), Epstein-Barr virus (EBV), pseudoradies virus (PRV) and herpes simplex virus (HSV) representing subgroups A, B, C, D and E respectively of the family Herpetoviridae.

Repeat sequences larger than 1000 bp in length, other than terminal reiterations are shown as dot filled rectangles. ⟶ indicates the orientation of the repeat sequences. Vertical lines and letters a_n and b_n signify multiple tandem repeat sequences. Numbers above the lines represent molecular weight ($x10^6$) while others above the vertical lines refer to unit length of the reiterated sequence.


Genome Type
Isomer
CCV
12
62
12
U
a b
a b
A
1
HVS
0·8
70
U
an
an
B
1
EBV
0·3
87
2
9
0·3
UL
bn
Us
an
C
1
PRV
66
10
10
UL
a' b'
Us
b a
D
2
HSV
6·3
68
6·3
4·3
4·3
9
UL
a b
b' a' c'
Us
c a
E
4

the genome, of reiterated DNA sequences of at least 100 bp, herpes viruses have been sub classified into 5 groups (A to D) (Roizman et al., 1981). Although most of the herpes viruses have been classified according to their biological properties, only a minority have been characterized sufficiently to group them by the criterion of genome structures.

(i) Group A: The genome of this group of viruses exemplified by the channel catfish virus (CCV) (Figure 1) is characterized by the presence of a single set of direct reiterated sequences at the termini (Chousterman et al., 1979) and hence only one isomer.

(ii) Group B: The genome of viruses in this group contains multiple copies of the same set of sequences at both termini in the same orientation e.g. herpesvirus saimiri (Bornkamm et al., 1976).

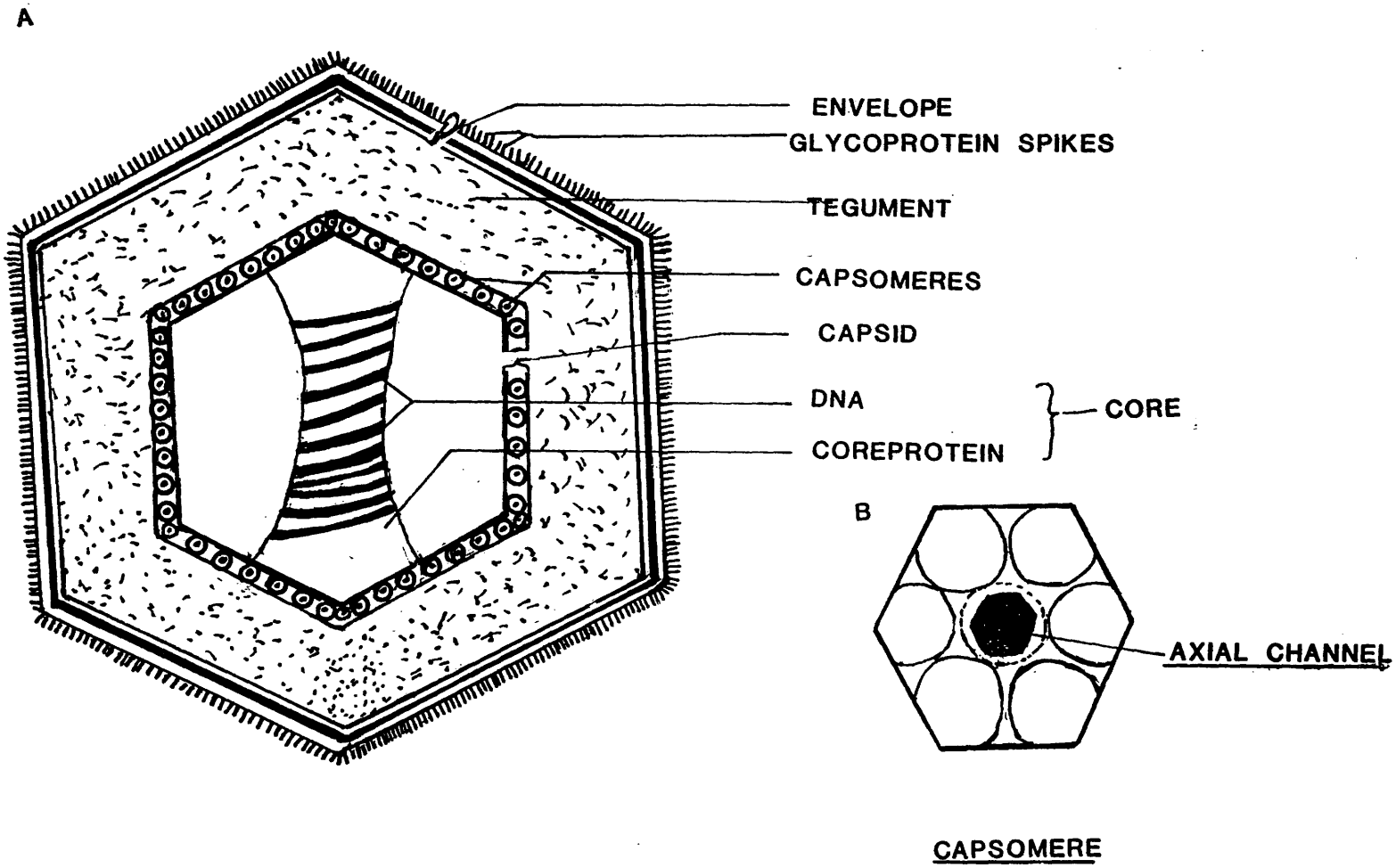
(iii) Group C: As exemplified by Epstein Barr Virus, the genome of this group of viruses is characterized by the presence of multiple reiterations of the same set of sequences at both termini in the same orientation and a variable number of tandem repeats of a different sequence internally. There is no isomerization of the genome (Raab-Traub et al., 1980).

(iv) Group D: The genome in this group of viruses e.g. PRV (Ben-Porat et al., 1979) occurs as two isomers due to internal reiteration in an inverted form of a single set of sequences from the terminus. The genome consists of two regions L and S. The unique sequences of S (U_S) are bracketed by inverted repeat sequences (IR_S and TR_S). The S component inverts relative to the L component which consists of a unique sequence in a fixed orientation thus giving rise to two isomeric forms.

FIGURE 2

A. Schematic diagram of a HSV virion. The virion is composed of four main structural elements - the core, capsid, tegument and envelope. Within the core, viral DNA 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.

B. Six-fold symmetry of the hexameric capsomeres. The hexamers are believed to have this symmetry, probably due to the presence of six molecules of the major capsid protein, Vmw 155.


A
ENVELOPE
GLYCOPROTEIN SPIKES
TEGUMENT
CAPSOMERES
CAPSID
DNA
COREPROTEIN
CORE
B
AXIAL CHANNEL
CAPSOMERE

DNA sequence analysis of the L-S joint and genome termini of VZV revealed that UL is flanked by an inverted repeat of 88.5 bp and hybridization studies indicated that 5% of VZV DNA molecules have an inverted L component (Davison, 1984). Thus VZV could be regarded as a member of group E. However the size of IR_L/TR_L is less than the minimum 100 bp considered as a prerequisite for classification purposes (Roizman, 1982).

(v) Group E: The genomes of viruses in this group have a single set of sequences from both termini reiterated in an inverted form internally. Thus the genome has two unique regions i.e. U_L and U_S each bracketed by a single set of inverted repeats. The inversion of both segments relative to each other produces four isomers in equal proportion. This group includes HSV-1, HSV-2, MDV and Bovine herpesvirus type 2 (BHV-2).

1.2. MORPHOLOGY OF HERPES SIMPLEX VIRIONS

Herpes simplex virions have four distinct morphological elements; (i) an electron-opaque core, (ii) an icosahedral capsid enclosing the core, (iii) an electron-dense amorphous material - the tegument surrounding the capsid and (iv) an envelope or lipoprotein membrane whose outer surface exhibits small spikes (Spear and Roizman, 1980; Dargan, 1986) (Figure 2).

The HSV core consists of viral DNA tightly spooled in the form of a toroid around a central cylindrical mass with a regular 4 to 5 nm spacing (Furlong et al., 1972). Absence of the virion polypeptide VP21 in empty nucleocapsids led Gibson and Roizman (1972) to suggest that the core is composed of the polypeptide VP21 (Vmw43). The cylindrical core structure appears to be connected to the inner poles of the capsid and may provide a mechanical support for the winding of the viral DNA (Nazerian, 1974).

An icosahedral capsid of approximately 100 nm in diameter and consisting of 150 hexameric and 12 pentameric capsomeres encloses the core (Wildy et al., 1960). Capsomeres are hollow, elongated polygonal prisms with tapering axial channels (Wildy et al., 1960; Steven et al., 1986). On electronmicroscopic examination of partially disrupted capsids, hexameres appear to be linked by intercapsomeric fibrils (Vernon et al., 1974; Palmer et al., 1975). The hexameres are believed to be composed of 6 molecules of the major capsid protein, Vmw 155 or p155 (Spear and Roizman, 1972; Marsden et al., 1976; Vernon et al., 1981; Steven et al., 1986), whereas pentamers are composed of the virion protein p50 (VP19, NC2) (Vernon et al., 1981). Polypeptides p50 and p155 have been shown to form a disulphide-linked complex in HSV-2 nucleocapsids (Zweig et al., 1979) . Studies with virus mutants containing a ts lesion within the gene for p40 also called VP22 (a member of the ICP35 family which also includes VP21) (Cohen et al., 1980; Braun et al., 1984a) has revealed that processing of this polypeptide is essential for encapsidation of viral DNA (Preston et al., 1983). The exact location of p40 in capsids is yet unresolved.

The tegument is an ill-defined layer located between the capsid and the envelope (Roizman and Furlong, 1974). In thin sections of budding particles or virions, the tegument has no distinctive structural features whereas in negatively stained virions it appears to have a fibrous structure (Wildy et al., 1960; Morgan et al., 1968; Schwartz and Roizman, 1969). Its thickness is genetically determined and varies considerably between different herpes viruses (McCoombs et al., 1971).The tegument is believed to play a role in envelopment of the virus (Vernon et al., 1982). The polypeptides constituting the tegument are of high molecular weight (more than 200,000 mw) and include VP1, VP2, VP3 and the virus transducing factor, Vmw 65

(Batterson and Roizman, 1983; Campbell et al., 1984).

A polypeptide of 10,000 mw the product of the US9 gene (McGeoch et al., 1985) has been located around the perimeters of capsids and can be precipitated from NP_{40} extracts of HSV-1 virions using an oligopeptide antiserum (Frame et al., 1986). The precise function of this polypeptide in the virus life cycle is unclear.

The herpes virus envelope, enclosing nucleocapsids containing full length genomes, is acquired by budding through the inner nuclear membrane (Darlington and Moss, 1968). It consists of a trilaminar membrane and has spikes of about 8 mm in length projecting from its surface (Wildy et al., 1960). It contains several viral glycoproteins which exhibit a number of important biological functions (see section 1.10.C)

1.3. GENOME STRUCTURE OF HERPES SIMPLEX VIRUS

1.3.a. General properties of the HSV genome

The genome of herpes simplex virus is a linear double stranded DNA molecule of 100 x 10^6 daltons about 152,260 base pairs (bp) (Becker et at; 1968; Kieff et al., 1971). A striking feature of the genome is its very high guanosine plus cytosine (G+C) content, 68-72% overall for HSV-1 (Kieff et al., 1971) and 69% overall for HSV-2 (Goodheart et al., 1968). These values differ widely from that of BHK C13 DNA which has a G+C content of 42% (Subak-Sharpe, , 1969). DNA sequence analysis has revealed that different parts of the HSV genome vary in their G+C contents. In particular the 6600 bp in length short repeats of HSV have a G+C content of 79.5%. Within this region, the gene coding for the transcriptional regulatory protein Vmw 175 has a base composition of 81.5% G+C (reviewed by McGeoch, 1987).

More than 50% of herpesvirus DNA is found to fragment upon denaturation with alkali even after careful extraction from virions

(Kieff et al., 1971; Frenkel and Roizman, 1972; Wilkie, 1973). This is believed to be due to the presence of ribonucleotides and/or single strand nicks or gaps (Hirsch and Vonka, 1974; Biswal et al., 1974). The single strand nicks are randomly distributed on both DNA strands (Wilkie, 1973). Though the presence of ribonucleotides in HSV DNA has been shown (Muller et al., 1979), their role in DNA fragmentation is not conclusive (Roizman, 1979).

The HSV genome is composed of two covalently linked segments, designated as Long (L) and Short (S). Each segment contains unique sequences (U_L and U_S) of 110,000 and 13,000 bp respectively each flanked by a pair of repeat sequences in opposite orientation (Sheldrick and Berthelot, 1974; Wadsworth et al., 1975, Figure 3). The terminal repeats (TR_L/IR_L) flanking the U_L segments are designated ab and b'a' (9200 bp each) while those (IR_S/TR_S) flanking U_S are designated a'c' and ca respectively (6600 bp each). Besides these a direct repeat sequence, called 'a' sequence, which varies in size from 200 to 550 bp among HSV strains (Davison and Wilkie, 1981 also see section - 1.4), is present at the genomic termini and in an inverted orientation at the L-S junction (Sheldrick and Berthelot, 1974; Grafstrom et al., 1974, 1975; Wadsworth et al., 1976; Davison and Wilkie, 1981). One to several copies of the 'a' sequence may be present at the L terminus and L-S junction, but only one copy is generally present at the S terminus (Wagner and Summers, 1978; Locker and Frenkel, 1979).

As a consequence of this genome structure, inversion of the L and S segments between the inverted copies of the 'a' sequence generates four isomeric forms of viral DNA in equimolar amounts (Sheldrick and Berthelot, 1974; Hayward et al., 1975; Clements et al., 1979; Delius and Clements, 1976; Skare and Summers, 1977). As depicted in Figure 3, these isomers are designated as protoype (P); inversion of the S

FIGURE 3

A. Genome arrangement of HSV-1. The long region (L) is composed of a unique region (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). Terminally redundant sequences are designated a (a'). The remaining sequences within TR_L/IR_L and TR_S/IR_S are designated b/b' and c/c' respectively.

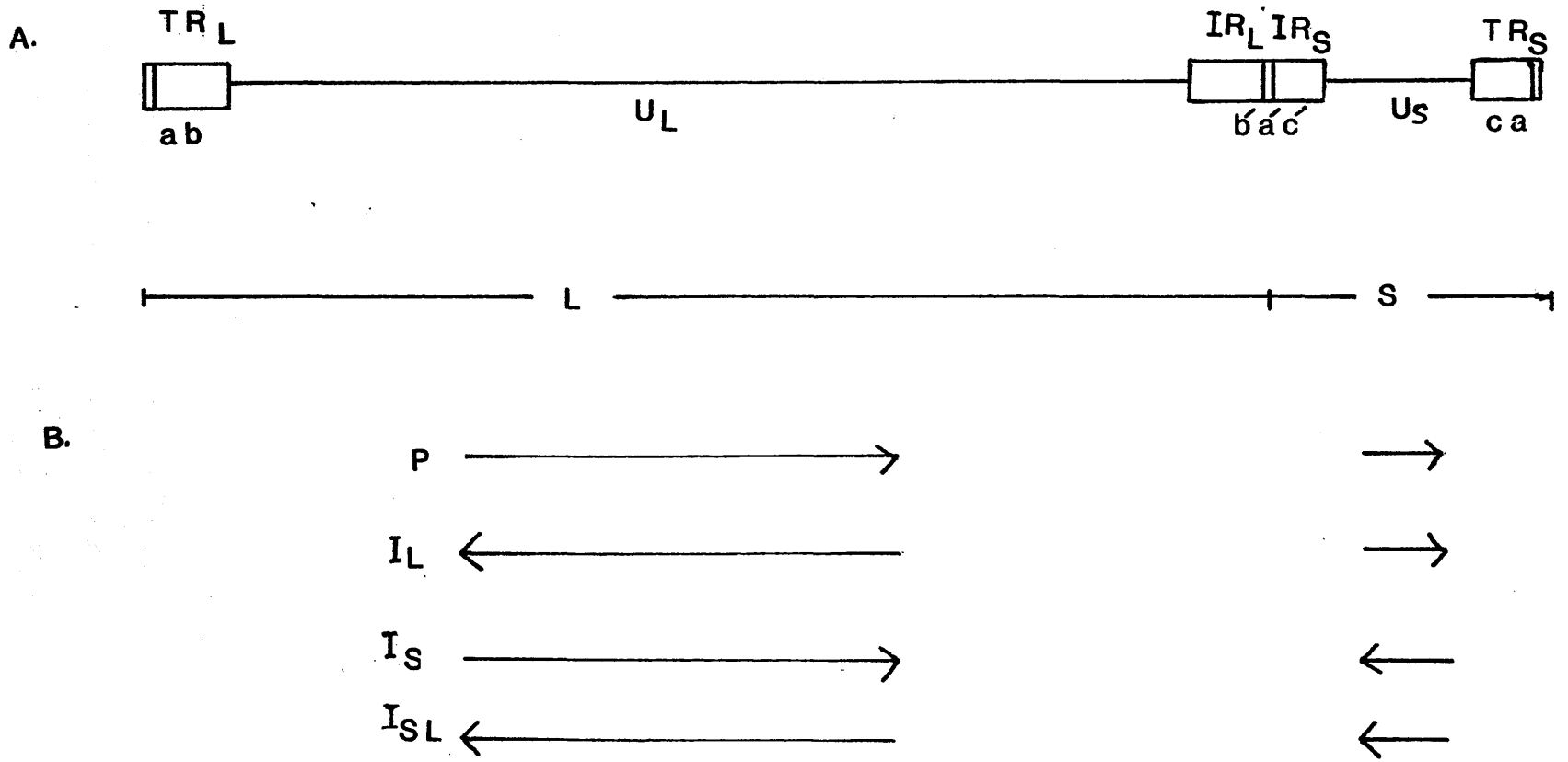
B. The four genome isomers:

P = proto-type orientation

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.
TR L
IR L
IR S
TR S
a b
U L
b́ á ć
Us
c a
L
S
B.
P
I L
I S
I SL

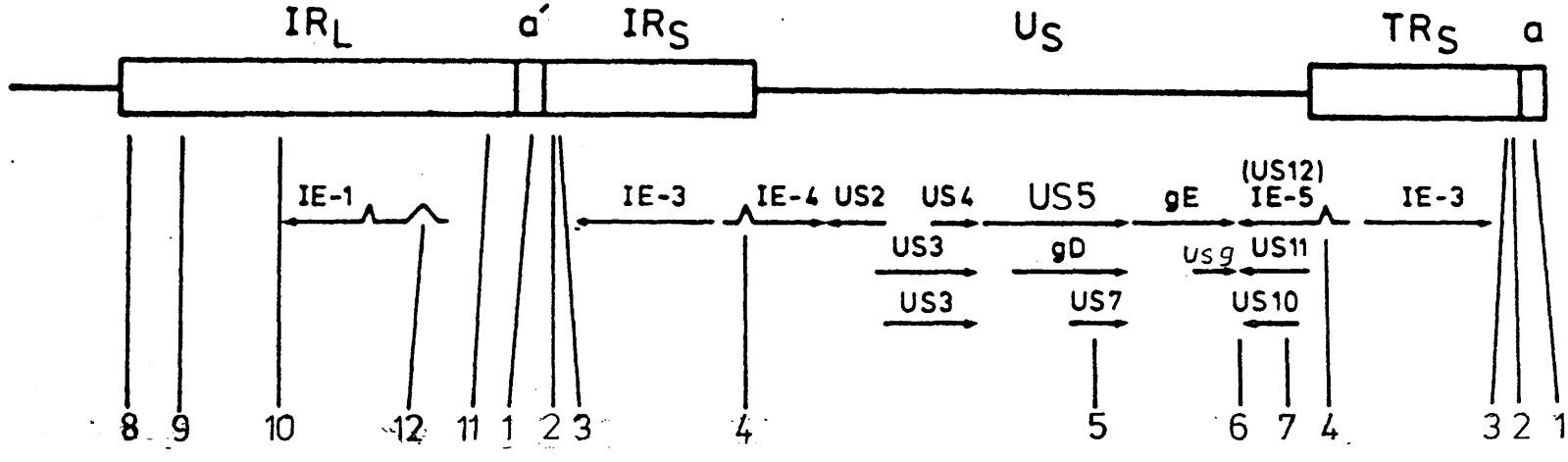
region (I_S), inversion of the L region (I_L), inversion of both S and L regions (I_{SL}) ,(Roizman, 1979; Hayward et al., 1975). Inversion of the genome is due to site specific recombination between the repeated sequence arrays (DR_2 and DR_4) within the 'a' sequence (see section 1.4.a). All four isomeric forms replicate to produce viable progeny (Davison and Wilkie, 1983b; Longnecker and Roizman, 1986). Isolation of viable virus mutants deleted in IR_S the internal 'a' sequence and most of the IR_L genes (Longnecker and Roizman, 1986; Jenkins and Roizman, 1986) indicates that besides the 'a' sequence, the repeat sequences flanking U_L and U_S also play a role in genome inversions. The S segment in these mutants is frozen in the I_S orientation while the L segment could invert at a frequency lower than that of the wild type.

The ability of the HSV genome to invert is not an essential prerequisite for virus growth as non-inverting viable populations of HSV have been isolated (Preston et al., 1978; Davison and Wilkie, 1981; Poffenberger et al., 1983; Poffenberger and Roizman, 1985; Jenkins and Roizman, 1986).

Sequence analysis of the HSV-1 genome has revealed the presence of sets of short, tandemly repeated sequences of variable length (McGeoch, 1984). Repeats of 12 to 54 bp in length have been reported in the non-coding sequences of HSV-1 (Murchie and McGeoch, 1982; Davison and Wilkie, 1981; Rixon et al., 1984; Perry et al., 1986; Chou and Roizman, 1986).The copy number of these high (G+C) content repeats varies between virus stocks and within cloned DNA fragments of the same stock. The locations of the short tandemly reiterated sequences occurring in the HSV-1 strain 17 genome are indicated in Figure-4. Three of these repeats are located in the U_S sequences i.e.(i) within the coding region of the US7 gene; (ii) in the intergenic region of

FIGURE 4

Locations of short tandemly reiterated sequences within the S segment and R_L regions of HSV-1 strain 17^+ DNA. Positions of reiterated sequences (numbered 1 to 12) are shown relative to the locations of mapped mRNA. (⌒) refers to the locations of spliced regions in immediate early (IE) gene -1, -4 and -5. Adapted from Watson et al., 1981; Rixon et al., 1984; Rixon and McGeoch, 1984, 1985; Perry et al., 1986.


IR$_L$
a'
IR$_S$
U$_S$
TR$_S$
a
IE-1
IE-3
IE-4
US2
US4
US5
gE
(US12)
IE-5
IE-3
US3
gD
usg
US11
US3
US7
US10
8
9
10
12
11
1
2
3
4
5
6
7
4
3
2
1

US 9 and US 10 and (iii) within the overlapping coding regions of genes US 10 and US 11 (McGeoch et al., 1985). The other repeats present in the S region are (i) in the introns of IE mRNA 4 and 5 (Murchie and McGeoch, 1982) and (ii) downstream of IE mRNA 3 (Rixon et al., 1984). Besides these, occurrence of repeats has also been reported in the 'a' sequences (Davison and Wilkie, 1981; Mocarski and Roizman, 1981) and downstream of mRNA of the IE 1 (Perry et al., 1986).

1.3.b. Organisation of HSV genes

The nucleotide sequence of the whole of the HSV-1 strain 17 syn^+ genome has now been determined (D.J. McGeoch, personal communication). The sequence of the whole of the short region and some parts of the long region of the genome has been published (Murchie and McGeoch, 1982; Rixon and Clements, 1982; Rixon and McGeoch, 1984; McGeoch et al., 1985; Perry et al., 1986). On the basis of sequence analysis HSV-1 strain 17 has been calculated to contain 72 genes (D.J. McGeoch, personal communication). The orientation of open reading frames of 56 genes termed UL 1 to UL 56 located in U_L and 12 genes termed US 1 to US 12 located in U_S along with the genes located in the repeats is shown in Figure 5 and Figure 6.

DNA sequence analysis of the HSV-1 genome has revealed: (1) There is no clustering of genes which are found in both orientations. (2) Each gene has its own promoter (McGeoch et al., 1985; Wagner, 1985). (3) There is frequent occurrence of 3' co-terminal families of mRNAs (Wagner, 1985). In this arrangement several adjacent genes are similarly aligned. For each gene transcription starts 5' to that gene's coding sequences and continues through the following genes of the family until a common distal polyadenylation site is reached. In these transcript species only the 5'-proximal coding region is

FIGURE 5

Open Reading Frames in HSV-1 (i).

Open reading frames in the left hand half 75 Kbp of HSV-1 strain 17 (D.J. McGeoch - personal communication). The box is repeated sequences. 'a' refers to the 'a' sequences. Genes in U_L are numbered UL1 to UL36. 15/1 and 15/2 refer to the two exons of gene UL15. Genes whose polypeptide product is known are labelled. Challberg indicates genes whose products are as yet unknown, but are necessary for plasmid based DNA replication. Location of Ori_L is marked. Dotted lines indicate that the open reading frame is continued to the following line.

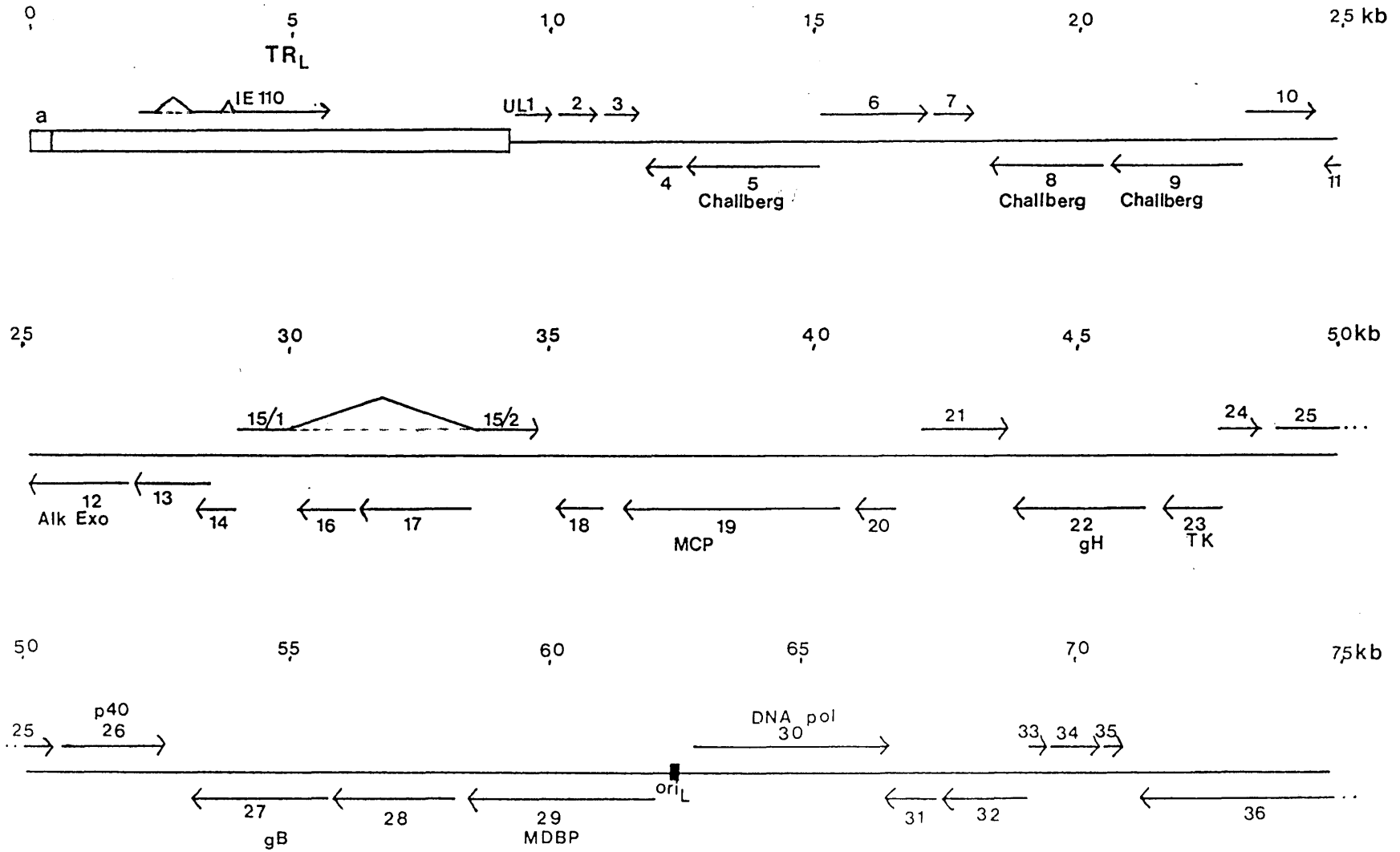
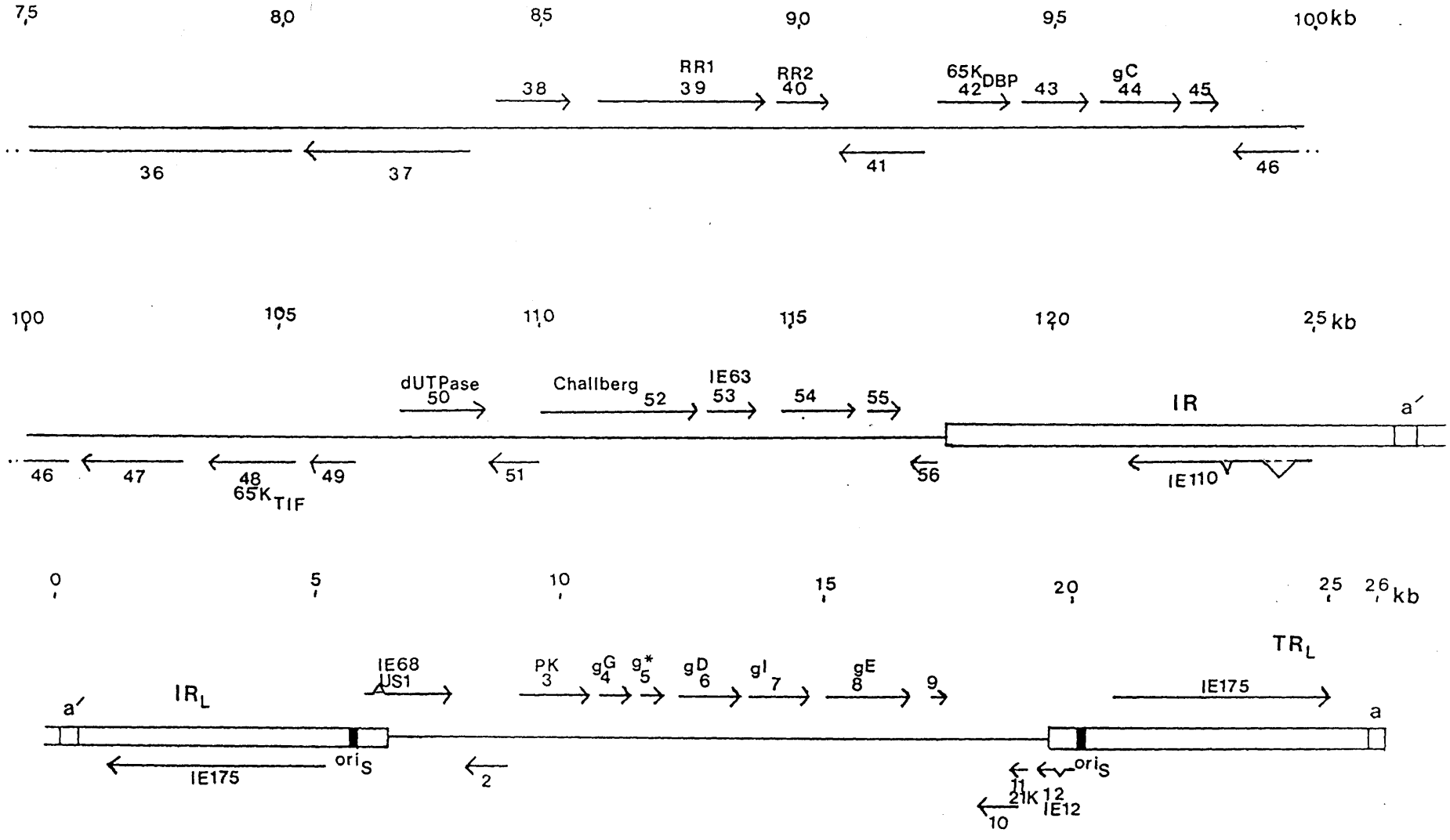

0
5
1,0
1,5
2,0
2,5 kb
TR$_L$
a
IE 110
UL1
2
3
6
7
10
4
5
Challberg
8
Challberg
9
Challberg
11
2,5
3,0
3,5
4,0
4,5
5,0kb
15/1
15/2
21
24
25
12
Alk Exo
13
14
16
17
18
19
MCP
20
22
gH
23
TK
5,0
5,5
6,0
6,5
7,0
7,5kb
25
p40
26
DNA pol
30
33
34
35
ori$_L$
27
gB
28
29
MDBP
31
32
36

FIGURE 6

Open Reading Frames in HSV-1 (2).

Open reading frames in the right hand half of HSV-1 strain 17 (D.J. McGeoch, personal communication). Genes in U_L are numbered UL36 to UL56 and in U_S are numbered US1 to US12. Locations of Ori_S sequences are marked. RR1 and RR2 are the large and small subunits of ribonucleotide reductase. $65K_{DBP}$ is the 65K DNA binding protein, which is distinct from the $65K_{TIF}$ (the tegument transactivator polypeptide). US5 is numbered g^* to indicate that it encodes a potential glycoprotein which has not yet been identified. Challberg refers to the genes, whose products are as yet unknown, but are necessary for plasmid based DNA replication.


7,5
8,0
8,5
9,0
9,5
10,0kb
RR1
39
RR2
40
65K
42
DBP
43
gC
44
45
38
36
37
41
46
10,0
10,5
11,0
11,5
12,0
12,5kb
dUTPase
50
Challberg
52
IE63
53
54
55
IR
a´
46
47
48
65K TIF
49
51
56
IE110
0
5
10
15
20
25
26kb
TRL
IE68
US1
PK
3
gG
4
g*
5
gD
6
gI
7
gE
8
9
IE175
a
IRL
a´
IE175
oriS
2
oriS
11
21K
12
IE12
10

translated (Rixon and McGeoch, 1984, 1985). (4) Introns are rare and have only been detected in immediate early (IE)- 1, -4, -5 and UL 15 gene transcripts (Rixon and Clements, 1982; Murchie and McGeoch, 1982; Costa et al., 1985a; Perry et al., 1986).

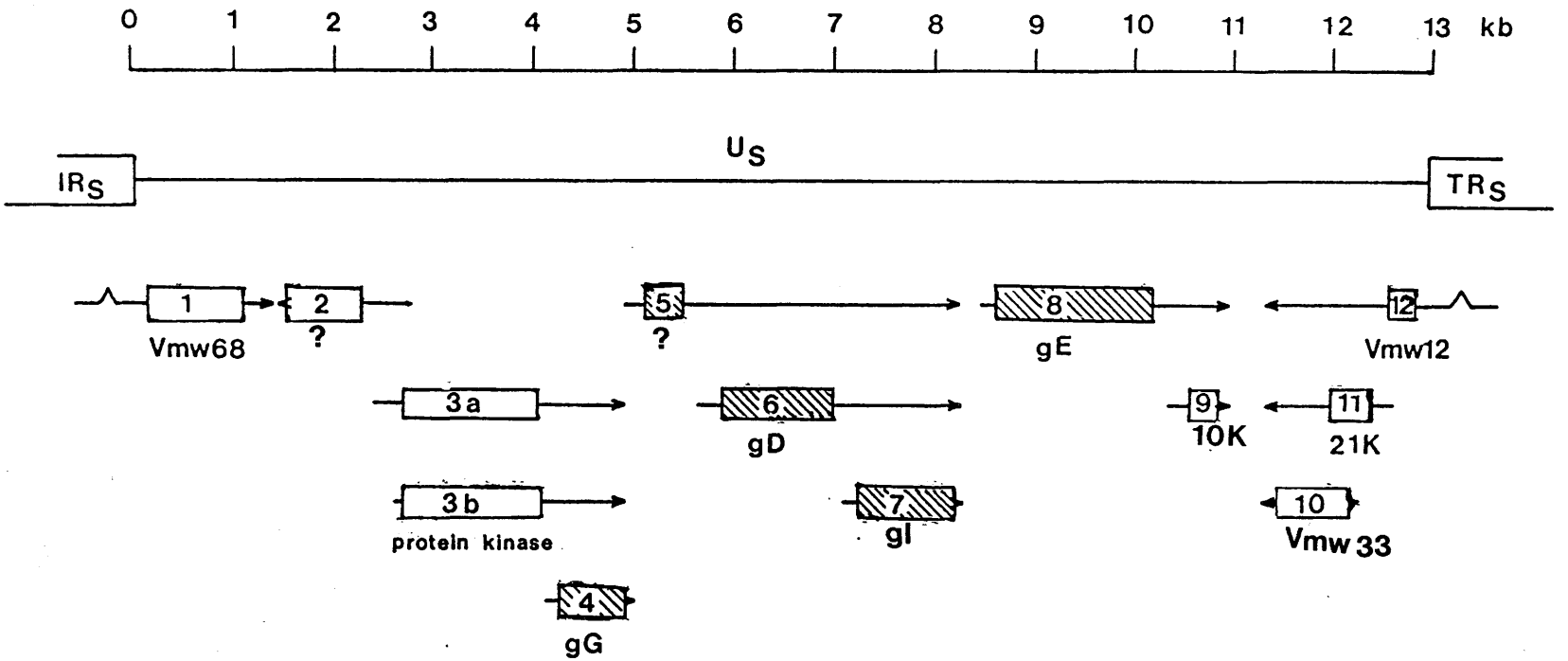
The immediate early genes are all located in or close to the repeat elements. the inverted repeats flanking U_L i.e. ab and b'a' contain one copy each of the IE-1 gene while those of U_S i.e. a'c' and c a, each contain a copy of the IE-3 gene (Watson et al., 1979; Mackem and Roizman, 1980). In HSV-1 strain F, the repeats flanking U_L are reported to contain one copy each of a gene encoding the ICP 34.5 protein (Chou and Roizman, 1986). TR_S/IR_S contain one copy each of the origin of virus DNA replication termed Ori_S (Stow, 1982; Stow and McMonagle, 1983) while ori_L is located in the centre of U_L (McGeoch, 1987). Ori_L is located between the divergently transcribed genes for the major DNA binding protein (DBP) and the DNA polymerase (Quinn and McGeoch, 1985; Weller et al., 1985) analogous to Ori_S which is located intergenically in the region of the divergently transcribed IE genes 3 and 4/5.

The U_S region of the genome as stated earlier contains 12 open reading frames termed US 1 to US 12 (Figure-7). A group of 5 consecutive genes within U_S (US 4 to US 8) encode known or potential membrane proteins. The functional significance of this clustering is uncertain. Mapping of the mRNA transcripts in the short region of HSV-1 (Rixon and McGeoch, 1984, 1985; McGeoch et al., 1985) has revealed the compact organisation of the HSV genome and clearly demonstrates that 3' co-terminal families of mRNA occur relatively frequently in HSV (Wagner, 1985). Sequence comparison of a portion of U_S of HSV-2 (HG52) has established the colinearity of the genes between the two strains (McGeoch et al., 1987).

HSV-1 mutants deleted in genes US 1 to US 5 and US 7 to US 12

FIGURE 7

Transcript map of the short unique region of the HSV-1 strain 17 genome adapted from McGeoch *et al* (1985). The U_S region, approximately 13,000 bp, contains 12 genes (US1 to 12) which are expressed as 13 mRNAs. The probable polypeptide coding (boxes) and non-coding (single lines) portions of mRNAs are indicated, and transcription is in the direction of the arrows. ▨ indicates membrane associated glycoproteins.


0 1 2 3 4 5 6 7 8 9 10 11 12 13 kb
IRS
US
TRS
1
Vmw68
2
?
5
?
8
gE
12
Vmw12
3a
6
gD
9
10K
11
21K
3b
protein kinase
7
gI
10
Vmw33
4
gG

(Post and Roizman, 1981; Umene, 1986; Longnecker and Roizman, 1986; Longnecker and Roizman, 1987; Weber et al., 1987), IE-3 and one copy of sequences encoding Ori_S in HSV-1 (Longnecker and Roizman, 1986) and HSV-2 (Brown and Harland, 1987) have been shown to grow normally in culture, indicating that these genes are dispensable for the lytic growth of virus. The functional significance of clustering of genes dispensable for lytic growth in cell culture in the [S] component of the HSV genome remains unknown.

1.4. THE HERPES SIMPLEX VIRUS 'a' SEQUENCE

1.4.a. General properties of the 'a' sequence

The HSV genome contains a 200 to 500 bp sequence termed the 'a' sequence which is present in the same orientation at the termini but in an inverted orientation at the L-S junction (Wadsworth et al. 1975, 1976). One to ten copies of this sequence may be present at the L-terminus and L-S junction but only one copy is invariably present at the S-terminus (Wagner and Summers, 1978; Locker and Frenkel, 1979 ; Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982b).

The size of the 'a' sequence has been shown to vary between strains as revealed by DNA sequencing of the 'a' sequence from several HSV-1 and HSV-2 strains (Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982b; Mocarski et al., 1985; Varmuza and Smiley, 1985). As shown in Figure-8, the HSV- 'a' sequence can be divided into several structural regions (Mocarski and Roizman, 1982a). These regions include unique (U) and directly repeated (DR) elements. Variation in copy number of the DR elements is responsible for differences in size between 'a' sequences from different strains. The 'a' sequence in HSV-1 strain F can be represented as DR_1-U_b-$(DR_2)_{19}$-$(DR_4)_3$-U_c-DR_1 (Mocarski and Roizman, 1982a). DR_1 is a 20 bp sequence present at both ends, U_b and U_c are unique 58 bp and 65 bp sequences

FIGURE 8

Structure of the HSV-1 'a' sequence.

A proto-type virus genome with, below, an expansion showing the structure of the 'a' sequence in the orientation found at the L-S junction.

U_b : a unique sequence located toward the b' sequence

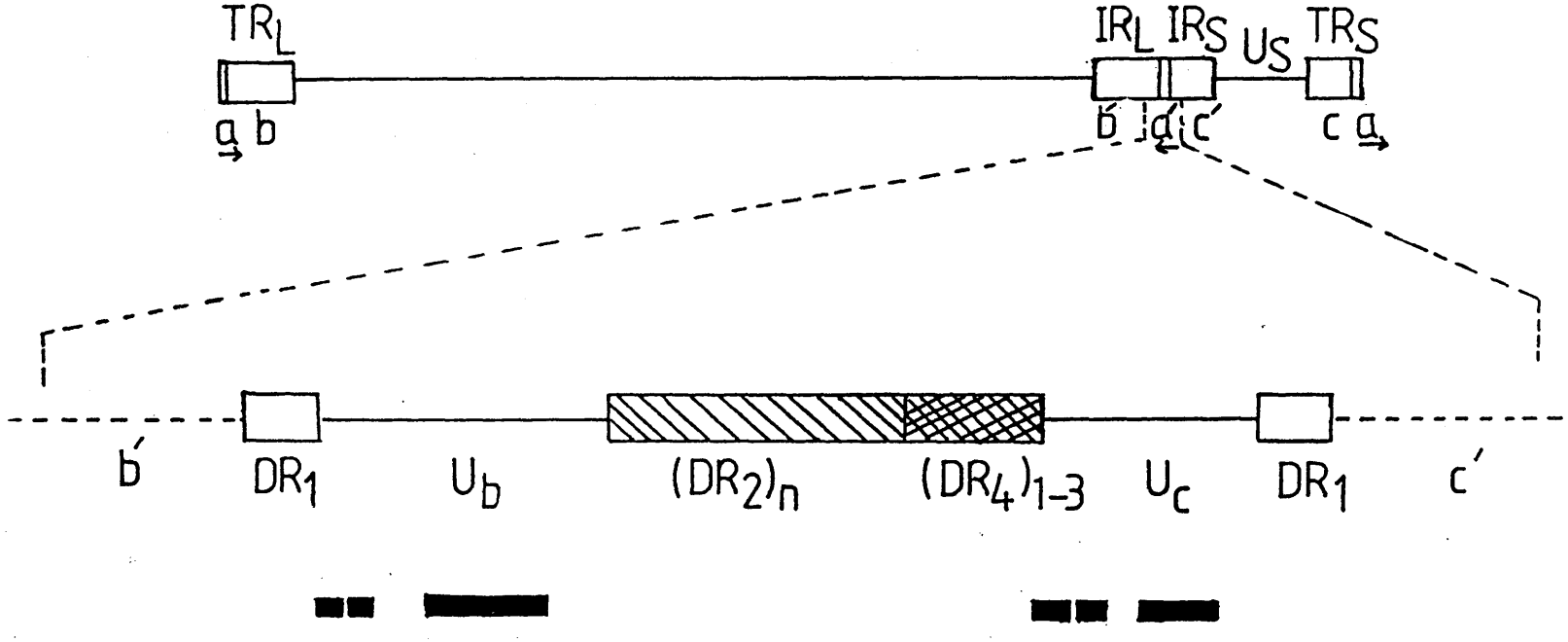
U_c : a unique sequence locted toward the c' sequence

DR_1 : a 17-21 bp element, present as a direct repeat at the ends of the 'a' sequence

DR_2 : a 12 bp repeat element, present in 1 to at least 22 copies

DR_4 : a 37 bp repeat element, present in 1 to 3 copies

▬ : represents regions of unique DNA sequence highly conserved between different strains of HSV.


TRL
IRL
IRS
US
TRS
a b
b́ á ć
c a
b́
DR1
Ub
(DR2)n
(DR4)1-3
Uc
DR1
ć

respectively, and are named by virtue of their proximity to the 'b' and 'c' repeated regions respectively; DR_2 is a 12 bp sequence present in 19-22 tandem copies; and DR_4 is a 37 bp sequence in 3 tandem copies.

The copy number of the DR_2 repeat element has been shown to vary both between and within strains. For example, the HG52 strain of HSV-2 has only one copy of DR_2 (Davison and Wilkie, 1981), while HSV-1 strain F has 19-22 copies (Mocarski and Roizman, 1981). Similar to DR_2, variations in copy number of DR_4 has also been observed. Many strains including HSV-1 strain 17 and HSV-2 strain HG52, contain only one copy of DR_4 which is regarded as part of U_c (Davison and Wilkie, 1981). HSV-2 strain HG52 could therefore be regarded as containing a single unique sequence bounded by a direct repeat (DR_1). Some strains of HSV-1 like strain Justin contain an extra direct repeat, called DR_3 occuring in two tandem copies (Mocarski and Roizman, 1981, 1982a; Mocarski et al., 1985). Despite variations in the number of copies of the DR_2 and DR_4 elements, some areas of the 'a' sequence are highly conserved in different strains. Within U_b and U_c there are short, well-conserved sequences of about 20 bp located approximately 40 bp and 35 bp from the ends of 'a' sequences respectively (Davison and Wilkie, 1981; Deiss et al., 1986). These homologous areas in U_b and U_c are shared by the 'a' sequence in CMV (Spaete and Mocarski, 1985) and the sequences found at the termini of other herpes viruses (Deiss et al., 1986).

Tandem reiterations of the 'a' sequence share the intervening DR_1 element. The S and L terminal 'a' sequences differ from the junctional 'a' sequence in that they contain an incomplete copy of the terminal DR_1. For example in HSV-1 strain 17, the L-terminus 'a' sequence contains 20.5 bp of the 21 bp DR_1 element with only 0.5 bp at the S-

terminus 'a' sequence (Davison and Rixon, 1985). Together the two partial sequences form a complete DR_1.

1.4.b. Functions of the 'a' sequence

The 'a' sequence has been implicated in a number of processes associated with genome organisation and viral DNA replication. Some of these functions of the 'a' sequence are described in the following sections.

(i) Circularization of the genome

Following infection the rapid circularization of the HSV genome is believed to be mediated by the 'a' sequence (Davison and Wilkie, 1983a; Poffenberger et al. 1983). The circularization of the genome is believed to occur by ligation of the two termini aided by the complementary single base 3' overhang (Mocarski and Roizman, 1982a,b; Davison and Rixon, 1985) as HSV genomes containing heterotypic 'a' sequences have been shown to circularize following infection (Davison and Wilkie, 1983a). Further circularization of both PRV and VZV genomes lacking terminal redundancy, following infection indicates that circularization occurs by ligation of the genomic termini (Davison, 1984; Wu et al., 1979).

(ii) Isomerization of the genome

As mentioned earlier in section 1.3a, a HSV DNA population consists of 4 equimolar isomers differing in the relative orientation of U_L and U_S (Hayward et al. 1975; Sheldrick and Berthelot, 1974; Delius and Clements, 1976). The inversion of the genome about 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 evidence that the 'a' sequence plays a part in inversion and isomerization of the genome has come from Chou and

Roizman (1985) who carried out a detailed deletion analysis of the 'a' sequence and observed that inversion of the genome was impaired on deletion of DR_4 sequences while it was abolished by deletion of DR_2 and DR_4 sequences; thus demonstrating the presence of *cis* acting signals for site specific recombination and inversion in the DR_2 and DR_4 elements. The constructs of Chou and Roizman, however, do not exclude a role for DR_1 in inversion since an extremely low frequency of inversion has been observed on deletion of both DR_1 elements (Varmuza and Smiley, 1985).

(iii) *Cleavage and packaging of the genome*

According to the rolling circle model of HSV replication, unit length genomes are generated from cleavage of head-to-tail concatamers and are subsequently packaged and encapsidated in the nuclei of infected cells (Ben-Porat *et al.*, 1976; Jacob *et al.*, 1979; Ben-Porat and Rixon, 1979). The *cis*-acting signals for cleavage/packaging of the viral genome have been shown to be present within the HSV 'a' sequence (Stow *et al.*, 1983; Deiss and Frenkel, 1986), in that plasmid constructs containing an HSV origin of DNA replication can be replicated but are only packaged if they contain an 'a' sequence. Varmuza and Smiley, (1985) and Deiss *et al* (1986) have localized the sequences involved in cleavage/packaging to homologous regions in U_b and U_c termed as pac-1 and pac-2 respectively (Deiss *et al.*, 1986). The cleavage of DNA appears to occur in a sequence independent manner at a set distance from the U_b and U_c cleavage/packaging signals. Deiss *et al* (1986) found that amplicon constructs lacking U_c did not result in the amplification of the input amplicons, whereas those lacking U_b were amplified and propagated at a low frequency only if a wild-type 'a' sequence from the helper virus was present. Based on these observations several models for amplification of the HSV 'a' sequence

and cleavage/packaging of DNA have been proposed. Some of these have been outlined in section (1.9).

(iv) Promoter activity

The U_b/DR_2 region of the 'a' sequence in HSV-1 strain F has been reported to contain the promoter of a gene encoding the polypeptide, ICP 34.5, whose coding region is in the long repeat (Ackermann et al., 1986; Chou and Roizman, 1986) The 5' terminus of the transcript maps to DR_1 and to the long repeat outside the 'a' sequence. The promoter of the gene encoding ICP 34.5 is atypical of the HSV genes in that there is no 'TATA' consensus in the normal position (i.e. around -25) and the best TATA homology (TTTAAA) lies at -15. Interestingly in HSV-1 strain 17, DNA sequence analysis of the corresponding region of the genome has not revealed any open reading frame coding for ICP 34.5 (D.J.McGeoch, personal communication).

1.5. RELATEDNESS OF HERPES SIMPLEX VIRUS TYPE I AND TYPE II GENOMES

The DNAs of HSV-1 and HSV-2 are approximately 50% homologous as revealed by hybridization studies (Kieff et al., 1972). Genetic studies have shown that recombination (Timbury and Subak-Sharpe, 1973; Halliburton et al., 1977; Morse et al. 1977; Preston et al. 1978) and complementation (Timbury and Subak-Sharpe, 1973; Esparaza et al., 1974; Schaffer et al., 1980) between different strains of the two serotypes can occur and that crossovers are distributed across the whole genome indicating homology throughout the genomes. This observation was augmented by the cross hybridization studies of Davison and Wilkie (1983c) involving the HSV-1 strain 17 and HSV-2 strain HG52 which showed that both genomes are co-linear and shared 50% homology. Similarly studies on cross reactivity of proteins specified by different strains and analysis of intertypic recombinants has indicated the mapping of viral genes at equivalent positions

(Marsden et al., 1978; Schaffer et al., 1973; Morse et al., 1978; Spear , 1985).

Recently, DNA sequence analysis has allowed precise comparison between some corresponding regions in the two serotype genomes. So far, these analyses have been limited in scope to individual genes and parts of genes, and to other relatively small, functional sequences, including promoters and origins of replication (Swain et al., 1985; McLauchlan and Clements, 1983; Whitton et al., 1983; Whitton and Clements, 1984a,b). The only detailed sequence comparison of HSV-1 and HSV-2 has been made in the short region of the genomes (McGeoch et al., 1985; 1987). The study revealed that throughout U_S both viruses (HSV-1 strain 17 and HSV-2 strain HG52) possess equivalent genes with homology at the amino acid level with the exception of the US4 gene. The US4 gene which encodes for the glycoprotein G-2 in HSV-2 is homologous to its counterpart in HSV-1 except for an insert of about 1460 bp in the coding region near the N-terminus in HSV-2.

1.6. LYTIC INFECTION WITH HERPES SIMPLEX VIRUS

1.6.a. Adsorption and penetration

Adsorption of the virus to the cell surface is the first step in the sequence of events leading to complex virus-host interactions. In permissive cells there is a rapid adsorption of HSV to cells (Hummeler et al., 1969; Vahlne et al., 1978) through a process which may involve specific cell surface receptors. The receptors for HSV-1 and HSV-2 appear to be different in that infection of cells with one strain causes interference with adsorption of a second strain of the homologous, but not the heterologous serotype (Vahlne et al., 1979; Addison et al., 1984).

Adsorption is followed by entry of the virus into the cell either by endocytosis (Hummeler et al., 1969) or by fusion of the

virus envelope with the cell membrane (Morgan et al., 1969). Following partial degradation of the viral capsids (Hochberg and Becker, 1968) in the cytoplasm, virus DNA is transported to the nucleus (Hummeler et al., 1969), where it is transcribed (Wagner and Roizman, 1969) by the host-cell polymerase II (Costanzo et al., 1977). During adsorption and penetration of the virus, the cell membrane has been shown to become less mobile, suggesting a multivalent attachment between virus and cell receptors while in the late stages of penetration it becomes more mobile than that of control infected cells (Rosenthal et al., 1984).

1.6.b. Effect of HSV infection on host-cell metabolism

Following infection of permissive cells with HSV, a characteristic suppression of host cell DNA, mRNA and protein synthesis occurs through a complex multistep process (Fenwick and Walker, 1978; Nishioka and Silverstein, 1978; Read and Frenkel, 1983). Host polyribosomes are disaggregated following infection (Sydiskis and Roizman,1968) and contain predominately virus encoded mRNAs (Stringer et al., 1977). Similar to inhibition of host protein synthesis host RNA sythesis is also reduced (Roizman et al., 1965; Pizer and Beard, 1976) and degradation of host mRNA released from the polysomes takes place (Nishioka and Silverstein, 1977). In addition to these changes, stimulation in expression of heat shock cellular genes during early infection (Notarianni and Preston, 1982; Lathangue et al., 1984; Patel et al., 1986) and inactivation of cellular promoters intergrated into the genome of biochemically transformed cells (Everett, 1985) has been reported. Generally infection with HSV-2 results in more rapid inhibition of host protein synthesis compared to HSV-1 (Powell and Courtney, 1975; Schek and Bachenheimer, 1985). However, HSV-2 strain HG52 has been shown to be deficient in early shut off of host macromolecular synthesis (Marsden et al. 1978).

At least two factors appear to be involved in the inhibition of host polypeptide synthesis - a virion component (s) and a protein (s) synthesised later in infection (Honess and Roizman, 1975; Fenwick and Walker, 1978; Read and Frenkel, 1983). The virion-associated component is non-essential in tissue culture (Fenwick and Clark, 1982; Read and Frenkel, 1983), while the delayed component can function in the absence of a functional virion component (Read and Frenkel, 1983), and is required for the full inhibitory effect on host protein synthesis.

The disaggregation of host polyribosomes, decrease in RNA synthesis and degradation of cellular mRNA all appear to involve a component of the virion (Fenwick and Walker, 1978; Nishioka and Silverstein, 1978; Schek and Bachenheimer, 1985), although in some cell types protein synthesis is required for the degradation of cellular mRNA (Nishioka and Silverstein, 1978). Whether one or more virion components are involved in inhibition of host polypeptide synthesis, is unclear.

The differential shut-off of host macromolecular synthesis caused by HSV-2 strain HG52 has been mapped to 0.52 to 0.59 m.u. (Morse et al., 1978).The inhibition of host cell DNA sythesis also appears to involve a virion component (Fenwick and Walker, 1978). The latter authors have mapped this function between 0.52 and 0.59 m.u. on the viral genome. However, the nature of the product, or products, involved in these functions is, as yet unknown.

A number of morphological changes also take place within a cell productively infected with HSV. The earliest changes are observed in the nucleus, and these include margination of host chromatin, reduplication of membrane /disaggregation of the nucleus, and the appearance of electron-translucent viral inclusions (Love and Wildy, 1963; Schwartz and Roizman, 1969; Dargan and Subak-Sharpe, 1983).

1.7. HERPES SIMPLEX VIRUS TRANSCRIPTION

HSV transcripts are synthesised in the host cell nucleus (Wagner and Roizman, 1969). The sensitivity of virus transcription to α-amanitin and the absence of any virus coded polymerase activity suggested that HSV transcription involved host cell polymerase II at all stages of virus infection (Alwine et al., 1974; Ben-Zeev et al., 1976; Costanzo et al., 1977). HSV transcripts like host cell mRNA are internally methylated and capped at their 5'-termini (Bartkoski and Roizman, 1976; Moss et al., 1977). The 3'-termini of most transcripts contain the polyadenylation sequence 'AATAAA' which is important for cleavage of pre-mRNAs (McKnight, 1980; Fitzgerald and Shenk, 1981). Further a (G+T) rich sequence - 'YGTGTTYY' located about 10 bp downstream from the poly-A site of several eukaryotic and HSV genes has been shown to be required for the efficient formation of the mRNA 3'-terminus (Taya et al., 1982; McLauchlan et al., 1985). Only a few HSV mRNAs have been shown to be spliced (Wagner, 1985); these include immediate early (IE) mRNAs -1, -4, -5 and the mRNA for glycoprotein C (gC) (Watson et al., 1981; Frink et al., 1981; Rixon and Clements, 1982; Frink et al., 1983; Perry et al., 1986). Following transcription HSV mRNA is transported to the cytoplasm where, after binding with ribosomes it is translated into proteins (Wagner and Roizman, 1969).

1.7.a. Temporal regulation of HSV transcription

During its lytic infectious cycle, HSV establishes an orderly programme of mRNA and hence viral protein synthesis. This programme is composed of three groups of viral proteins that have been classified according to their temporal order of synthesis (Honess and Roizman, 1974, 1975; Clements et al., 1977; Jones and Roizman, 1979). The three groups are termed the immediate early (IE or α), early (DE, E or β) and late (L or γ) proteins. The sequential appearance of these classes

is regulated in a cascade fashion. That is, IE proteins are required for the induction of E protein synthesis, and in turn E proteins are required to facilitate L protein synthesis. is a schematic diagram which depicts the synthesis of HSV-1 proteins during the lytic cycle.

1.7.b. Immediate early (IE) gene expression

IE mRNAs are the first set of transcripts to be seen following infection of the cell with HSV. IE protein synthesis is detectable by one hr post infection in the absence of de novo protein synthesis (Honess and Roizman, 1974), peaks at 3 to 4 hr post infection, and then declines markedly by 6 hr post infection. Low levels of IE protein synthesis continue to be detectable after 6 hr post infection (Honess and Roizman, 1974; Harris-Hamilton and Bachenheimer, 1985). When the lytic cycle is blocked by inhibitors of protein synthesis (such as cycloheximide), IE mRNAs accumulate to the exclusion of mRNAs from the other temporal classes (Clements et al., 1977).

The five IE-genes designated IE-1, -2, -3, -4 and -5 encode for polypeptides Vmw 110 (ICPO), Vmw 63 (ICP 27), Vmw 175 (ICP 4), Vmw 68 (ICP 22) and Vmw 12 (ICP 47) respectively (Preston et al., 1978; Clements et al., 1979; Watson et al., 1979; Marsden et al., 1982). Genes encoding IE-1 and IE-3, because of their location in the repeat sequences of the long and short components of the genome respectively, are present in two copies each. Soon after synthesis, the IE polypeptides except Vmw 12 are phosphorylated and transported to the cell nucleus where they are found tightly associated with the cell chromatin. Vmw 12 occurs as a non-phosphorylated form in the infected cell cytoplasm (Pereira et al., 1977; Preston, 1979a; Hay and Hay, 1980; Marsden et al., 1982).

A variety of studies have shown that at least three of the five IE proteins regulate ensuing steps of HSV-1 protein synthesis cascade. The first example comes from genetic studies of viral strains carrying ts alleles of the gene encoding an IE protein Vmw 175. At the restrictive temperature, these mutants fail to make the transition from IE to DE protein synthesis (Preston, 1979b; Dixon and Schaffer, 1980; Watson and Clements, 1980). Moreover, if the temperature is shifted to the restrictive condition after the induction of E or L protein synthesis, IE mRNA production resumes and E and L gene expression ceases (Watson and Clements, 1980). These studies indicated that Vmw 175 (ICP4) activity is required continuously for the maintenance of both E and L gene expression. Deletion mutants lacking one copy of the IE-3 gene have been shown to grow normally in tissue culture (Longnecker and Roizman, 1986; Brown and Harland, 1987), demonstrating that one copy of the IE-3 gene is sufficient for virus growth.

Similar to Vmw 175, at the restrictive temperature, viruses carrying a ts allele of the gene IE-2 encoding Vmw 63 (ICP 27) express IE and E proteins but fail to synthesise appropriate levels of a number of L proteins (Sacks et al., 1985). However, MacLean and Brown (1987b) have described a deletion variant of HSV-1 strain 17 which fails to synthesise Vmw 63 under immediate early conditions, although the production of the polypeptide under early and late conditions remains to be determined. The precise role of Vmw 63 in the virus life cycle, has yet to be established.

An alternative approach has suggested a regulatory function for Vmw 110 (ICP0), the product of the IE-1 gene. Plasmid borne copies of the IE-1 gene have been shown to induce expression from cloned E and L genes in transient co-transfection experiments. Moreover, a similar activity of the cloned IE-3 gene is enhanced when it is transfected

into cultured cells along with the IE-1 gene (Everett, 1984a; O'Hare and Hayward, 1985; Quinlan and Knipe, 1985; Mavromara et al., 1986). No ts mutants have been isolated in IE-1 gene. One copy of this gene is required for normal virus growth and gene expression in tissue culture (Brown et al., 1984; Harland and Brown, 1985; MacLean and Brown, 1987b). Stow and Stow (1986) have constructed a virus which contains a large deletion in both copies of IE-1. This deletion mutant of HSV-1 is capable of growing on a number of cell lines, although the virus yield was reduced 20 to 100-fold compared to wild-type HSV-1. Its ability to plaque was more significantly impaired than its yield, indicating that initiation of infection was poor. Infected cell polypeptide synthesis in this mutant was normal compared to wild-type virus. Sacks and Schaffer (1987) have also isolated two deletion mutants in IE-1, which exhibit very similar properties to that of Stow and Stow (1986). Sandri-Goldin et al. (1987) have constructed cell lines expressing an antisense IE-1 message. Expression of Vmw 110 was reduced to less than 10% of wild-type levels, with little effect on the expression of HSV early and late genes or virus yield.

Thus, it appears that Vmw 110 plays an important though possibly not essential role in productive cell culture infections. Its precise requirement in vivo remains to be determined, although preliminary studies suggest it is non-essential in latency in the mouse model (G.B. Clements, personal communication).

The product of the IE-4 gene, Vmw 68, is complemented by host cell factors as demonstrated by the impaired growth of a deletion mutant in some cell lines and not in others (Sears et al., 1985). Deletion mutants in the IE-5 gene encoding for Vmw 12 have also been shown to grow normally in a number of cell lines (Mavromara-Nazos etal., 1986; Umene, 1986; Brown and Harland, 1987). These studies have

clearly demonstrated that both Vmw 68 and Vmw 12 are not essential for virus growth.

Immediate early gene transcription though not requiring de novo protein synthesis, is stimulated in trans by a component of the virus particle (Post et al., 1981; Batterson and Roizman, 1983), identified as the major tegument protein, Vmw 65 (Campbell et al., 1984). Transcriptional stimulation by Vmw 65 has only been observed for genes containing IE promoters. The role of specific cis-acting DNA sequences present in the promoter or 5'-upstream regulatory regions which are characteristically unique to each temporal class of genes have been suggested to take part in the control of HSV transcription. Each IE gene promoter has been found to contain (i) a TATA box located 25 to 30 bp 5' to the mRNA start site. This is required for the accurate initiation of transcription; (ii) multiple binding sites for the cellular factor Spl (Jones and Tjian, 1985); (iii) proximal promoter sequences located at -37 to -108 bp which are required for transcriptional initiation in the absence of further upstream sequences; (iv) far upstream sequences (-174 to -331), containing one to multiple copies of the sequence, 'TAATGARATT' (Mackem and Roizman, 1982b; Whitton and Clements, 1984b). The highly conserved sequences 'TAATGARATT' is unique for all the immediate early and not the early and late gene promoters (Mackem and Roizman, 1982a; Whitton et al., 1983; Whitton and Clements, 1984a) and mediates responsiveness to the virion trans-inducing factors Vmw 65 (Preston et al., 1984). Recently the interaction between 'TAATGARATT' elements and Vmw 65 has been shown to occur via a cellular polypeptide (Kristie and Roizman, 1987; Preston et al. submitted for publication). The (G+C) rich region flanking the 'TAATGARATT' sequences appears to bind to Spl and modulates transcription from IE genes (Jones and Tjian, 1985).

1.7.c. Early (E) gene expression

The early or β polypeptides are characterized by their absolute dependence on the prior synthesis of immediate early polypeptides (Honess and Roizman, 1974). The synthesis of E proteins begins by 3 hr post infection, peaks at roughly 6 hr post infection and declines through subsequent time points (Honess and Roizman, 1974; Harris-Hamilton and Bachenheimer, 1985, also see Figure 9).

The early class of polypeptides induces many enzymes involved in virus nucleic acid metabolism and several structural polypeptides. The two classes of early polypeptides, B_1 and B_2, have been distinguished on the basis of their synthesis in the presence of different amino acid analogues (Pereira et al., 1977) and the analysis of polypeptides induced by a ts mutant in Vmw 175, (ts K) at the non permissive temperature (Preston, 1979b).

Several studies on analyses of early gene promoter regions have been undertaken with the aim of identifying cis- and trans- acting transcriptional control signals. Investigation of the thymidine kinase (tk) gene promoter region by both deletion analysis and insertion of clustered sets of point mutations at random locations, enabled three distinct cis-acting control signals to be identified within the 106 bp upstream sequence required for efficient transcription of the tk gene (McKnight and Kingsbury, 1982; McKnight et al., 1984). These included a proximal TATA homology and two upstream distal signals which exhibited sequence homology and were required for quantitative transcriptional control. Experiments designed to determine early gene promoter sequences which respond to transactivation by virus immediate early polypeptides yielded conflicting results. Zipser et al. (1981) and Elkareh et al. (1985) reported that functional domains which had differential roles in constitutive and virus-induced expression of the gene, could be defined within the tk promoter region. Everett (1984b)

and McKnight et al., (1984), however using genes containing the gD and tk promoters respectively in trans-induction assays, could not detect any specific sequences required for transactivation by immediate early polypeptides.

Recent experiments revealed that the same early gene upstream sequences are required for efficient expression of early genes in constitutive (frog oocyte) and regulated (HSV-infected fibroblast cells) environments (Eisenberg et al., 1985). Taken together with evidence that the mammalian transcription factor Spl binds strongly to IE and early gene promoters (Jones and Tjian, 1985), these findings indicate that IE polypeptides transactivate early genes in conjunction with, or by modification of, cellular transcription factors.

1.7.d Late (L) gene expression

The late genes are transcribed late in viral infection and require functional immediate early and viral DNA replication for their efficient expression (Honess and Watson, 1977; Jones and Roizman, 1979; Holland et al., 1980). The two sub groups of the class termed γ_1, or 'leaky late' and γ_2 or 'true late' are distinguishable by the fact that γ_2 gene expression is barely detectable in a normal lytic infection before viral DNA replication, whereas γ_1 gene expression is readily detectable (Roizman and Batterson, 1985; Wagner, 1985). The polypeptides encoded by the late genes comprise a number of structural proteins such as the major capsid protein (Vmw 155, VP 5), glycoprotein C (gC), and the US-11 gene product, Vmw 21 (Honess and Roizman, 1973; Marsden etal, 1976; Costa et al., 1981; Frink et al., 1983).

The overall mechanism for late gene expression is poorly understood. Early and late genes can be distinguished in an in vitro transcription system prepared from uninfected cells (Frink et al.,

1981). In the absence of transactivation, early genes are expressed whereas late genes remain transcriptionally silent. One possible explanation is that late promoters are intrinsically weak, requiring transactivation for efficient expression.

Efficient transcription from a true late gene promoter requires fewer regulatory sequences compared to immediate early genes. Only the 'TATA' box region appears to be required (Homa et al., 1986; Johnson and Everett, 1986b). The presence of an active origin of replication in cis has been shown to be necessary for efficient expression from the promoter of a late gene (US 11). The necessary DNA sequences for the efficient, regulated expression of US 11 lie within 31 bp of the RNA cap site (Johnson and Everett, 1986a). A similar sequence requirement has been demonstrated for the gC gene promoter - another true late gene (Homa et al., 1986). The requirement for DNA replication for the efficient expression of late genes may reflect more than an increase in copy number compensating for a weak promoter. An alteration in the genome structure or in the factors binding to viral DNA during replication may influence late gene expression (Silver and Roizman, 1985; Johnson and Everett, 1986a). Late genes may also be subject to negative control by the major DNA binding protein Vmw 136 (ICP 8), since ts mutants with lesions in ICP 8 exhibit increased levels of late gene expression at the restricted temperature (Godowski and Knipe, 1983; 1985).

1.8. HERPES SIMPLEX VIRUS DNA REPLICATION

The mechanistic details of HSV DNA replication are not fully understood, however, the cis- and some of the trans- acting functions have now been identified. Following infection, the viral DNA rapidly accumulates in the host cell nuclei where it is rapidly circularized presumably by ligation of the terminal 'a' sequences (Davison and

Wilkie, 1983a; Poffenberger and Roizman, 1985). Electronmicroscopic studies of the replicating DNA (Friedman et al., 1977) and analyses of defective virus genomes (Frenkel et al., 1975; Kaerner et al., 1979) have revealed that cis- acting sites for the initiation of virus DNA replication exist.

HSV DNA replication in BHK cells is first detected at about 3 hr post infection, reaching a peak in 9 to 11 hr and being completed by 16 hr post infection at 37°C (Rixon, 1977). Only 5% of the infecting HSV DNA enters the replicating pool (Jacob and Roizman, 1977) whereas in PRV up to 80% of the input genomes undergo replication (Jean and Ben-Porat, 1976; Ben-Porat et al., 1976).

The nature of the replicating form of HSV DNA is unknown. The large size and fragility of virus DNA complicates the analyses. Ben-Porat and Rixon (1979) described replicating HSV DNA as 'large, tangled masses'. The rapidly sedimenting form of HSV DNA relatively lacking in termini seen in later stages of infection has indicated that the DNA is either circular or in head-to-tail concatamers (Jacob et al., 1979; Jongeneel and Bachenheimer, 1981). It is unclear whether DNA replication first undergoes a template amplification as circular monomers before packaging into progeny virus. Late in infection a rapidly sedimenting form of the replicated DNA, thought to be an extensive concatameric form, indicates that replication of DNA, at least at this stage is by a rolling circle mechanism (Jacob et al., 1979). The newly synthesized DNA is further processed, cleaved/packaged into nascent nucleocapsids in the cell nuclei (Vlazny et al., 1982).

1.8.a. Origins of HSV DNA replication

Electronmicroscopic examination of HSV DNA extracted from infected cells revealed that the virus genome might possess one or

more specific sites which may act as start sites for DNA replication (Friedman et al., 1977). The location of cis-acting sites for DNA replication was determined by analyses of the nature of two classes of defective HSV genomes which accumulate in virus stocks passaged at high multiplicities of infection. Both classes of defective genomes contain sequences from the terminus of TR_S. Class I molecules contain in addition further sequences from the S segment while Class II molecules contain sequences from near the centre of U_L (Kaerner et al., 1979; Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982). The ability of both classes of defective molecules to be replicated and packaged with the help of wild-type virus indicated the presence of cis-acting signals for DNA synthesis and processing within the defective genomes. The cis-acting site located in TR_S/IR_S is termed Ori_S (Stow and McMonagle, 1983) while that in the centre of U_L is termed Ori_L (Gray and Kaerner, 1984; Weller et al., 1985).

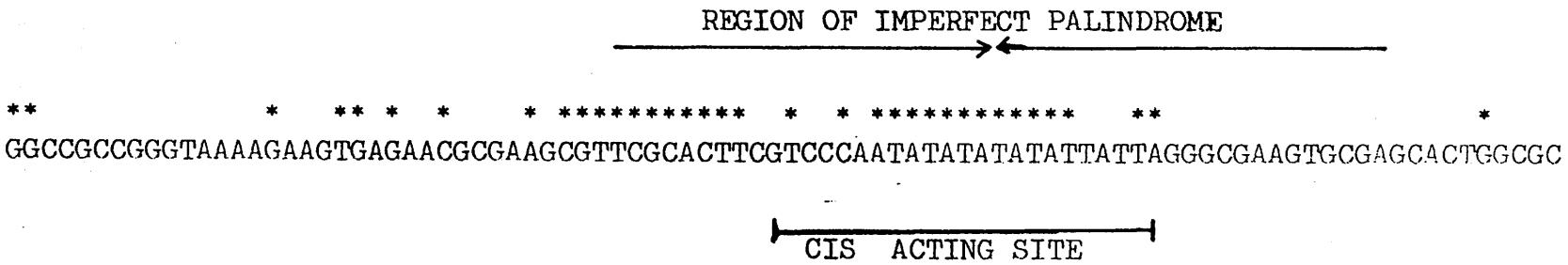
(i) Ori_S

Stow and McMonagle (1983) using a plasmid based system, have localised the origin of DNA replication in the R_S sequences of HSV-1. The HSV-1 Ori_S a 90 bp fragment is located between the 5' ends of the divergent transcripts for IE3 and IE4/5. There are thus 2 copies of Ori_S, one in IR_S and the other in TR_S (Figure 10). The Ori_S in HSV-1 strain 17, contains an imperfect palindrome of 42 bases with an $(AT)_6$ sequences at the centre. The $(AT)_6$ rich central region and the sequences surrounding the palindrome are essential for DNA replication (Stow and McMonagle, 1983; Stow, 1985).

In HSV-2 strain HG52, a sequence similar to OriS has been identified in R_S. The HSV-2 Ori_S differs from that of HSV-1 in that there are two copies of Ori_S sequences, contained within almost identical direct repeats of 137 bp (Whitton and Clements, 1984a).

FIGURE 10

The 90 bp minimal Ori_S sequence. The sequence of the 90 bp fragment containing the *cis*-acting sequences required for Ori_S function (Stow and McMonagle, 1983) is shown. This represents the upper strand, inthe 5'—> 3' direction, of the Ori_S sequence within the short internal repeat. ——> <—— represents the 45 bp imperfect palindrome within this region. * refers to bases conserved between the VZV and HSV Ori_S fragments. ⟝ indicates the sequence deleted by Stow (1985), resulting in a plasmid which fails to replicate. ⊡ represents the bases on this strand protected by the Ori_S-binding protein (Elias *et al.*, 1986).


REGION OF IMPERFECT PALINDROME
** * ** * * * ********** * * ********** ** *
GGCCGCCGGGTAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGCGC
CIS ACTING SITE
PROTEIN BINDING SITE

The nuclei from HSV-1 infected cells are found to contain a specific Ori_S DNA binding protein which protects an 18 bp region across one end of the palindrome from DNase digestion (Figure 10) (Elias et al., 1986). The 11 bp out of the 18 bp which bind to protein are conserved between HSV-1 and VZV (Elias et al., 1986). Hubenthal-Voss et al (1987) by cloning and DNA sequence analysis of cDNA from the Ori_S containing region of HSV-1(F) have reported that each Ori_S sequence is contained in an open reading frame designated Ori_S ORF. The transcription of Ori_S ORF is initiated approximately 860 nucleotides upstream from that of the IE-3 genes and 162 nucleotides downstream but on the opposite strand from the transcription initiation site of the IE-4 or IE-5 genes within the inverted repeat C sequence. The Ori_S ORF transcript is 3' coterminal with the mRNA of the IE-3 genes, poly adenylated but not spliced and codes for a 34,000 mw protein. The insensitivity of Ori_S ORF transcripts to phosphonoacetic acid indicates that it is regulated as either an early or delayed early gene.

(ii) Ori_L

On the basis of analysis of defective genomes, the cis-acting site for HSV DNA replication located in U_L i.e. Ori_L was mapped between 0.360 and 0.419 m.u. (Spaete and Frenkel, 1982). The systematic deletion analyses of Ori_L were hindered because of the occurrence of a 100 to 600 bp deletion within this region (0.360 to 0.419 m.u.) upon cloning in a standard plasmid vector. The deleted fragments contained no origin activity. However, following serial passage in the presence of wild-type helper virus, the deleted sequences were restored presumably through recombination with helper virus and the plasmids were capable of replication (Spaete and Frenkel, 1982; Weller et al., 1985).

Successful cloning of Ori_L in an undeleted form was achieved in a yeast plasmid vector which was capable of replicating in a plasmid-based system following superinfection with HSV (Weller et al., 1985). Sequence analysis of Ori_L from HSV-1 Ang (Grey and Kaerner, 1984), HSV-1 strain 17 (Quinn and McGeoch, 1985) and HSV-1 strain KOS (Weller et al., 1985) has revealed the presence of a perfect 144 bp palindrome which is responsible for deletion and cloning instability (McGeoch, 1987).

Lockshon and Galloway (1986) have identified and sequenced Ori_L in HSV-2. The almost perfect palindrome of 136 residues found in Ori_L of HSV-2 shows a strong degree of sequence homology to that of HSV-1 Ori_L (Lockshon and Galloway, 1986). Stow and Davison (1986) could not find any sequence counterpart of HSV Ori_L in the equivalent genome location of VZV.

Of the 90 bp sequence homology seen in HSV-1 Ori_S and Ori_L, greatest homology is found near and to the left of the centre of symmetry (Figure 11). The short inverted repeat sequences found within each arm of the Ori_L palindrome are believed to be the recognition sites for a DNA binding protein (Weller et al., 1985). Two of the three inverted repeat sequences located on the left side of the palindrome are shared by Ori_S. This may reflect the bi-directional mode of replication from Ori_L whereas that from Ori_S is unidirectional (Quinn and McGeoch, 1985; Weller et al., 1985).

The functional Ori_L in HSV-1 mapped between 0.398 and 0.413 m.u. is situated between the divergent transcripts for the DNA polymerase and the major DNA-binding protein (Quinn and McGeoch, 1985; McGeoch, 1987), Figure 12). The significance of the location of both Ori_L and Ori_S between divergent transcripts encoding polypeptides with a major role in virus replication and transcription is unknown.

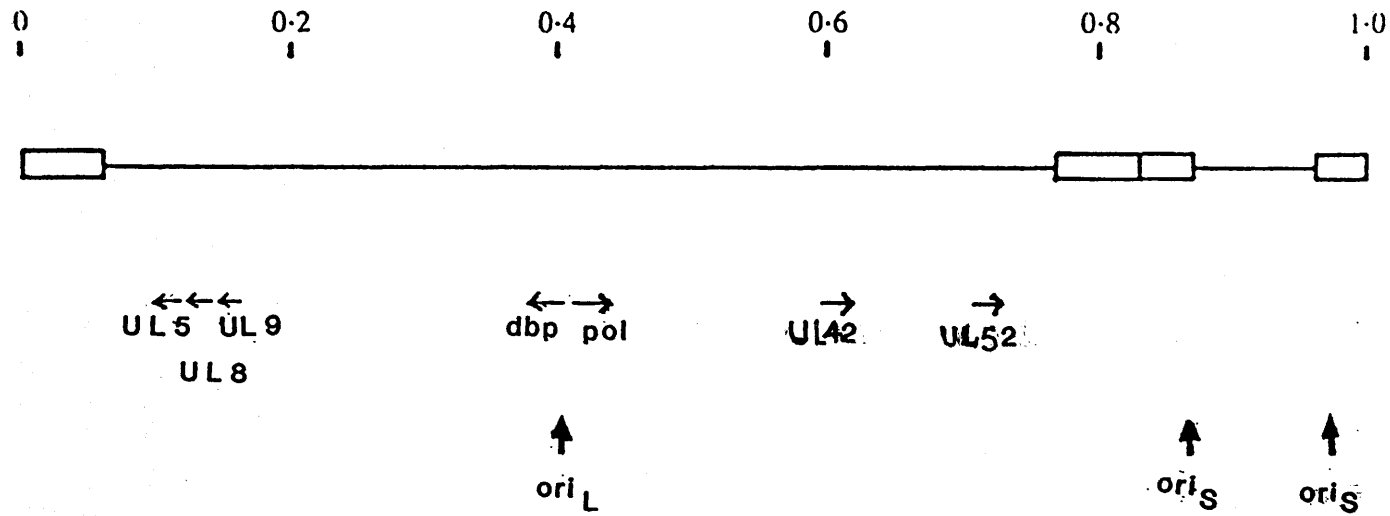
FIGURE 11

Comparison between Ori_L and Ori_S of HSV-1. The 144 bp perfect palindrome from Ori_L is compared with the similar region in Ori_S. Conserved bases are shown below the two sequences (Weller et al., 1985). The positions of the 3 inverted repeat sequences present in the arms of the Ori_L palindrome are indicated ⟶ . Two similar inverted sequences, present only on the left hand side, are shown below. ▨ refers to the 18 bp region protected from DNAase I digestion by the Ori_S-binding protein.


-10 -20 -30 -40 -50 -60 -70 10 20 30 40 50 60 70
ori_L AGCCGGTGGGCGTGGCCGCTATTATAAAAAAAGTGAGAACGCGAAGCGTTCGCACTTTGTCCTAATAATATA TATATTATTAGGACAAAGTGCGAACGCTTCGCGTTCTCACTTTTTTTATAATAGCGGCCACGCCCACCGGCT
ori_S CCCAAGGGGGCGGGGCCGC-CGGGTAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATA-TATA TATATTATTAGGGCGAAGTGCGAGCACTGGCGCCGTGCCCGACTCCGCCCCGGCCCCGGGGGCGGGCCCGGG
C C GGGCG GCCCGC TAAAA AAGTGAGAACGCGAAGCGTTCGCACTT GTCC AATA TATA TATATTATTAGG C AAGTGCGA C CT C T C GC CC G
ori_L
ori_S
ori_S-binding protein

FIGURE 12

Locations of genes required for HSV-1 DNA synthesis. The prototype HSV-1 genome is shown, with the scale in fractional map units. The locations and orientations of seven genes necessary for virus DNA synthesis are indicated, together with the positions of Ori_L and Ori_S.


0
0·2
0·4
0·6
0·8
1·0
UL5 UL9
UL8
dbp pol
UL42
UL52
ori L
ori S
ori S

1.8.b. Function of HSV origins of DNA replication

Although the exact role of various sequences in the Ori elements is not fully understood, it is believed that binding of Ori-specific proteins at the conserved locus in Ori sequences provides a site for assembly of the replicative complex, while the $(A+T)_n$ region comprises a site for strand separation prior to synthesis of a new DNA strand. Also it is not known whether one origin of replication is used in preference during virus replication. Similarly the functional significance of the presence of two copies of Ori_S is unknown; although viruses lacking one copy of Ori_S grow normally in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987). Polvino-Bodnar et al (1987) have recently reported a virus mutant lacking Ori_L which is capable of replication in cell culture.

1.8.c. Genes involved in HSV DNA replication

Recently, Challberg (1986) using a plasmid assay system involving cotransfection of cloned HSV genome fragments and Ori_S containing plasmids has identified cis-acting loci in the HSV genome, necessary for plasmid replication. This approach in conjunction with DNA sequence information has resulted in identification of seven genes essential for virus DNA replication (Figure 12). These genes include DNA polymerase (136000 Mr), the major DNA binding protein (128,000 Mr), UL 9 (94,000 Mr) and UL 52 (115,000 Mr). The functions of the gene products encoded by the last five genes is yet unknown. The genes for ribonucleotide reductase, IE-1, -2 and -3 are required for optimal plasmid amplification but are not absolutely essential. Some of the properties of the proteins encoded by the genes needed for HSV DNA replication are described in section 1.10.b.

1.9. CLEAVAGE/PACKAGING OF HERPES SIMPLEX VIRUS DNA

The head-to-tail concatamers formed during replication of HSV DNA are cleaved into unit length genomes and packaged into capsids in the nuclei of infected cells (Jacob et al., 1979; Kaerner et al., 1981; Vlazny and Frenkel, 1981). The processes of cleavage and packaging of the genome are tightly linked (Ladin et al., 1980; Deiss and Frenkel, 1986; Addison, 1986).

The signals for cleavage and packaging have been located in the 'a' sequences (Vlazny and Frenkel, 1981; Vlazny et al., 1982; Spaete and Frenkel, 1982; Stow et al., 1983; Varmuza and Smiley, 1985; Deiss and Frenkel, 1986; Deiss et al., 1986). The presence of partial copies of the DR_1 sequences at both termini of a mature virus genome led to the belief that the cleavage event might have occurred by a single double-strand breakage within DR_1.

Varmuza and Smiley (1985), using deleted sub-fragments of the 'a' sequence inserted into the viral thymidine kinase gene (tk), observed that two separate cleavage/packaging signals are present within the U_b and U_c segments of the 'a' sequence with cleavage occurring in a sequence independent manner at a fixed distance from these sites. Since sequences from an intact U_b and an intact U_c were inactive individually, the functioning of these two signals in concert was indicated. This observation was strengthened by the deletion analysis studies of Deiss et al (1986) in which an HSV amplicon containing 'a' sequences with deletions within U_c failed to be packaged whereas those within U_b were only packaged if they first acquired an insert of a wild-type 'a' sequence from a helper virus.

The presence of highly conserved sequences at the termini of fully packaged HSV genomes from different herpes viruses and from different strains within the same type (Davison and Wilkie, 1981;

Davison and Rixon, 1985; Deiss et al., 1986) in conjunction with the ability of different strains of HSV to package efficiently heterologous 'a' sequence containing DNA (Vlazny and Frenkel, 1981; Deiss and Frenkel, 1986) led Deiss et al (1986) to conclude that these highly conserved sequences are the cis-acting sites for cleavage/ packaging of the virus genome. The conserved region of homology in U_b was termed pac-1 and that in U_c, pac-2 (Deiss et al., 1986) (Figure 13). On the basis of these observations different models for cleavage/ packaging of HSV DNA proposed by various workers are described in the following sections.

1.9.a. Staggered nick-repair model

As outlined in Figure 14, in the 'staggered nick-repair model' of Varmuza and Smiley (1985), the L- and S-termini are generated by staggered single stranded nicks followed by repair synthesis resulting in duplication of the 'a' sequences between adjacent viral genomes. Ligation of these termini then results in tandem reiteration of the 'a' sequence. In this model it is believed that junctions having two or more tandemly repeated 'a' sequences might be processed by a double strand cleavage, as a result of cooperation between two adjacent recognition signals. However, the model fails to explain the production of the 3' single nucleotide extensions found at the L- and S-termini of the standard genome.

1.9.b. Wastage or theft model

According to the wastage model of Varmuza and Smiley (1985) the genome termini are generated by double strand (ds) cleavage at any L-S junction containing a single 'a' sequence (Figure 15). Following packaging of a full length genome, cleavage occurs at the next 'a' sequence which is stolen from the adjoining DNA. As a consequence, two unpackaged termini lacking an 'a' sequence are also generated. Since

FIGURE 13

Conserved regions within the 'a' sequence

(a) Structure of the HSV-1 'a' sequence

(b) Unique DNA sequences conserved between different strains of HSV

(c) Pac-1 and pac-2 regions (Deiss et al., 1986). These regions are conserved between a number of herpes viruses as indicated in the text. For HSV, pac-1 has the sequence:

$[C_5]\ [G_6]\ [TGTGTTT^{C}_{T}]\ [G_8]$ ... HSV-1

$[C_5]\ [G_8]\ [TGTTTTTT]\ [G_9]$ ... HSV-2

For HSV, pac-2 has the sequence:

$[CGCCGCG]-n_{31}-[T_6]$ ... HSV-1

$[CGCCGCG]-n_{29}-[T_6]$ ... HSV-2

where n = any nucleotide.

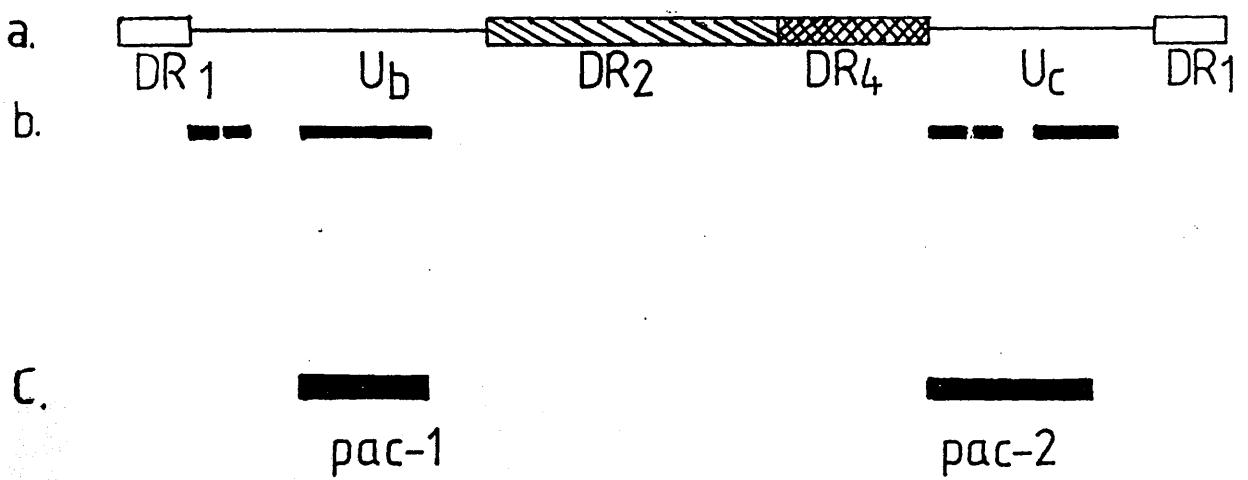

a.
DR 1
Ub
DR2
DR4
Uc
DR1
b.
c.
pac-1
pac-2

FIGURE 14

A) The staggered nick-repair model of HSV DNA maturation proposed by Varmuza and Smiley (1985). Open and filled triangles represent the S and L terminus recognition complexes bound to signals located in U_b and U_c respectively.

B) A model whereby junctions bearing two or more tandemly repeated 'a' sequences give rise to termini, as a result of co-operation between L and S recognition complexes in adjacent 'a' sequences producing a double stranded break.

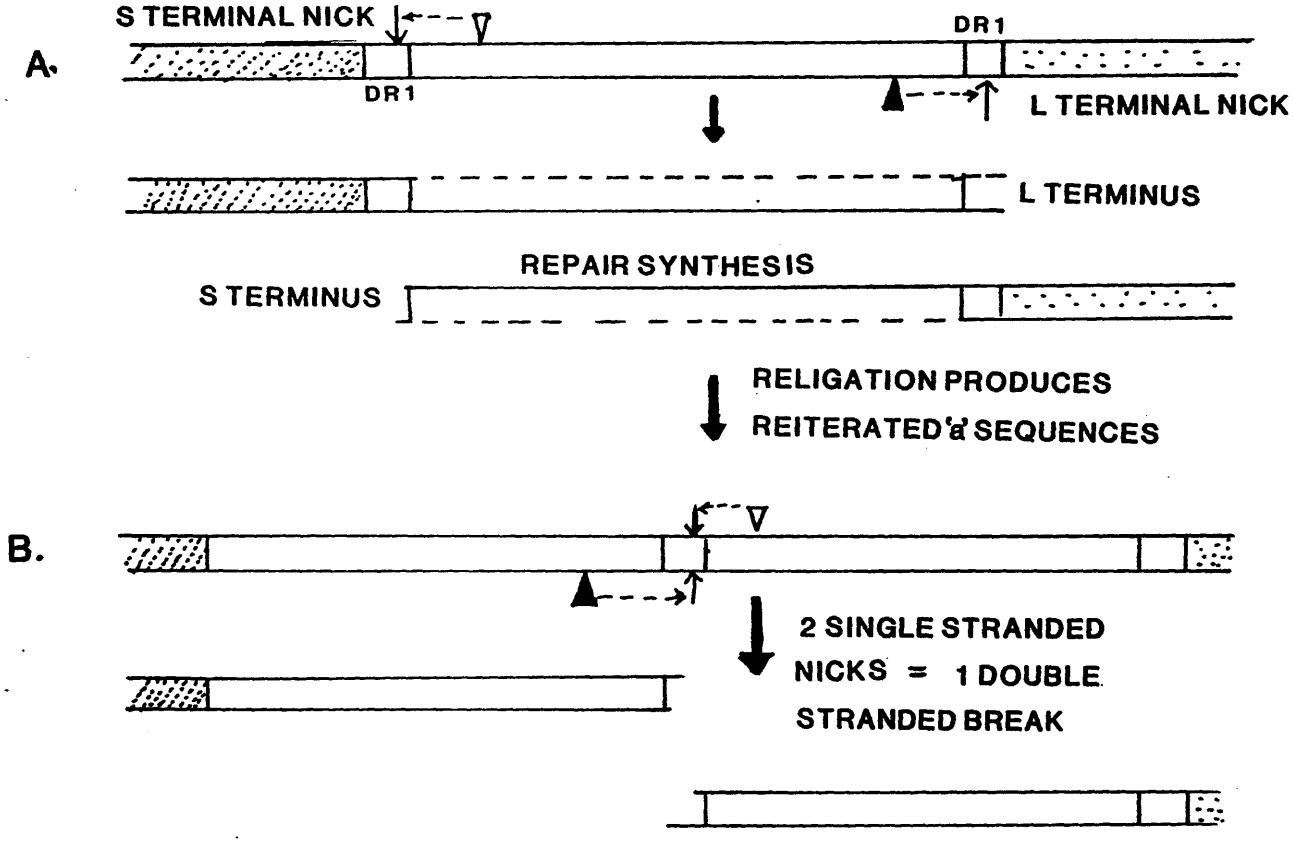

A.
S TERMINAL NICK
DR1
DR1
L TERMINAL NICK
L TERMINUS
REPAIR SYNTHESIS
S TERMINUS
RELIGATION PRODUCES
REITERATED 'a' SEQUENCES
B.
2 SINGLE STRANDED
NICKS = 1 DOUBLE
STRANDED BREAK

FIGURE 15

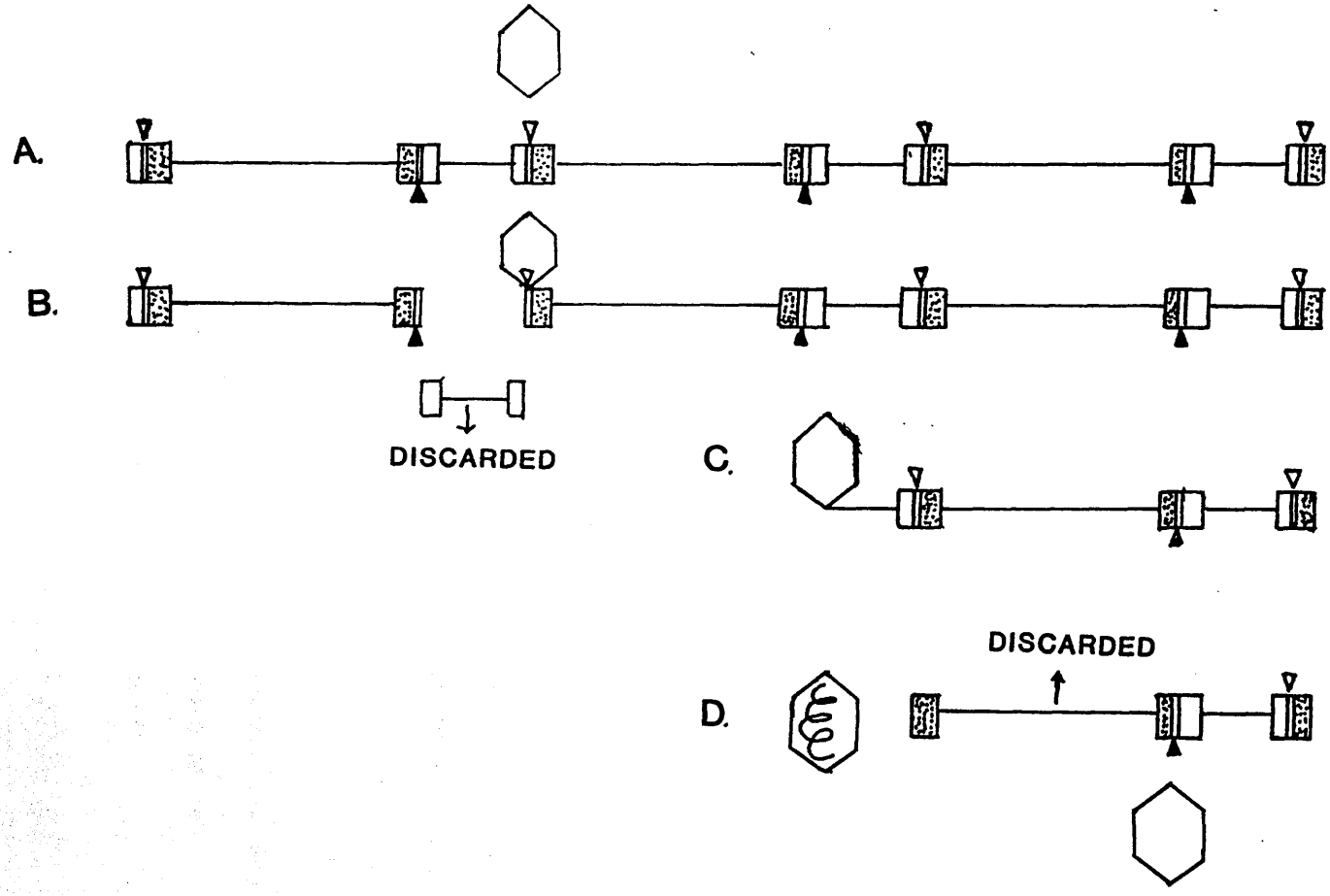
'Theft' model for cleavage and packaging of HSV DNA proposed by Varmuza and Smiley (1985). Open and filled triangles represent packaging signal(s) in alternate phases. The cleavage/packaging complex is indicated as an empty capsid particle.

(A) Double stranded cleavage occurs at an L terminus cleavage signal. This creates an L terminus carrying an 'a' sequence and an S terminus lacking an 'a' sequence.

(B) Packaging proceeds until the next appropriately oriented L-S junction is encountered, at which point,

(C) a double stranded S terminus cleavage is made, generating an S terminus with an 'a' sequence and a L terminus lacking an 'a' sequence.

(D) The model proposes that ends lacking 'a' sequences are discarded and rapidly degraded.


A.
B.
DISCARDED
C.
D.
DISCARDED

these terminal fragments lacking an 'a' sequence have never been detected experimentally (Mocarski and Roizman, 1982a; Deiss and Frenkel, 1986), they must be rapidly degraded.

1.9.c. Directional cleavage - packaging model

Deiss et al (1986) have proposed a directional cleavage-packaging model which is essentially similar to the 'theft or wastage model' proposed by Varmuza and Smiley (1985), except in the former model a polarity in the cleavage-packaging process is included. The first cleavage (producing a 3' single-base overhang) of the concatameric HSV DNA occurs at the DR_1, element at a fixed distance proximal to the first U_c sequence. The directional packaging begins at the generated L-terminus (which contains at least one 'a' sequence) whereas the generated S-terminus (which is devoid of 'a' sequence) is rapidly degraded. While packaging in the L-S direction, the DNA is scanned for the next directly repeated junction, skipping the inverted junction. A second cleavage occurs at a constant distance from the first U_b sequence encountered in the second junction, leaving a 3' single-base overhang and producing a packaged molecule which carries a ca terminus and an unpackaged terminus which is degraded or forms a substrate for a new round of packaging into a separate capsid.

The directional model like the theft model of Varmuza and Smiley (1985) also predicts the generation of S-termini devoid of 'a' sequences. Since such S-termini have never been observed experimentally they must be rapidly degraded.

1.9.d. Double-strand break and gap repair model

In order to explain some of the anomalies observed in "the theft model" and "the directional cleavage-packaging model", Deiss et al (1986) have proposed a second model based on the double-strand break-

repair model of Szostak et al (1983). According to this model interaction of two directly repeated junctions results in amplification of the 'a' sequence by a gene-conversion-like mechanism (Figures 16, 17). The resultant junctions containing double 'a' sequences are then cleaved with 3' single base extensions to yield the genomic termini. The cleavage/packaging process, as outlined in Figures 16 & 17, thus includes the following steps: (i) the packaging complex travels at random until it reaches the first U_c signal (junction A). No cleavage occurs; (ii) Again, packaging occurs in the L-to-S direction until the packaging complex meets a directly repeated junction (junction B); (iii) the two junctions are juxtaposed (Figure 17,1) and a U_c signal-directed double-stranded cleavage occurs within the DR_1 element of either 'a' sequences (Figure 17,2); (iv) the resultant 3' terminus invades the homologous sequence in the other junction and is extended by copying the 'a' sequence in that juncton while displacing the equivalent strand (Figure 17,3,4); (iv) this displaced strand serves as the template for repair synthesis of the second strand of the invading 'a' sequence (Figure 17,5); (iv) the process terminates by the resolution of the two Holliday structures (Holliday, 1964) - this will be expected to occur at or near DR_1; (vii) if the other junction also cotained a single 'a' sequence, then the process would be repeated in the other direction. Thus, the result is amplification of the 'a' sequence in both junctions to generate a double 'a' sequence in which DR_1 is flanked by both U_b and U_c packaging signals; (viii) both double 'a' sequence junctions are cleaved (generating a 3' single base extension) between the newly inserted and original 'a' sequence. This cleavage could also be the result of staggered single-strand cleavages generated by U_b and U_c of adjacent 'a' sequences.

FIGURE 16

The double-strand gap/repair model (Deiss et al., 1986). Briefly DNA is packaged prior to cleavage, the two directly repeated 'a' sequences then align and are amplified, by a double strand break/ repair mechanism, to generate two double 'a' sequence-containing junctions Cleavage between the double 'a' sequences then generates 4 'a' sequence - containing termini. ▬ refers to 'a' sequences. The detailed steps of this model are outlined in Figure 17. Asapted from MacLean, 1987.

DOUBLE-STRAND GAP/REPAIR MODEL

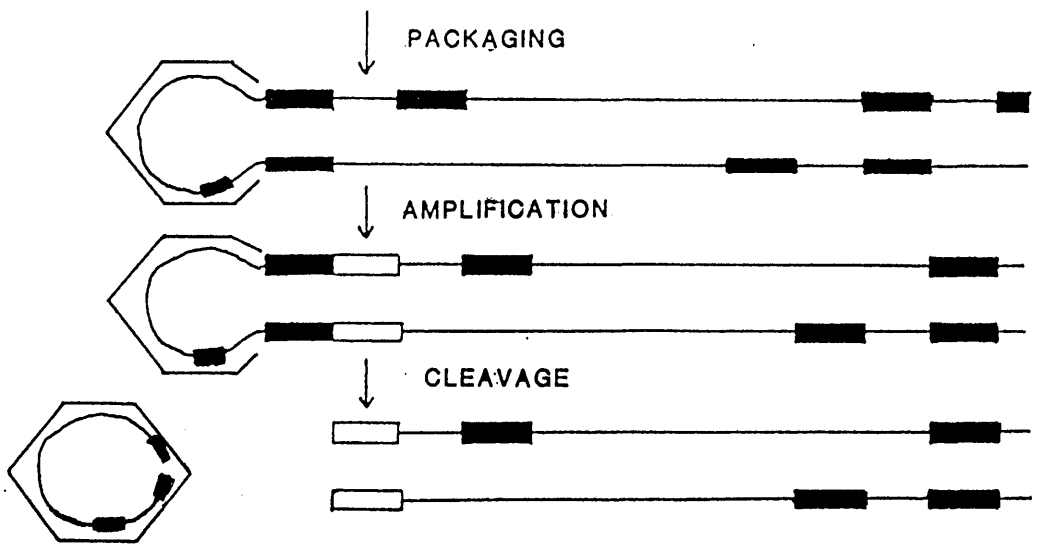
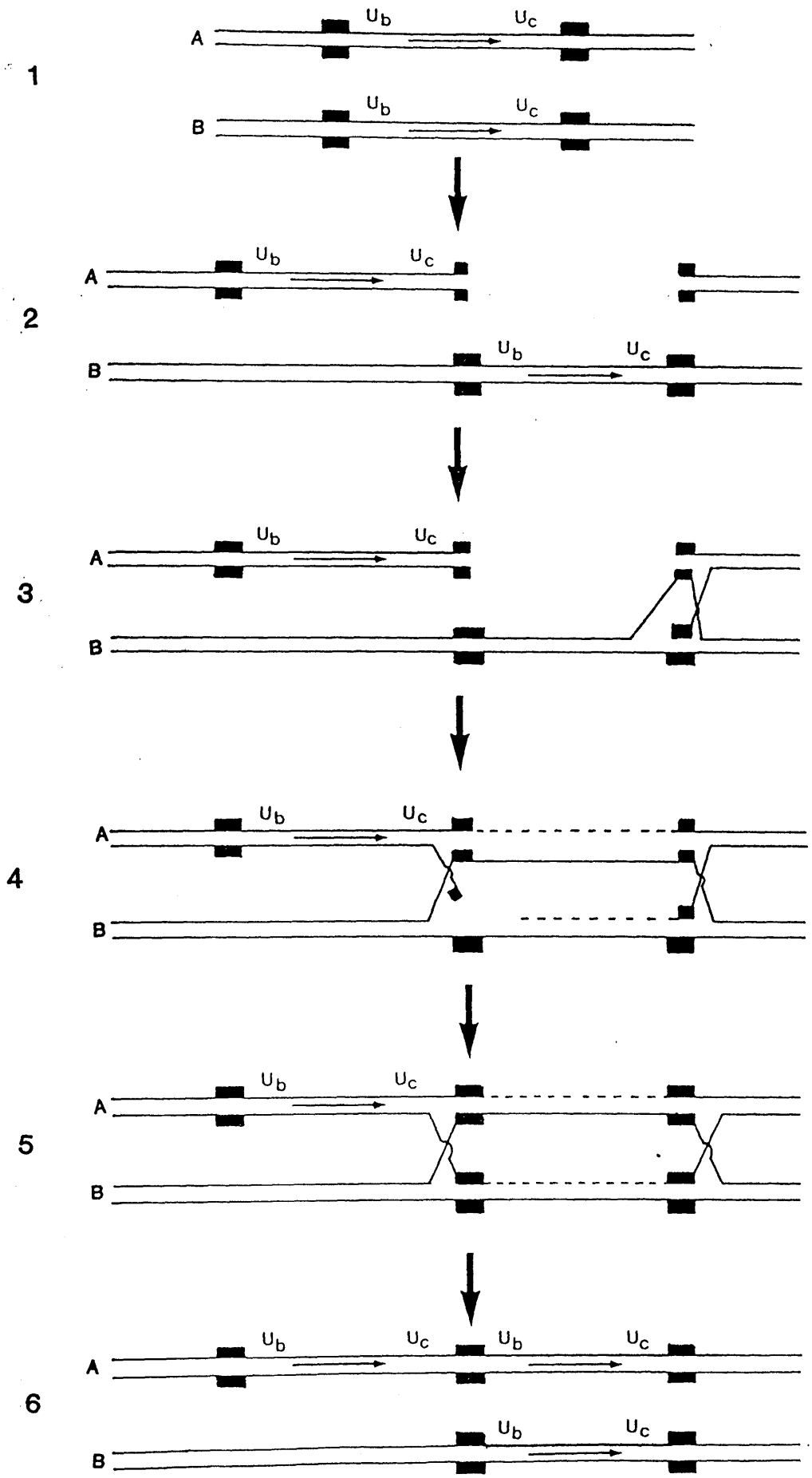


FIGURE 17

Double-strand gap/repair model as proposed by Deiss et al (1986)

1. DNA is packaged prior to cleavage, and the directly-repeated 'a' sequences which will form the L and S termini then align (junctions A and B, respectively).
2. A U_c-directed double-strand cleavage occurs through the DR_1 (▬) of junction A. The cleaved ends align with the DR_1 elements of junction B.
3. One strand from junction A then invades junction B, displacing the equivalent strand. (For simplicity this diagram is drawn with the top and bottom strands of junction B reversed).
4. The displaced strand from junction B aligns with the partial DR_1 elements of junction A, and serves as a template for repair synthesis.
5. Similarly, the non-displaced strand from junction B serves as a template for repair synthesis between the invading partial DR_1 elements.
6. Resolution of the Holliday structure results in the presence of two directly repeated copies of the 'a' sequence at junction A.
7. A similar process is then envisaged to take place for junction B. Cleavage between the adjacent 'a' sequences then generates the L and S termini of the packaged DNA.


1
A
B
U_b
U_c
2
3
4
5
6

This model again predicts that around 80% of the packaged genomes (initial encounter of packaging complex in L) will carry ba and ca termini regardless of the number of 'a' sequences in the cleaved junctions, whereas the remaining packaged genomes (initial encounter of packaging complex in S) will be terminated with ca and ba_n sequences. The termini of the free molecules generated by cleavage would be of the type ba_n and ca_n or ca, and might be either degraded or serve as a target for new packaging cycles.

This model accounts for all the features of HSV cleavage/ packaging explained by the directional model (Deiss et al., 1986; Section 1.9.c), and also some of the other anomalies: (i) There are no termini devoid of 'a' sequences produced. (ii) It also explains the finding of multiple 'a' sequence-containing S-termini seen in defective-containing stocks (Deiss and Frenkel, 1986). However, the drawback of the model is its dependence on the sequence homology at the DR_1 elements of the 'a' sequence for the correct copy of the second 'a' sequence.

1.10. HERPES SIMPLEX VIRUS INDUCED POLYPEPTIDES

Following infecton of cells with HSV a large number of polypeptides are induced. These include IE polypeptides involved in HSV transcription, virus-induced enzymes and DNA-binding proteins involved in virus genome replication, structural polypeptides and glycoproteins involved in virus structure composition. Approximately 50 HSV induced polypeptides have been detected on SDS-PAGE (Honess and Roizman, 1973, 1974; Powell and Courtney, 1975; Marsden et al., 1976), while on two-dimensional SDS-PAGE at least 230 distinct polypeptide species have been recognised (Haarr and Marsden, 1981). This increased number may be in part due to the resolution of families of polypeptide species related through post-translational modification. The apparent

difference in mobilities on SDS-PAGE of some *in vitro* synthesised viral polypeptides compared to those from infected cell extracts reflects the occurrence of post translational modification of some of the HSV-induced polypeptides (Preston, 1977). The four main post-translational modification events of HSV induced polypeptides include phosphorylation (Marsden *et al.*, 1978; Wilcox *et al.*, 1980); glycosylation (Haarr and Marsden, 1981; Palfreyman *et al.*, 1983; Hope and Marsden, 1983); sulphation (Hope *et al.*, 1982) and proteolytic cleavage (Eisenberg *et al.*, 1984; Balachandran and Hutt-Fletcher, 1985).

1.10.a. HSV-induced enzymes involved in DNA replication

(i) DNA polymerase

HSV induced DNA polymerase is a polypeptide of approximately 150,000 mw (Powell and Purifoy, 1977). This virus encoded enzyme has been shown to be essential for virus DNA replication and because of its sensitivity to phosphonoacetic or phosphonoformic acid has been mapped to 0.40 to 0.42 m.u. of the genome (Hay and Subak-Sharpe, 1976; Chartrand *et al.*, 1979, 1980; Crumpacker *et al.*, 1980). Coen *et al.*, (1982) showed that HSV polymerase contains at least two functional domains and possesses both DNA polymerase and a 3' to 5' exonuclease activity for proof reading (Weissbach *et al.*, 1973; Knopf, 1979). *In vivo* the viral polymerase is thought to form a functional complex with other viral proteins like the major and other DNA-binding proteins and the alkaline exonuclease (Purifoy and Powell, 1981; Littler *et al.*, 1983; Chiou *et al.*, 1985).

(ii) Alkaline exonuclease

Infection of cells with HSV induces a polypeptide of approximately 85,000 mw (Marsden *et al.*, 1978) associated with high levels of alkaline exonuclease activity (Keir and Gold, 1963; Morrison

and Keir, 1968; Hay et al., 1971; Hoffmann and Cheng, 1978). This virus coded enzyme has been mapped by marker rescue of ts leisions to 0.145 to 0.185 m.u. of the genome (Preston and Cordingley, 1982). It possesses an endonuclease activity besides its 5' and 3' exonuclease activities (Hoffmann and Cheng, 1979; Hoffmann, 1981) and appears to be essential for viral replication (Moss, 1986). Its function in vivo either in DNA replication in association with DNA polymerase (Francke and Garrett, 1982) or in degradation of host cell DNA during viral infection (Wildy et al., 1961; Hoffmann and Cheng, 1978) is not yet clear.

(iii) Topoisomerase

Topoisomerases produce either transient single strand breaks or double strand breaks and thus interconvert the topological isomers of DNA. Topoisomerases in general are believed to be involved in DNA replication, transcription and recombination (Gellert, 1981). HSV-1 induced topoisomerase activity has been reported (Biswal et al., 1983; Leary and Franke, 1984; Muller et al., 1985), and has been suggested to be a component of the virion envelope or tegument structure (Muller et al., 1985).

(iv) Thymidine kinase (tk)

This enzyme is encoded by a 1.2 kb coding sequence mapping between 0.30 and 0.31 m.u. The virus coded thymidine kinase (tk) (Dubbs and Kit, 1964; Munyon et al., 1971) has been shown to phosphorylate thymidine, deoxycytidine (Jamieson and Subak-Sharpe, 1974; Jamieson et al., 1974) and thymidylate (Chen and Prusoff, 1978). It is also associated with a nucleoside phosphotransferase activity in converting thymidine to thymidylate which possibly requires a component of the cellular thymidine kinase system (Jamieson et al.,

1976; Falke et al., 1981). Virus mutants lacking tk activity have shown that the enzyme, though non-essential in tissue culture, is essential for growth of the virus in serum starved cells (Jamieson et al., 1974; Fisher and Preston, 1986). The tk enzyme is, therefore, important in cells with a low level of de novo pyrimidine synthesis. Similarly, viruses with no tk activity have been shown to have reduced pathogenicity in experimental animals (Field and Wildy, 1978).

(v) Ribonucleotide reductase

A ribonucleotide reductase activity different from that of the cellular enzyme has been demonstrated following infection with both HSV-1 and HSV-2 (Cohen, 1972; Ponce de Leon et al., 1977; Averett et al., 1983; Huszar et al., 1983). The enzyme catalyses the reduction of all four ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphates and thus is essential for DNA replication in both prokaryotes and eukaryotes (Thelander and Reichard, 1979). Infection of cells at the NPT with the HSV-1 mutant, ts 1207 which has a mutation in the gene resulted in 100-fold reduced growth over a 24 hr period, indicating that ribonucleotide reductase is essential for virus replication and is virus coded (Dutia, 1983; Preston et al., 1984). Like its cellular counterpart, ribonucleotide reductase appears to be composed of a complex of two polypeptides of approximately 136,000 (RR1) and 38,000 (RR2) mw both of which are thought to be essential for the enzyme activity (Frame et al., 1985; Bacchetti et al., 1986). Both RR1 and RR2 polypeptides encoded by HSV-1 and HSV-2 have been sequenced (McLauchlan and Clements, 1983; Swain and Galloway, 1986; Nikas et al., 1986). Virus-induced enzyme activity encoded by sequences between 0.51 and 0.587 m.u. of the HSV genome has been shown to be abolished by a synthetic oligopeptide corresponding to the carboxyterminus of the 38,000 mw polypeptide component (Frame

et al., 1985). The lack of enzyme activity is believed to be due to the inhibition of an interaction between the two sub-unit polypeptides (Cohen et al., 1986; Dutia et al., 1986).

(vi) Deoxyuridine-5'-triphosphate nucleotidohydrolase (dUTPase)

The enzyme dUTPase catalyses the hydrolysis of dUTP to dUMP and pyrophosphate. In cells it reduces the intracellular concentration of dUTP and, as a consequence, minimises incorporation of dUTP into DNA. Thus it provides the cell with a pool of dUMP which is converted to TMP by thymidylate synthetase.

Virus specific dUTPase activity is induced in cells following infection with HSV-1 or HSV-2 (Wohlrab and Francke, 1980; Williams, 1984). Unlike the host cellular dUTPase, most of the HSV-1 induced enzyme activity is found in the nuclear fraction of cells and is active at 4°C (Wohlrab and Francke, 1980; Caradonna and Cheng, 1981) while HSV-2 induced activity is found predominantly in the cytoplasmic extracts of fractionated cells (Wohlrab et al., 1982). dUTPase activity in HSV-1 has been mapped to a gene between 0.69 and 0.70 m.u. (Preston and Fisher, 1984) which codes for 39,000 mw polypeptide. HSV-1 mutants lacking dUTPase activity have been shown to grow normally in cell culture (Fisher and Preston, 1986).

(vii) Uracil-DNA glycosylase

A virus-specific uracil-DNA glycosylase activity has been detected in cells infected with HSV (Caradonna and Cheng, 1981). Though the virus-induced enzyme differs from the host cell enzyme on a biochemical basis (Caradonna and Cheng, 1981), it is yet to be shown to be virus encoded. The host cell counterpart has been shown to be involved in removal of deaminated cytosine residues produced as a result of deamination of incorporated deoxycytidine monophosphate (dCMP) residues from DNA. The enzyme is thought to act as an

antimutator or editing enzyme.

(viii) Protein kinase

DNA sequencing studies have revealed that the US3 genes of HSV-1 and HSV-2 (McGeoch et al., 1985, 1987) and the corresponding gene of varicella zoster virus (Davison and Scott, 1986) encode proteins that are clearly homologous with the members of the protein kinase family of eukaryotes (McGeoch and Davison, 1986b). The predicted mw of polypeptides encoded by the US3 genes of HSV-1 and HSV-2 are 53,000 mw. Recently Frame et al (1987) using an oligo-peptide induced antiserum specific for the HSV-1 US3 have identified a 68,000 mw protein from cells infected with HSV-1.

1.10.b. HSV-induced DNA-binding proteins

Among the various proteins induced following HSV infection, atleast 17 have been reported to have DNA binding properties, as monitored by DNA-cellulose affinity chromatography (Bayliss et al., 1975; Powell and Purifoy, 1976). Some of these like the major DNA-binding protein (MDB) (Littler et al., 1983), the viral DNA polymerase (Powell and Purifoy, 1977) and the 65,000 (65K) mw DNA binding protein (Marsden et al., 1987; MacLean et al., 1987) are well characterized. The functions of most of the other DNA binding proteins are not yet known.

(i) Major DNA binding protein (MDB)

The 128,000 mw major DNA-binding protein (ICP8), an abundant delayed early gene product is implicated as an important regulatory factor in HSV replication (Lee and Knipe, 1983). It has been extensively purified, found to be nonphosphorylated (Powell et al., 1981) and to bind preferentially to single-stranded DNA (Ruyechan and Weir, 1984; Lee and Knipe, 1985). The gene coding for the enzyme has

been mapped to 0.38 to 0.41 m.u. (Rafield and Knipe, 1984). The enzyme has been shown to be essential for viral DNA replication and for normal levels of synthesis of late polypeptides (Conley et al., 1981) as mutants in the gene gave altered patterns of delayed early and late gene products (Godowski and Knipe, 1983). Genetic studies of Leinbach et al (1976) and Chiou et al (1985) have demonstrated that the MDB functionally interacts with DNA polymerase, the alkaline exonuclease, and 65,000 mw DNA binding protein. Temperature sensitive mutants within or near the gene fail to synthesise HSV DNA at the NPT (Conley et al., 1981; Littler et al., 1983; Weller et al., 1983) indicating its absolute requirement for virus DNA replication.

(ii). The 65,000 mw (65K) DNA-binding protein

Plasmid borne assays to identify essential genes for HSV replication have indicated that the 65K DNA binding protein is one of the seven genes essential for replication of plasmid molecules containing an HSV-1 origin of DNA replication (Challberg, 1986, reviewed by McGeoch, 1987). The 65K DNA binding protein is distinct from the 65K virion transactivating factor (Marsden et al., 1987). It has a strong DNA binding property and can bind to DNA in the absence of other proteins, except HSV DNA polymerase (Bayliss et al., 1975; Powell and Purifoy, 1976; Vaughan et al., 1985). The gene coding for the 65K DNA binding protein is located between 0.574 and 0.682 m.u. on the HSV-1 genome and the polypeptide is post-translationally modified by phosphorylation. The exact function of this polypeptide is not yet fully understood.

Besides the above a number of other virus polypeptides have been reported to interact with virus DNA. These include the IE polypeptide Vmw 175 which specifically interacts with the promoter-regulatory regions of a number of HSV genes (Beard et al., 1986; Kristie and

Roizman, 1987); Vmw 21 and Vmw 22 which specifically interact with the HSV 'a' sequence (Dalziel and Marsden, 1984; MacLean et al., 1987). Some of the structural polypeptides like Vmw 145K, 87K, 43K, the major capsid protein of HSV have been reported to have DNA binding activities (Bayliss et al., 1975; Powell and Purifoy, 1976). The precise functions of these polypeptides are not yet known. Elias et al (1986) from nuclear extracts of HSV infected cells, have partially purified a polypeptide which specifically binds to Ori_S in a nitrocellulose filter-binding assay. The exact role of this polypeptide remains to be determined.

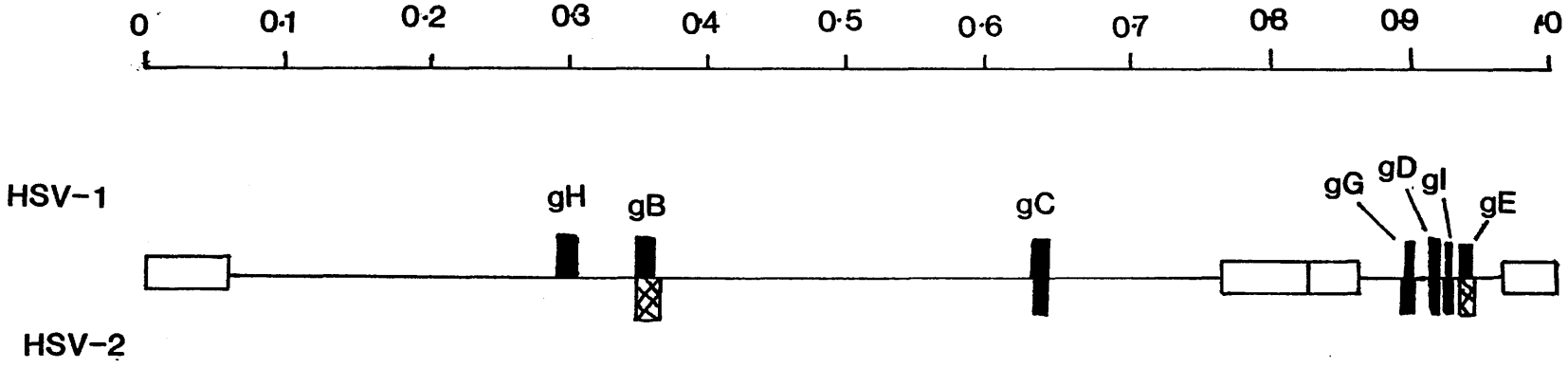
1.10.c. HSV-induced glycoproteins

HSV induced glycoproteins besides polyamine and spermidine (Gibson and Roizman, 1971) form the major constituents of the virus envelope. Of seven glycoproteins i.e. gB, gC, gD, gE, gG, gH and gI (Spear, 1976; Marsden et al., 1978, 1984; Bauke and Spear, 1979; Buckmaster et al., 1984; Roizman et al., 1984; Longnecker et al., 1987; McGeoch et al., 1987), encoded by HSV-1, all except gI, have been shown to be constituents of the virus envelope. An HSV-2 counterpart for gB, gC, gD, gE and gG has been reported on the basis of biochemical or immunological criteria or on DNA sequence analysis (reviewed by Spear, 1985; Marsden, 1987). The map locations of genes encoding various glycoproteins is indicated in Figure 18.

Studies with ts mutants and deletion mutants in genes coding for various glycoproteins have indicated that gC, gE, gG and gI are dispensable for growth of virus in tissue culture (Hoggan and Roizman, 1959; Heine et al., 1974; Cassai et al., 1975/76; Holland et al., 1984; Zezulak and Spear, 1984; Longnecker and Roizman, 1986, 1987; Longnecker et al., 1987; Weber et al., 1987; Harland & Brown, 1988). Inability of ts mutants, having mutations in genes encoding for gB and

FIGURE 18

Location of the genes encoding HSV glycoproteins (Marsden, 1987). The HSV genome is represented in the prototype orientation with fractional genome length (m.u.) above the line. The approximate map locations of the glycoproteins are indicated. ▮ refers to genes which have been sequenced. ▨ indicates genes which have not been sequenced. HSV-1 encoded glycoproteins are indicated above the line while HSV-2 encoded counterparts are indicated below the line.


0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
HSV-1
gH
gB
gC
gG
gD
gI
gE
HSV-2

gH, to grow at the non-permissive temperature (Manservigi et al., 1977; Little et al., 1981; Weller et al., 1983) indicates that gB and gH are essential for lytic growth of the virus. The requirement for gD in a lytic infection with HSV is at present unknown.

Some of the important properties of HSV glycoproteins are summarized in Table 1. Glycoproteins gB and to a lesser extent gD are involved in adsorption of virus to cells as antibodies to gB and to a lesser extent to gD and gC, inhibited the attachment of virosomes (liposomes containing purified virion polypeptides) to cells (Johnson et al., 1984). Fuller and Spear (1985) have also demonstrated that antibodies against gC and high concentrations of the Fc fragments of immunoglobulin (Ig)G can inhibit adsorption. However, it is unlikely that either gC, gE or gI are essential components of the virus receptor because all the three have been shown to be non-essential for virus growth, at least, in tissue culture.

Glycoproteins gB and gD have also been shown to play a role in virus penetration. Temperature sensitive mutants containing mutations in the gB gene although adsorbing to cells, fail to penetrate (Sarmiento et al., 1979; Haffey and Spear, 1980; Little et al., 1981). Recently Fuller and Spear (1987) and Highlander et al (1987) while investigating, by immuno gold electron microscopy the fate of infectious virus and virus neutralized with monoclonal antibodies against gD, concluded that the neutralizing antibodies inhibited fusion of the virion envelope (penetration) with the plasma membrane but not its attachment (adsorption).

A number of glycoproteins have been reported to be involved in syncytium formation following infection of cells with virus. At least seven, and possibly eight, genetic loci have been mapped for syncytium formation (reviewed by Spear, 1985; Marsden, 1987). The glycoproteins

TABLE 1

Properties of HSV-encoded glycoproteins

Glycoprotein/map location		Possible functions
gB	0.35 - 0.37	Essential for lytic growth; implicated in adsorption, penetration and cell fusion.
gC	0.63 - 0.64	Dispensable for growth in tissue culture. Implicated in adsorption, penetration and cell fusion. gC-1 can act as a C_3b-receptor.
gD	0.91 - 0.927	Implicated in adsorption, possibly penetration, and in cell fusion. No ts mutants have yet been described.
gE	0.924 - 0.951	Dispensable for lytic growth, implicated in adsorption, interacts with gI to form a Fc-receptor.
gG	0.89 - 0.9	Dispensable for growth in tissue culture.
gH	0.27 - 0.312	Essential for virus replication in tissue culture. Implicated in cell fusion and cell-to-cell spread of infectious virus.
gI	0.919 - 0.927	Dispensable for growth in tissue culture. Interacts with gE to form a Fc-receptor.

involved in this process include gB, gD and gH (reviewed by Marsden, 1987).

A possible role of gH in egress of virus from infected cells has also been shown. A monoclonal antibody against gH has been shown to inhibit plaque formation suggesting the involvement of gH in cell-to-cell spread of infectious virus (Buckmaster et al., 1984).

Glycoprotein gC-1 and not gC-2 has been shown to act as the receptor for the C_3b component of complement (Friedman et al., 1984). This property of gC-1 has been attributed to a sequence of 28 amino acids which are absent in gC-2 (Frink et al., 1983; Dowbenko and Lasky, 1984; Swain et al., 1985).

Glycoprotein gE has been shown to bind to the Fc portion of IgG (Bauke and Spear, 1979; Johansson et al., 1984).

On immunizing animals with HSV virions or virus infected cells, all the HSV glycoproteins except gI have been shown to elicit the production of neutralizing antibodies (reviewed by Marsden, 1987). Monoclonal antibodies specific for gB, gC, gD, gE and gH have been reported to have neutralizing activity (Pereira et al., 1980; Balachandran et al., 1982; Holland et al., 1983) and have been shown to confer possible protection on laboratory animals against a potentially lethal dose of HSV (Marsden, 1987).

HSV encoded glycoproteins also appear to be involved in cell mediated immunity. Passive protection conferred by the non-neutralizing antibodies to gB, gC, gD, and gE against a lethal HSV challenge indicates that these glycoproteins act as targets for antibody-dependent cell-mediated cytotoxicity (Balachandran et al., 1982; Schrier et al., 1983). Rector et al (1984) demonstrated that antigB and gD antibodies which did not recognise cell-free virus were able to confer passive protection in mice against a lethal challenge dose of HSV, again demonstrating the role of HSV glycoproteins in

induction of cell mediated immunity.

1.11. HERPES SIMPLEX VIRUS GENETICS

The genetics of HSV has for the most part begun with the isolation and characterization of virus mutants. These mutants enable individual genes to be identified and their functions analysed. Isolation of a wide variety of conditional lethal mutants such as temperature sensitive (ts), drug resistance and host range and other mutants such as deletion mutants and restriction enzyme site deletion mutants has, helped in our understanding of HSV gene functions. The various classes of HSV mutants isolated and analysed are summarised in this section.

1.11.a. Temperature sensitive (ts) mutants

Temperature sensitive mutants are generally produced by missense mutations, which alter the nucleotide sequence of the wild type virus in such a way that the resulting protein is unable to assume or maintain its correct functional configuration at the non-permissive temperature (NPT). Because of the very low level of spontaneous ts mutations in wild-type virus stocks, most ts mutants of HSV have been obtained by artificially induced mutagenesis with a variety of agents like bromodeoxyuridine, nitrous acid, hydroxylamine, nitrosoguanidine or UV light (Schaffer et al., 1970; Timbury, 1971; Schaffer et al., 1973; Brown et al., 1973; Manservigi, 1974; Esparza et al., 1974). Although, theoretically, ts mutations can be introduced into any gene, the screening procedure results in the selection of ts mutants with defects in essential genes only.

Complementation analysis and marker rescue of ts mutants with purified DNA fragments has identified and mapped more than 35 genes (Schaffer et al., 1978; Stow et al., 1978; Stow and Wilkie, 1978).

Interestingly, no ts mutants have so far been reported in the U_S region of the genome. Characterization of ts mutants of HSV has helped the understanding of molecular events involved in various stages of the virus replication cycle such as penetration (Sarmiento et al., 1979; Little et al., 1981; Addison et al., 1984), uncoating (Batterson et al., 1983), regulation of virus gene expression (Preston, 1979b; Sacks et al., 1985) and DNA encapsidation (Preston et al., 1983). Grouping of various ts mutants of HSV into different complementation groups and their recombination analyses has led to construction of linkage maps (Brown et al., 1973; Brown and Ritchie, 1975a,b; Schaffer et al., 1974; Timbury and Calder, 1976).

1.11.b. Drug resistant mutants

Use of antiviral drugs such as 5-bromo-2-deoxyuridine (BUDR) has resulted in production of mutants of HSV and indirectly the identification of virus genes conferring resistance to these drugs.

Virus growth in the presence of the thymidine analogues BUDR (Kit and Dubbs, 1963; Dubbs and Kit, 1964) or the deoxycytidine analogue, 5-bromo-2'-deoxycytidine (BCDR) (Brown and Jamieson, 1977; Stow et al., 1978) in tk- cells gives rise to thymidine kinase negative (tk^-) mutants which utilise the de novo pathway of thymidine synthesis and thus avoid the incorporation of lethal quantities of nucleoside analogues into the virus DNA. Mutants resistant to acycloguanosine (ACG or Acyclovir) have been reported (Field et al., 1980). These mutants are generally tk^-, but tk^+ ACG-resistant virus specifying a DNA polymerase which fails to recognise the phosphorylated form of ACG have been isolated (Elion et al., 1977; Crumpacker et al., 1980; Darby et al., 1981; Larder and Darby, 1985). Mutations in the HSV DNA-polymerase gene also confer resistance to ACG (Schnipper and Crumpacker, 1980; Coen and

Schafer, 1980).

The drug, phosphonoacetic acid, directly interacts with the pyro phosphate binding site on the DNA polymerase (Leinbach et al., 1976) and has been shown to inhibit not only in vivo replication of HSV but also its activity in vitro (Mao et al., 1975). Mutants of both HSV-1 and HSV-2 showing resistance to PAA have been isolated (Hay and Subak-Sharpe, 1976; Purifoy and Powell, 1981; Lee et al., 1978).

1.11.c. Host range mutants

Two host range mutants of HSV which are able to grow in some cell lines but not in others, have been documented. (1) A mutant of HSV-1 strain MP able to grow in non-permissive dog kidney cells after several weeks of continuous propagation of wild-type virus (Roizman and Aurelian, 1965) has been shown to have overcome a block at the nucleocapsid envelopment stage of virus morphogenesis observed in infection with the wild-type virus (Spring et al., 1968). (2) A ts host range mutant of HSV-2, which in contrast to the parental virus, fails to replicate in hamster embryo fibroblasts and mouse fibroblasts cells at 39°C, has been reported (Komet and Rapp, 1975a,b). The growth of the ts host range mutant in vivo corresponded with its in vitro growth properties, as the mutant was virulent in mice but attenuated in hamsters.

Recently a number of cell lines which carry stably integrated HSV genes such as the IE-3 gene (Davidson and Stow, 1985; DeLuca et al., 1985) and the IE-1 gene (Stow and Stow, 1986) have been constructed. In contrast to their parent cell lines, these biochemically transformed cell lines, because of induction of the resident wild-type gene, allow the propagation of mutant virus having a deletion in that gene. Analysis of such host range mutants helps in the study of virus induced polypeptide functions and their essentiality in the virus

replicative cycle.

1.11.d. Plaque morphology mutants

In contrast to the individual cell rounding (syn$^+$ plaques) normally associated with the cytopathic effect of HSV, variants have been isolated which produce cell fusion or syncytia (syn plaques) formation (Roizman, 1962; Ejercito et al., 1968; Brown et al., 1973; Timbury et al., 1974). The plaque morphology phenotype has been used as an unselected marker in three-factor reciprocal genetic crosses (Brown et al., 1973). Formation of heteroduplex molecules during genetic recombination has been speculated to generate mixed plaques containing both syn and syn$^+$ morphologies (Brown and Ritchie, 1975a,b). In doubly infected cells, the syn$^+$ allele is dominant, but as the plaque grows, syn$^+$ and syn segregate and form sectors (Keller, 1976). Eight different loci involved in syncytium formation have been mapped on the HSV genome (reviewed by Marsden, 1987).

1.11.e. Immune cytolysis-resistant mutants

The immune cytolysis-resistant mutants of HSV are characterized by their ability to render mutant virus-infected cells resistant to complement-mediated immune cytolysis with antisera directed against virus specific glycoproteins, due to altered synthesis, processing or incorporation of glycoproteins into infected cell membranes (Machtiger et al., 1980; Glorioso et al., 1980; Pancake et al., 1983).

Similarly monoclonal antibody-resistant (mar) mutants possess mutations affecting the antigenic sites of glycoproteins exposed on the virion envelope, and hence are resistant to these monoclonal antibodies plus complement (Holland et al., 1983).

1.11.f. Restriction endonuclease site deletion mutants

Mutants of both HSV-1 and HSV-2 deleted in XbaI restriction

endonuclease sites have been isolated (Brown et al., 1984; Harland and Brown, 1985; MacLean and Brown, 1987a; Harland and Brown, 1988). These mutants have been shown to exhibit normal growth characteristics in tissue culture and have been used in super-infection experiments to rescue viral DNA information from latently infected cultures (Cook and Brown, 1987). The mutants will be useful in the analysis of the mechanisms and kinetics of HSV recombination. The use of restriction endonuclease sites as unselectable markers in conjunction with selected ts markers to study both intertypic and intratypic recombination in HSV will elucidate the role in recombination of parental and progeny molecules and genome homology.

The restriction site deletion mutants by virtue of their structure allow the possibility of introducing new restriction sites at desired positions for use as eukaryotic vectors or as recipients for mutagenized cloned viral fragments.

1.11.g. Deletion mutants

Although ts mutants have been useful in determining essential HSV genes, they have a number of drawbacks. Firstly, ts mutants are only isolated in essential genes; secondly, the leakiness or reversion of the ts mutation to the parental phenotype makes their analyses more difficult. These problems have been overcome by the use of deletion mutants sometimes in conjunction with complementing cell lines.

Removal of the entire or a large part of the open reading frame of a gene in deletion mutants will unambiguously determine its essentiality and no reversion is possible. Deletion variants which arose spontaneously or by deliberate construction have determined several non-essential genes in vitro including IE-1 (Stow and Stow, 1986; Sacks and Schaffer, 1987); two genes at the right of U_L, UL55 and UL56 encoding for polypeptides of 20K and 22K respectively (Perry

et al., 1986; MacLean and Brown, 1987b); tk (Sanders et al., 1982) and all the genes located in U_S except US6 which codes for gD (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987; Longnecker et al., 1987; Weber et al., 1987).

1.11.h. Complementation in HSV

Complementation is a non-genetic interaction between the products of the different genomes in cells simultaneously infected with two mutants. On the basis of complementation tests most ts mutants of HSV have been assigned to different cistrons (Timbury, 1971; Brown et al., 1973; Crombie, 1975; Schaffer et al., 1978). Two types of complementation namely intergenic and intragenic have been reported. In intergenic complementation, mutants with defects in different genes are, at the restrictive temperature, able to supply the functional product required by the other mutant while in intragenic complementation the function of the defective gene may be restored, at least in part, by the formation of a hybrid protein containing two defective products from mutants which have the mutation at different sites in the same gene. This type of complementation has been observed between tk^- mutants of HSV-1 where it was detected biochemically (Jamieson and Subak-Sharpe, 1978).

Of the two main quantitative complementation tests namely the progeny yield test and the infectious center test (Brown et al., 1973; Messer, 1978) the former is generally considered more reliable since fewer recombinant viruses are generated. Complementation indices more than 2 or 4 are considered positive which means that the mutants under test lie in different genes (Timbury, 1971; Brown et al., 1973). Intertypic complementation between HSV-1 and HSV-2 has also been demonstrated (Timbury and Subak-Sharpe, 1973).

1.11.i. Recombination HSV

Using lesion morphology on chorio-allantoic membranes and virulence of progeny virus in mice as indicators, recombination in HSV was first demonstrated by Wildy (1955). Recombination in HSV was later confirmed by Subak-Sharpe (1969) by detecting ts^+ recombinants among the progeny of crosses between pairs of ts mutants. Genetic linkage maps based on the analysis of recombination frequencies between ts mutants, were constructed for HSV-1 (Brown et al., 1973; Schaffer et al., 1974) and HSV-2 (Benyesh-Melnick et al., 1974; Timbury and Calder, 1976). These investigators observed that efficient recombination occurred between mutants which complemented well whereas mutants in the same complementation group either failed to recombine or recombined poorly. Mostly recombination analysis involved two factor crosses whereas Brown et al (1973) and to a limited extent Timbury and Calder (1976) by incorporating a plaque morphology marker, were able to use reciprocal three factor crosses. The linkage maps have generally been correct in the orders of ts lesions but the genetic distance between the markers did not accord with their physical map locations possibly due to multiple cross-overs between distant markers (Stow et al., 1978; Wilkie et al., 1978). The close correlation between the genetic and physical maps was observed but the case of HSV-2 strain HG52 (Timbury and Calder, 1976; Wilkie et al., 1978), was an exception to this observation.

Intertypic recombination has also been observed between HSV-1 and HSV-2 as evidenced by isolation of ts^+ virus from crosses of ts mutants of HSV-1 and HSV-2 (Timbury and Subak-Sharpe, 1973; Esparza et al., 1974). Such recombinants contain DNA sequences derived from both parental viruses and specify both HSV-1 and HSV-2 polypeptides and antigens (Halliburton et al., 1977; Wilkie et al., 1977; Morse et al.,

1977, 1978; Preston et al., 1978; Marsden et al., 1978). Intertypic recombinants have been invaluable in mapping virus genes encoding specific polypeptides but of little use in understanding recombination in HSV per se.

Site specific recombination has also been observed in HSV. The sequences within the 'a' sequence promote recombination that results in inversion of the U_L and U_S components of the genome leading to the generation of four isomers in equal proportions. Evidence for this has come from the studies indicating that (1) insertion of an additional 'a' sequence into the tk gene results in novel rearrangements of the genome, consistent with inversion of any genome segment bounded by inverted copies of the 'a' sequence (Chou and Roizman, 1985; Mocarski et al., 1980; Mocarski and Roizman, 1981, 1982a). (2) deletion of sequences containing the 'a' sequence from the junction between the L and S components prevents inversion of the L and S components (Poffenberger et al., 1983). The functional significance of inversions in the HSV genome is yet obscure.

Sequences present in the BamHI fragment 1 (0.706 to 0.744 m.u.) have also been shown to cause inversions (Pogue-Geile et al., 1985) in that insertion of a second copy of these sequences in an inverted orientation into the tk gene results in inversion of unique sequences bounded by these inverted repeats due to recombination between copies of the BamHI 1 sequences. This recombination event was later on found to be intramolecular since the genetic marker (syn^{-1} or syn^{-1+}) originally present in one copy of BamHI 1 appeared in progeny at both normal and inserted loci at a high frequency (Pogue-Geile and Spear, 1986).

The mechanism of recombination in HSV is yet relatively poorly understood. Very little, if at all, is known about the virus factors such as genome sequences (hot spots) involved in recombination and

what role, if any, the different genomic isomers play in recombination (Honess et al., 1980). It is also unclear whether recombination involes both progeny and parental molecules. The role played by host factors, if any, is also far from clear (Das Gupta and Summers, 1980).

Intratypic recombination between two strains of HSV-1 using restriction enzyme sites as unselected markers, has indicated that no area of the virus genome has an excess of recombination from the overall recombination frequency of 0.007 per Kbp (Umene, 1985). Honess et al (1980) while studying recombination between pairs of selected and unselected markers in two different strains observed two-factor recombination frequency of 2 to 40% and no satisfactory correlation with distance between the markers was found. In this case a lack of homology in different areas of the genome or incompatibility of gene products from different strains may have influenced the recombination process.

Occurrence of four isomeric forms of the HSV genome complicates the analysis of recombination. Although analysis of recombinants involving markers within U_S or U_L is not affected, the analysis of recombinants between markers located in the two different segmens of the genome is complicated by the genome isomerization. Honess et al (1980) while investigating recombination between structural and regulatory genes of HSV-1, using a variety of markers of known physical map locations in addition to ts mutations suggested a circular recombination map. This could be explained by involvement of either all the 4 isomers, or recombination between 2 circular molecules formed following infection (Davison and Wilkie, 1983a; Poffenberger et al., 1983; Poffenberger and Roizman, 1985) or between the long concatamers of head-to-tail molecules formed during replication (Jacob et al., 1979). Taken together these reports

indicate that more than one isomer and probably either circular or concatameric molecules are involved in recombination.

It is not yet resolved whether both progeny and parental molecules take part in recombination. Ritchie et al (1977) demonstrated that recombination in HSV increases with time, indicating that both parental and progeny molecules are involved. The evidence for this was obtained from two and three factor crosses with ts mutants and syncytial plaque morphology (syn) mutants of HSV. Under the conditions of a standard genetic cross until about 20 hr post infection about 5% of plaques were of a mixed syn/syn$^+$ morphology. These mixed morphology plaques remained constant and did not increase with time unlike the proportion of ts$^+$ recombinants. However, in the presence of 5-fluorodeoxyuridine, an inhibitor of DNA synthesis, a several fold increase in the frequency of recombinants and mixed morphology plaques was observed. These observations are in contrast to those in PRV in which only parental molecules were shown to be involved in recombination (Ben-Porat et al., 1982). Recombination studies with restriction site deletion mutants of HSV (Brown et al., 1984; Harland and Brown, 1985; MacLean and Brown, 1987a) will allow not only investigation of the role of parental and progeny molecules in recombination but also the contribution of specific viral factors such as specific DNA sequences/genes.

1.12. MORPHOLOGICAL TRANSFORMATION BY HERPES SIMPLEX VIRUS

Transformation of cultured cells by HSV was first reported by Duff and Rapp (1971) using UV-inactivated HSV-2 to morphologically transform mouse 3T3 cells. Subsequently specific fragments of HSV-1 and HSV-2 DNA were shown to induce morphological transformation of rodent cells in culture (Camacho and Spear, 1978; Reyes et al., 1979; Galloway and McDougall, 1981; reviewed by Macnab, 1987). An unexpected

outcome of this work was the finding that most of the viral DNA was lost from the transformed cells (Galloway and McDougall, 1983). Skinner (1976) suggested a 'hit and run' mechanism to describe this type of transformation in which there was no continuous expression of virus DNA. Later on this was shown to be due to successive loss of HSV DNA sequences on passage of transformed cells (Minson et al., 1976).

Three diffeent regions of HSV DNA have been implicated in morphological transformation of cultured cells. i) MTRI (morphological transformation region of HSV-1 (Camacho and Spear, 1978) maps in XbaI f (0.29 to 0.45 m.u.) and BglII i (0.311 to 0.415 m.u.) (Reyes et al., 1979). ii) MTRII is the morphological transformation region of HSV-2 which maps in BglII n (0.58 to 0.62 m.u.) (Reyes et al., 1979; Macnab and McDougall, 1980; Galloway and McDougall, 1981; Cameron et al., 1985); iii) MTR III or second region of HSV-2 (Pe)den et al., 1982) maps in HSV-2 BglII c (0.54 to 0.58 m.u.) (Jariwalla et al., 1983).

A small stem/loop structure of 737 bp in HSV-2 BglII n was proposed to induce morphological transformation (Galloway et al., 1984). This subfragment is not large enough to encode a transforming protein and lies completely outside the coding sequences of both the large and small units of the ribonucleotide reductase gene. The transforming ability of the sub-fragment has been attributed to its apparent potential to adopt a stem/loop structure like that of insertion-like sequences and may cause activation of cellular genes through enhancer-like activity (Galloway et al., 1984). HSV-1 lacks a similar structure in the corresponding region of the genome.

Similarly Jariwalla et al (1986) suggested that the Bgl II c fragment of HSV-2 contains two independent transforming and immortalizing functions. The tansforming function was mapped to a 480 bp subfragment which was subsequently sequenced (Jones et al., 1986). The sequences within this subfragment are considered to adopt a

stem/loop structure and may be involved in activation of cellular genes. No DNA sequences of more than 500 bp from the corresponding region of HSV-1 are consistently retained in transformed cells (Cameron et al., 1985). The insertion like sequences reported by Galloway et al (1984) have frequently been found in other regions of HSV DNA; proof of a biological function therefore, for these is required (reviewed by Macnab, 1987).

The precise mechanism of transformation by HSV is still unclear but two observations whereby specific fragments of HSV could transform cells in culture, are as follows:

1. Mutagenesis: Infection with inactivated HSV-1 was mutagenic for the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene of HSV replication permissive human rhabdosarcoma cells (Schlehfer and zur Hausen, 1982). Similarly, infection with HSV-2 in non-permissive XC cells increases the natural mutation frequency of the cellular HGPRT gene by up to 10 times (Pilon et al., 1985). Such mutagenic activity may be an important role of HSV in oncogenesis but the molecular mechanisms deserve further investigation.

2. Gene amplification: Infection of some SV-40 transformed hamster cells with HSV-1 induces amplification of SV40 DNA in these cells (Schlehofer et al., 1983) and is dependent upon the presence of a functional HSV-1 DNA polymerse (Matz et al., 1984). Infection with HSV-1 or HSV-2 also causes upregulation of some cellular polypeptides, some of which accumulate to higher levels in transformed cells than in controls (Macnab et al., 1985; [illegible]; reviewed by Macnab, 1987).

However, the fragments of HSV-1 and HSV-2 DNA shown to transform cells are not homologous (Reyes et al., 1979) and do not contain the

gene for DNA polymerase. The mechanism by which these small pieces of DNA carry out transformation, either by their ability to mutagenise cells, to amplify cellular DNA sequences or by upregulating host cell proteins, is yet to be determined.

1.13. HERPES SIMPLEX VIRUS AND CERVICAL CARCINOMA

The association between HSV-2 and cancer of the cervix has long been speculated upon (Naib et al., 1966; Nahmias et al., 1969). The basis of these observations had been the presence of high titers of antibodies to HSV-2 in patients with an increased risk of developing cervical carcinoma. HSV-2 DNA has been detected at least once in a cervical carcinoma biopsy (Frenkel et al., 1972). Besides the presence of HSV-2 DNA in preneoplastic lesions and carcinomas (McDougall et al., 1980, 1982; Park et at 1980, Eglin et al., 1981); the causal relationship between the presence of viral RNA in biopsies and the severity of disease has not been established (Maitland et al., 1981). It is very likely that more than one factor is involved in the development of cervical cancer. Several types of human papilloma viruses have been detected in cervical carcinomas (Durst et al., 1983). It is probable that HSV-2 may be a cofactor (co-carcinogen) in causation of cervical carcinoma by other infectious agents (Vonka et al., 1984).

1.14. HERPES SIMPLEX VIRUS LATENCY

Unlike many other human viruses which are eliminated by host-mediated defence mechanisms soon after primary infection, HSV-1 and HSV-2 have evolved a complex strategy of replication and survival wherby they persist in a latent state within the host for extended periods of time and avoid immune clearance. The complex and little understood mechanisms underlying establishment, maintenance and control of HSV latency have been the subject of many reviews

(Kirchner, 1982; Wildy et al., 1982; Blyth and Hill, 1984; Hill, 1985). In spite of elegant advances in molecular virology made in recent years, our definition of HSV latency still remains an operational one i.e. infectious virus can not be recovered from latently infected ganglia or peripheral tissues following homogenisation. However, recovery of infectious virus can be made following explantation or co-cultivation of the tissues which harbour latent virus (Wildy et al., 1982).

In the present chapter an attempt has been made to provide a brief over-view of recent developments underlying the establishment, maintenance and control of HSV latency with reference to its molecular biology. Much of the information on latency has been derived from studies in experimental animals and where available corresponding information from the human disease is incorporated. Infection with HSV-1 is not considered separately from that with HSV-2. Information from human studies and from animal models is included in the first section whereas that from in vitro models of latency is in the second section of this chapter.

Section I

1.14.a. SITES OF LATENCY

1.14.a.I. Neural sites

(i) Peripheral nervous system (P.N.S.)

The role of the peripheral nervous system in harboring latent HSV was first postulated by Goodpasture (1929) in observations of the pathogenesis of ocular HSV infections in rabbits. In human patients undergoing surgery of the trigeminal tract, Carton and Kilbourne (1952) observed that 2 to 4 days after section of the root of the fifth cranial nerve, oral or facial herpetic lesions (but not ophthalmic) occurred in about 90% of individuals provided that the

ganglion was not destroyed. These observations of Carton and Kilbourne (1952) were interpreted as indicating that virus was latent in the trigeminal ganglion and reactivation of latent virus in the skin was caused by the surgery.

Experimental evidence that HSV can indeed establish a long-term 'silent-infection' in neurones began to appear in the 1970s when Plummer et al (1970) demonstrated that 9 to 11 months after the intramuscular injection of HSV-2 into rabbits, virus could be recovered from their central nervous system (brain) and sensory ganglia by inoculation of trypsinized suspensions of these neural tissues onto cell monolayers. Such observations were confirmed and much extended in mice (Stevens and Cook, 1971) and rabbits (Stevens et al., 1972) where recovery of the latent virus was achieved 7 to 14 days after cultivation of sensory ganglia proximal to the site of inoculation, explanted 4 months after the primary infection.

Using explantation methods, HSV was isolated from 9 to 86% of human trigeminal ganglia taken at post-mortem (Bastian et al., 1972; Baringer and Swoveland, 1973; Rodda et al., 1973; Plummer et al., 1973; Warren et al., 1977). Lumbosacral ganglia have been shown to have latent HSV-2 infections in mice (Walz et al., 1977), monkeys (Reeves et al., 1976) and humans (Baringer, 1974) who had previously recovered from experimental or natural HSV-2 vaginal infections.

Cook and Stevens (1976) rigorously tested many tissues of mice that had been intravenously injected with herpes simplex virus and could only get reactivating virus from sensory ganglia, brain tissues and adrenal glands. Nesburn et al., (1972) made similar observations in rabbits. Puga et al., (1978) using solution hybridization techniques detected HSV specific nucleotide sequences in sensory ganglia from a large number of latently infected mice.

In addition to the sensory ganglia, latent virus has been

reactivated from the autonomic nerve ganglia of humans. Warrren et al., (1978) recovered latent virus from vagus ganglion (part of the autonomic nervous system) explants of humans. Autonomic ganglia of mice (Price et al., 1975; Price and Schmitz, 1978; [illegible] 1980) and rabbits (Martin et al., 1977) have also been shown to harbour latent HSV infections.

Within ganglia the neuronal body has been presumed to harbour latent HSV. The evidence for this presumption came from studies in mice and rabbits in which virus was regularly reactivable from ganglia (which contains neuronal bodies) and never from nerve roots which do not contain nerve bodies (Baringer and Swoveland, 1973; Cook et al., 1974). Cook et al., (1974) while following the time course of reactivation of virus from ganglia from latently infected mice [illegible] implanted in millipore chambers found, that virus antigen as detected by immunofluorescence, virus particles as detected by electron microscopy and thymidine incorporation into the virus DNA as detected by autoradiography, all commenced in neurones before being detectable in satellite cells, thus indicating that neuronal cell bodies harbor latent virus. The fact that neuronal cell bodies harbor latent virus, was unambiguously revealed by studies in mice with a ts mutant of HSV (McLennan and Darby, 1980). Some weeks after the primary infection explanted ganglia were incubated at either the permissive temperature (PT) or non-permissive temperature (NPT). Reactivation was regularly observed at the PT but at the NPT only the development of viral antigen as detected by immunofluorescence was observed in single neurones. Confirmation of this observation was also achieved by reactivation in vivo after nerve section.

(ii) Central nervous system (CNS)

Studies in experimental animals have revealed that HSV can be

latent in brain tissues. Cook and Stevens (1976) reported the isolation of virus from brain explants of latently infected mice in which virus had been administered intravenously. Similarly recovery of latent virus has been reported from co-cultivation of tissue explants from the CNS of latently infected rabbits (Plummer et al., 1970) and guinea-pigs (Tenser and Hsiung, 1977). Openshaw (1983) demonstrated recovery of the virus from tissue explants of retinas of mice that had survived the acute corneal infection. Embryologically and anatomically, the retina forms a part of the CNS.

Fraser et al (1981) using DNA-DNA hybridization techniques detected virus specific DNA in 7 of 11 human brains and in most cases complete virus genomes were present. Recently Efstathiou et al (1986) have confirmed the findings of Fraser et al (1981) by demonstrating virus specific DNA hybridization in CNS and TG of latently infected mice. Similarly Cabrera et al (1980) demonstrated DNA homologous to viral DNA in the brains of 30% of mice in which the incidence of latent infection by the explantation method in the brain was 5% and in the trigeminal ganglia 95%. It was argued that the apparently low level of incidence of latency on explantation of brain tissue may be due to difficulties in demonstrating its presence by the usual explantation techniques.

1.14.a.II. Extra neural sites

(i) Skin

Peripheral tissues such as skin which exhibit recurrent herpes lesions have long been suspected as sites of latent infection (Carton and Kilbourne, 1952; Carton, 1953) but attempts to culture virus from explants of human skin from areas prone to develop HSV lesions have been unsuccessful (Smith and McLaren, 1977). However, studies on the pathogenesis of HSV in experimental animals have indicated that the

virus can be latent in the skin. Scriba (1981) has shown that HSV-2 could be isolated from foot pad explants of the majority of latently infected guinea pigs in the absence of clinical lesions and any nerve supply from the lumbosacral ganglia. In guinea pigs infected with HSV-1, virus was found to persist almost entirely in the skin tissue i.e. at the site of inoculation, with little or no latent infection in the lumbosacral ganglia (Scriba, 1977; Scriba and Tatzer, 1981).

Evidence that HSV can establish a latent infection of skin has come from studies by Al Saadi et al (1983) in which mice were injected in the foot pad with HSV-2 strain HG52 and various of its ts mutants. Three months after primary infection, mice foot pads (site of inoculation of virus) and lumbosacral ganglia were explanted and infectious virus was recovered from both (skin and ganglia) several days after explantation. No infectious virus was detected in the cell free homogenates of ganglia or foot pad immediately after explantation.

It is not yet certain which cell types in skin harbor latent virus. In limited studies on foot pads of latently infected mice, viral RNA, has been demonstrated by in situ hybridization in the cells lying at the base of the hair follicles as early as 3 days after explantation (Clements,G.B.; personal communication). However, the precise cell type harboring the viral RNA has not been identified.

(ii) Cornea

Besides skin, latent virus has also been recovered from the corneal explants of latently infected rabbits (Cook et al. 1987 and this thesis) simulating the findings of latent virus in human corneal explants removed during the course of treatment for chronic stromal keratitis (Shimeld et al., 1982; Tullo et al., 1985; Easty et al., 1987).

Cook and Brown (1986, 1987) have shown that in vitro cultures of the 3 corneal cell types i.e. epithelial cells, keratocytes and endothelial cells of rabbits support latent infection with HSV induced by high temperature (42°C). The recovery of virus at 37°C from the three cell types cultured in the presence of the virus inhibitor acycloguanosine at 42°C demonstrated that corneal cells can support HSV latency and not merely virus persistence.

1.14.b. ESTABLISHMENT OF A LATENT INFECTION WITH HERPES SIMPLEX VIRUS

(i) Definition

Functionally, the latent phase of HSV infections is established when infectious virus can only be isolated from explant cultures but not from cell free homogenates of tissues or organs. Before coming to this final phase in the process, there are other important and distinct but interrelated phases, namely growth of virus in the peripheral tissues such as skin, transport of virus to the ganglion and acute infection of the ganglion which need to be mentioned.

(ii) Virus replication during primary infection

The first stage of peripheral infection with HSV in mice involves replication of virus at the site of inoculation (Hill et al., 1975) which results in characteristic lesions (Klein, 1985).

Following inoculation of virus in the ear (Hill et al., 1975) or footpad (Stevens and Cook, 1971), virus titers reach a peak in 3 to 4 days and by 14 days virus is no longer detectable from the cell free homogenates of infected skin. Kristensson et al (1971) and Cook and Stevens (1973) demonstrated that infectious virus can be isolated from the ganglia 2 days after infction of the skin or cornea even with relatively small doses (10^4 to 10^5 pfu) of virus. Studies of Field and Hill (1974) with PRV which has a similar pattern of pathology to HSV

in mice, have shown that replication of virus in the peripheral tissues is not an absolute necessity for latent infection of the nervous tissue, since with large inocula of PRV, virus was found in the ganglia as early as 17 hr after inoculation into the skin. However in HSV symptomatic primary disease has been shown to result in rapid and widespread latent infection of the CNS (Harbor et al., 1981).

(iii) Acute ganglionic infection

Following peripheral inoculation of mice, HSV can be demonstrated in spinal ganglia within 20 to 24 hr post infection. The amount of infectious virus that can be detected in the corresponding ganglia increases for the first 4 to 7 days post infection and then subsequently declines. The increase in amount of virus in the ganglia leads to the assumption that a productive neuronal infection occurs during the acute phase (Cook and Stevens, 1973). The topical application of 2% phosphonoacetic acid at the inoculation site has been shown to suppress virus multiplication at the site of inoculation with subsequent drop of virus titer in corresponding ganglia (Klein and Destefano, 1981), thus indicating that local antiviral treatment could reduce the viral titer in ganglia by halting continuous virus supply from the site of primary infection. However, the possibility that productive virus infection may occur to a limited extent has not been totally excluded. On the other hand good evidence has indicated that a productive infection is not necessary for the establishment of latent infection in the neurones of ganglia. Using HSV-1 ts mutants, Lofgren et al (1977) showed that there is no apparent relationship between the capacity of the mutants to induce encephalitis and the subsequent establishment of latent infection in brains of mice. Similarly, McLennan and Darby (1980) from their studies with ts mutants in mice concluded that there is no absolute requirement for a

productive infection during establishment of latency in neurones. Al-Saadi et al (1983) were able to recover virus from the ganglionic explants and footpad skin of mice latently infected with ts mutants of HSV-2, thus demonstrating that replication of virus was unnecessary for latent infection of ganglia as the core temperature (39°C) of the mouse is non-permissive for replication of ts mutants.

(iv) Virus pathways to the nervous system

The failure to detect HSV in the distal and proximal sections of the sciatic nerve (during the first two days after infection) and the fact that HSV is first detected in the ganglia and only afterwards in the nerves (Wildy, 1967; Kristensson et al., 1971) suggested that an alternative (haematogenous or lymphatic) route may be involved in the dissemination of virus to the nervous system. Studies of Cook and Stevens (1973) involving passive administration of anti-HSV sera in mice, demonstrated that latent infection of the ganglia could be established even in the presence of significant levels of neutralizing antibodies, thus indicating that haematogenous or lymphatic routes are not essential in spread of virus to the nervous system. Kristensson et al (1971, 1978) could not detect any virus in the blood of mice after subcutaneous inoculation of virus in the footpad, while in some other studies HSV was detected in the blood of new born mice following intranasal inoculation of virus (Kern et al., 1975). It is not known whether virus invades the nervous system subsequent to viraemia or whether viraemia is due to spill over from the peripheral nervous tissues. Direct evidence for the possible involvement of the circulatory system is the demonstration that HSV-1 selectively establishes latent infections in mouse neural tissues, particularly sensory ganglia, after intravenous inoculation. This was shown conclusively when inoculation of virus in tail veins was immediately

followed by tail amputation at 2.5 cm proximal to the infection site (Cook and Stevens, 1973). However, it is possible that nervous tissue may not be infected directly by virus in the blood stream but indirectly by virus travelling in the nerves associated with vessels or other organs that became first infected through viraemia.

Wildy (1967) showed that sciatic nerve sectioning three days prior to virus inoculation, prevents HSV spread to the spinal ganglia in 80% of mice. Moreover, Kristensson et al (1971) reported that colchicine treatment, ligature or freezing of sciatic nerve 0 to 4 days before virus inoculation into the foot pad of mice reduced the mortality rate to 10% compared to 100% if the treatment was carried out after inoculation. In neither of the above reports were attempts made to recover the virus from ganglia. Scriba (1981) demonstrated that total surgical section of the sciatic and femoral nerves completely prevented the establishment of latent HSV infection in sensory ganglia of guinea pigs inoculated into the foot pad as detected by the explantation technique. Similar experiments in mice (Klein, 1982) revealed that establishment of acute infection in spinal ganglia was prevented by section of both femoral and sciatic nerves in about 80% of mice. Free virus, however, was regularly demonstrated on the 4th and 7th day after infection in spinal ganglia of mice in which either the sciatic or femoral nerves were severed.

Further evidence supporting the axonal transport hypothesis has come from the in vitro study of attachment of HSV to rabbit astrocytic glia, neuronal perikarya and synaptosomal (nerve terminal) fractions (Vahlne et al., 1978). HSV preferentially adsorbs to the synaptosome fraction rather than neuronal perikarya. However it remains unclear whether this difference in virus attachment was due to the presence of specific HSV receptors on the synaptosomes or merely reflected a high endocytic activity of the synaptosomes.

Another observation is the rate of HSV transloction from the skin to the ganglia which has been measured for HSV (Kristensson et al., 1978; Cook and Stevens, 1973) and for PRV (Field and Hill, 1974). In all cases rates obtained were in the range of 2 to 10 mm per hr which is consistent with the rates of movement of macromolecules and organelles by retrograde axonal transport (reviewed by Kristensson, 1978).

Taken together it seems that axonal transport is the most important method of virus translocation but the virus may also travel by other routes. Irrespective of the way the virus travels to the nervous system, the subsequent result is the establishment of latent infections.

(v) Establishment per se

Following primary infection, the amount of detectable HSV in cell free ganglion homogenates decreases gradually, and by 14 days post infection, virus can no longer be detected. However, HSV can still be recovered by explantation or cocultivation of ganglia. At this point the acute phase is terminated and the latent phase of the infection begins (Openshaw et al., 1979b; Klein, 1982). The transition from the 'acute' to the 'latent' phase of infection, coincides with the appearance of immune responses particularly high levels of antibodies. It has been proposed that immune factor(s) play a major role in the switch from the acute to the latent phase of infection (Openshaw et al., 1979a, 1981), though the mechanism by which these immune responses could produce transition from the acute to the latent state of infection is not clear. Openshaw et al (1981) have proposed some hypothetical models to explain the role of the immune response in modulation of HSV infection. These models are: (1) The immune modulation model which assumes that ganglion cells are permissive but the immune response to HSV modulates the infection at the level of the

ganglion; converting a potentially lytic infection into the non-lytic or latent infection. (2) The immune elimination model; there are two populations of ganglionic cells: permissive and non-permissive. A productive HSV infection occurs in the permissive cells and a non-lytic or latent infection occurs in the non-permissive cells. The host immune response strictly has nothing to do with the establishment of latency, but just eliminates only acutely infected cells. (3) The third model assumes, that ganglion cells are ordinarily non-permissive for HSV replication, however they become permissive after receiving 'signals' induced by inflammatory reactions in the skin due to virus replication. The host immune response then turns off these signals by decreasing virus replication in the skin, returning ganglion cells to the non-permissive state hence latency is established.

The above three models rely solely on immune mechanisms to account for the transition from the acute to the latent state of infection. Another model has been proposed by Klein (1982) which assumes that ganglion cells are non-permissive; colonization of the ganglia is achieved by continuous virus supply from the site of primary infection. Immune responses restrict virus replication in mucocutaneous tissue. It is suggested that the majority of virions in neurones are inactivated by cellular enzymes and only a minority will be in a latent form in cellular structures.

It must be emphasised, that the above models are merely hypothetical and there is no hard evidence to support them. Besides, the models do not consider the possible establishment of latent infection in tissues other than ganglion cells.

1.14.c. MAINTENANCE OF HERPES SIMPLEX VIRUS LATENCY

The factors involved in maintenance of HSV latency have not been fully identified. Studies with immunized and nude mice showed that

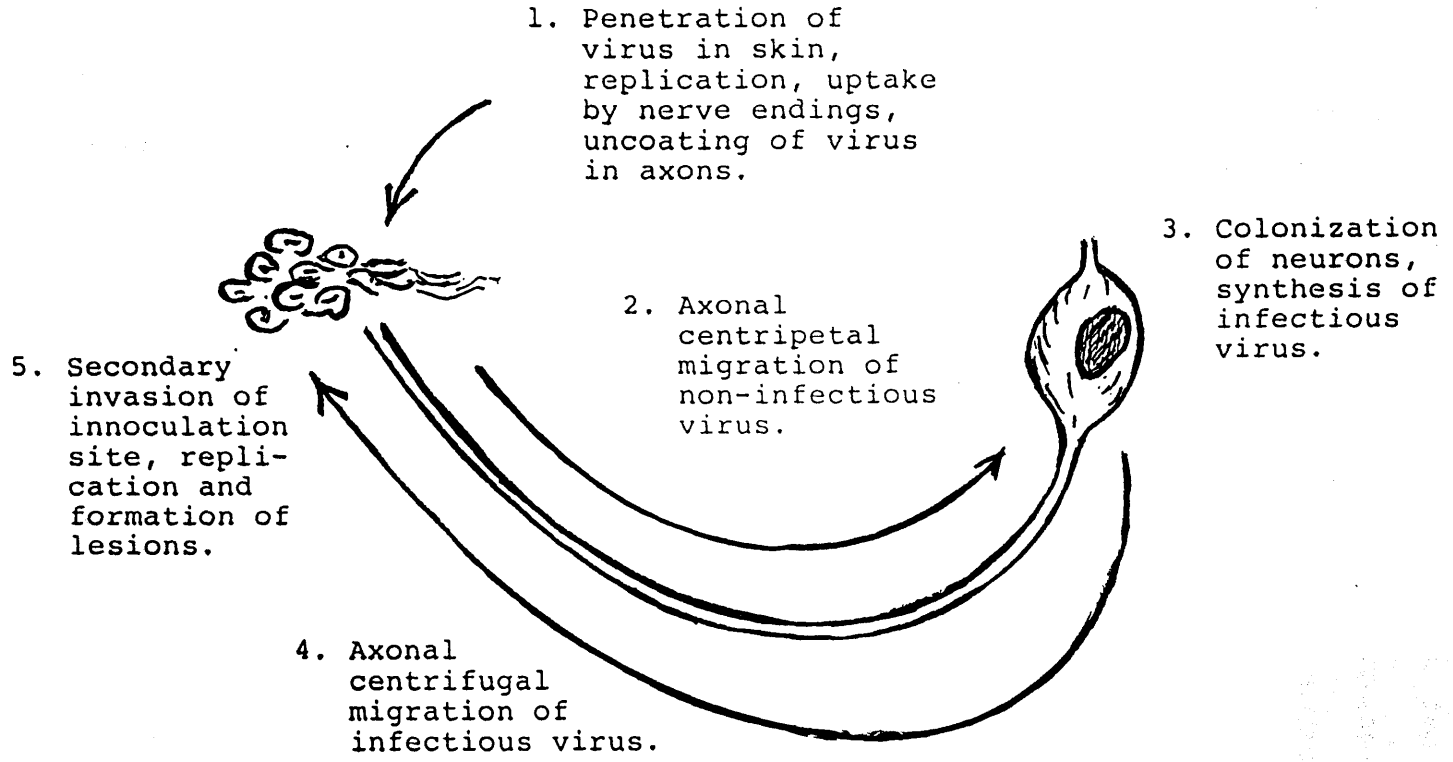
the immunological status of the host is one of the factors that controls the number of cells in ganglia that become infected (Walz et al, 1976). However, in vitro studies (Openshaw et al, 1979a; Wohlenberg et al, 1979) have shown that neither antibodies or interferon treatment prevented reactivation. Sekizawa et al. (1980) was able to establish latent HSV infection in mice passively immunized with rabbit anti-HSV serum administration at various times post infection. A few months later, neutralizing antibody was undetectable in passively immunized mice which remained latently infected (ganglionic homogenates were negative but ganglionic explants yielded virus). Persistence of virus in the absence of neutralizing antibody suggests that once latency is established, serum neutralizing antibody does not appear to be required to maintain the latent state (Openshaw et al., 1981). However, a role for non neutralizing antibodies has not been excluded (Nash, 1981).

Klein (1976) has proposed two mechanisms for the maintenance of latent infections with HSV. In both mechanisms namely "one-way" transmission and the "round-trip" transmission it is assumed that the neurone is destroyed and reactivation occurs in all latently infected neurones. The "one way" trip proposed that the number of latently infected neurones is fixed, and the virus is reactivated from the neurone, travels to the skin. However, the information for renewed recurrence is never lost from latently infected neurones. The "round-trip", mechanism proposes that infection of a new set of neurones is possible. After reactivation, some virions escape the elimination process at the neural level, and travel to the skin where they produce lesions. The virus synthesised in the skin migrates towards the neurone and re-establishes a latent infection.

Recent studies of Gordon et al; (1987) counter argue the "round-

FIGURE 19

The round trip of herpes simplex virus during the acute phase of infection. Details described in the text. Arrows indicate direction of spread of virus from skin to the ganglia and vice versa.


1. Penetration of virus in skin, replication, uptake by nerve endings, uncoating of virus in axons.
2. Axonal centripetal migration of non-infectious virus.
3. Colonization of neurons, synthesis of infectious virus.
4. Axonal centrifugal migration of infectious virus.
5. Secondary invasion of innoculation site, replication and formation of lesions.

trip" mechanism of maintenance of latency proposed by Klein (1976). Gordon et al; (1987) infected rabbits with a tk negative strain of HSV-1 in the eye and the phenotype of the emergent virus following iontophoresis of adrenalin was taken as a criterion to test the "round-trip" hypothesis. Repeated adrenalin induced ocular shedding of latent HSV-1 was observed in 100% of rabbits. It was observed that the tk positive isolates and syncytial variants of the tk negative inoculating strain recovered at the ocular surface after the initial iontophoresis could not be demonstrated following subsequent trials of iontophoresis, indicating a lack of the "round-trip" hypothesis of latency.

1.14.d. STATE OF THE VIRUS DURING A LATENT INFECTION

(i) Infectious virus

It is not known whether the virus on establishing a latent infection, exists in a static state or replicates at a very low level (dynamic) state (Roizman, 1965,). Baringer and Swoveland (1974) by electron microscopy of serial sections of trigeminal ganglia from latently infected rabbits found a very small number of productively infected cells (presumably neurones), indicating a dynamic state of virus latency. However, in other studies treatment of latently infected rabbits and mice with various potent antiviral drugs did not decrease the incidence of ganglionic latency (Field et al,, 1979; Klein et al., 1979; Field and DeClercq, 1981; Svennerholm et al., 1981; Nesburn et al., 1983) indicating a non-dynamic or static state of latency, as such drugs only affect replicating virus.

(ii) The viral genome

Lonsdale et al, (1979) studied infected cell polypeptides and the DNA restriction profiles of 31 HSV-1 isolates from the trigeminal, superior cervical and vagus ganglia from 17 individuals. Analysis of

the DNA restriction profiles indicated that virus isolates from the trigeminal, superior cervical and vagus ganglia of the same individual, or virus isolates from the left or right ganglia of the same individual or multiple isolates from different explants of a single ganglion were indistinguishable, indicating that only one strain of the virus is predominately latent in the ganglia of each individual. Studies of Centifanto et al; (1982) supported these findings in humans by demonstrating that initial infection of rabbit corneas with a less virulent HSV strain prevents subsequent ganglionic superinfection by a more virulent strain of HSV-1.

The recovery of infectious virus from explanted ganglia clearly indicates that the complete virus genome must be contained within the cells of the ganglia. However, Brown et al; (1979) and Lewis et al; (1984) have shown that by super infection with ts mutants of HSV, viral information could be rescued from explanted human ganglia that had spontaneously failed to yield infectious virus after a period of time in culture. This indicated that some ganglia that are "latency negative" by the standard criteria contain non-inducible viral genomes. A low incidence (0.1 genome equivalents per cell) of viral DNA in latently infected mouse ganglia has been detected by hybridization techniques (Puga et al., 1978; Cabrera et al., 1980; Rock and Fraser, 1983).

The state of viral DNA in latently infected neurones is unknown. Southern blot hybridization of BamHI restriction enzyme digested DNA from latently infected mouse brain gave signals with HSV-1 cloned fragments from the joint region of the genome and not with the cloned termini, indicating that the virus DNA is present in an endless configuration (Rock and Fraser, 1983, 1985). The endless configuration of virus DNA has also been observed in latently infected human ganglia

(Efstantiou et al., 1986). It is not clear whether the endless form of HSV DNA detected in latently infected cells is due to the integration of the viral genome into cellular DNA or its maintenance extrachromosomally in a circular or concatameric form. However, in a recent limited study based on analysis of caesium chloride buoyant density gradient banding patterns of host and viral DNAs, HSV-1 specific DNA from latently as well as acutely infected mouse brains was found to band at the buoyant density of virion DNA. These results indicated an extra chromosomal state of latent HSV-1 DNA in the mouse model (Mellerick and Fraser, 1987).

(iii) HSV transcription in latency

It is not conclusive whether HSV gene expression occurs during a latent infection. Initial attempts using liquid-phase hybridization techniques to detect viral transcripts in mouse ganglia either soon after the establishment of latency or at later times were unsuccessful (Puga et al., 1978). However, using in situ hybridization, limited transcription from the left hand end of the HSV genome has been detected in thoracic and lumbo-sacral ganglia of humans (Galloway et al., 1979, 1982), in trigeminal ganglia of latently infected guinea pigs (Tenser et al., 1981) and lumbar ganglia of latently infected mice (Stroop et al., 1984). The presence of Vmw 175 in the ganglia of latently infected rabbits was demonstrated by immunofluorescence (Green et al., 1981). However, the presence of Vmw 175 in the ganglia of latently infected rabbits was thought to be due to reactivation of the latent virus.

The occurrence of the hypomethylated form of HSV-1 DNA in the CNS of latently infected mice suggests the transcriptionally active nature of the viral DNA (Dressler et al., 1987). A more interesting recent

finding by in situ hybridization and Northern blotting techniques is the identification in the spinal ganglia of mice latently infected with HSV-1 KOS-M, of transcripts co-mapping with the IE-1 gene region. The transcripts are from the anticomplementary strand of the virus DNA (Puga and Notkins, 1987; Stevens et al., 1987) and the putative polypeptide is thought to play a role in the maintenance of the viral genome in a latent state in host neurones.

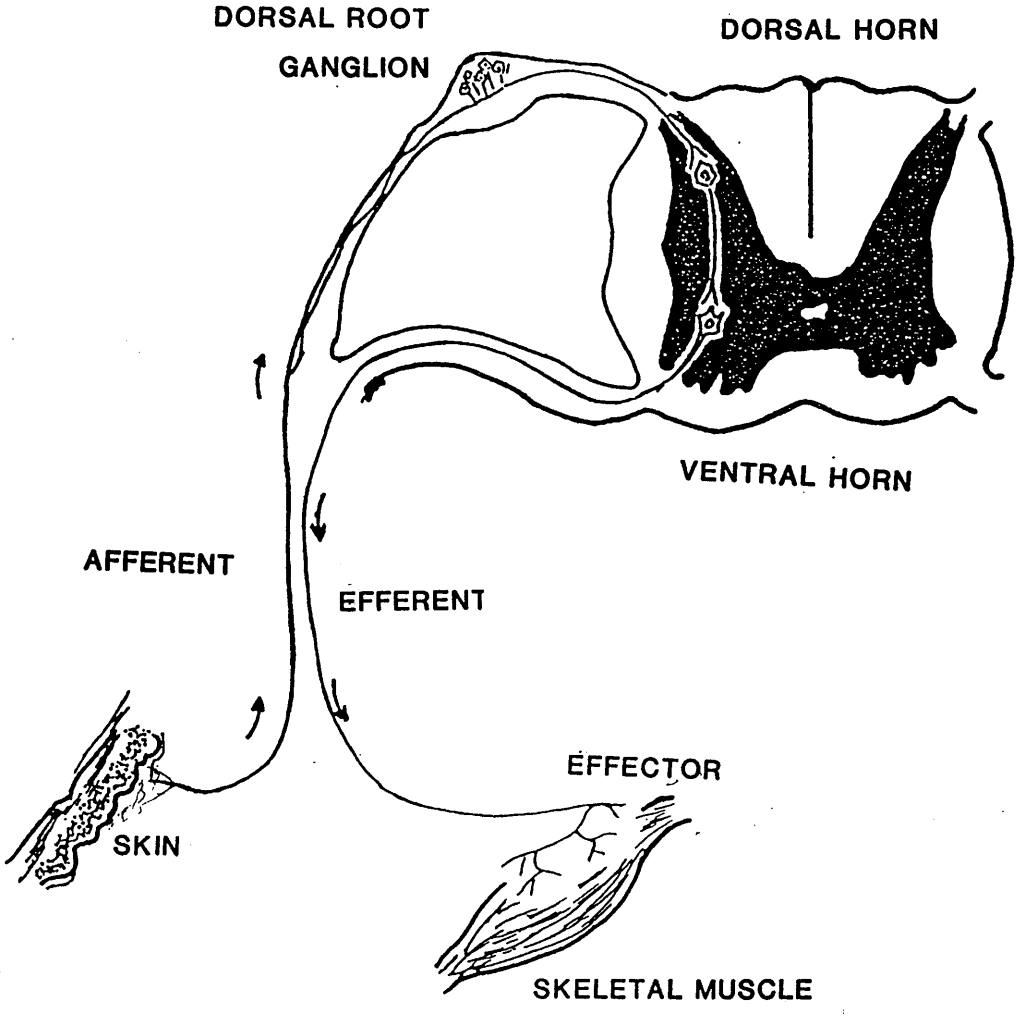
1.14.e. REACTIVATION OF HERPES SIMPLEX VIRUS FROM LATENCY

Reactivation as defined by Wildy et al (1982) is the reawakening of virus from the latent state either spontaneously or as a result of external stimuli, so that infectious virus may be isolated. Following reactivation, the released virus may produce observable lesions (recrudescent lesions) in the neurodermatome (Figure 20) related to the sensory ganglion, or alternatively the virus travels to the periphery (where it probably multiplies and can be isolated) without the development of a noticeable lesion, called recurrence (Wildly et al., 1982).

Various physical and chemical methods have been used to induce reactivation. Good and Campbell (1948) were able to reactivate HSV in rabbit brain (with subsequent development of encephalitis) by subjecting the latently infected rabbits to anaphylactic shock (by intravenous injection of 0.2 to 0.6 ml of egg white), or by the injection of synthetic adrenalin (Schmidt and Rasmussen, 1960) intramuscularly with a total dosage of 2.0 mg. Electrical stimulation of the trigeminal ganglion has been described as inducing in vivo reactivtion of HSV in tear films (Green et al., 1981). Epinephrine iontophoresis of the cornea has also been used to induce reactivation (Hill et al., 1981). Reactivation of HSV-1 (Sekizawa et al., 1980) and HSV-2 (Kurata et al., 1978) has been successfully demonstrated in mice

FIGURE 20

Diagrammatic representation of relations of sensory neurones (Dorsal root ganglia) to other types of tissues. Adapted from Al Saadi, 1984


DORSAL ROOT
GANGLION
DORSAL HORN
VENTRAL HORN
AFFERENT
EFFERENT
EFFECTOR
SKIN
SKELETAL MUSCLE

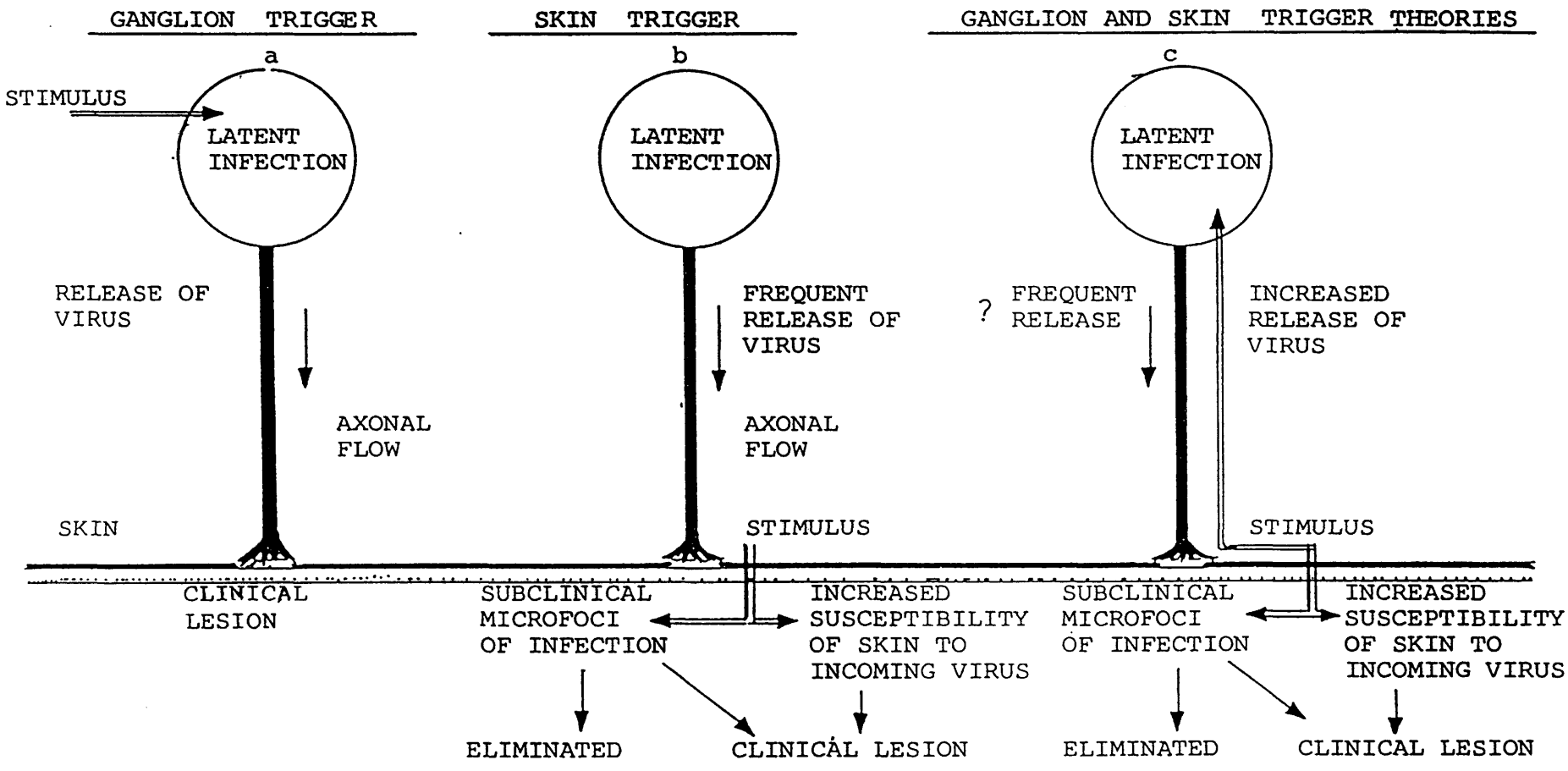
(inoculated by lip or by the corneal route) by the use of cyclophosphamide. Reactivation has been shown to precipitate by burning with CO_2 (Openshaw et al., 1979b), hair plucking (Hurd and Robinson, 1976), U.V. and x-ray treatment (Blyth et al., 1976; Openshaw et al., 1979b). Sectioning of the nerve has been shown to reactivate virus in the spinal ganglia of mice (Price and Schmitz, 1978; McLennan and Darby, 1980; Klein, 1982). It is not known whether reactivation positive stimuli act directly on the latent virus or through mediator, or whether they only promote the development of recurrent lesions by virus already reactivated (Wildy et al., 1982).

Two theories have been proposed to explain the process of reactivation. (1) According to the first theory called the "Ganglion Trigger" theory (Hill and Blyth, 1976) (Figure 21a), a stimulus acts on the latent infection in the ganglion to "switch on" virus from the latent state. The virus then travels down to the peripheral nerve, and epidermal cells are infected so that a skin lesion develops (Cook and Stevens, 1973; Lehner et al., 1975). The theory is widely accepted, however, it does not explain (at least in man) the development of a lesion in a relatively short time after stimulation or injury, or how certain stimuli like exposure of surface skin in U.V. light can affect virus latent in the ganglion. Moreover, reactivation of virus in ganglia does not automatically produce recurrent disease (Hill and Blyth, 1976).

(2) "The Skin Trigger" theory (Hill and Blyth, 1976; Figure 21b) assumes that a reactivating stimulus acts on virus in the skin rather than in the ganglion. This postulates that virus is often produced in the ganglion and as a result reaches the skin cells via a nerve perhaps every few days, then microfoci of infection develop which are usually eliminated by host defence mechanisms; most of these infections may be abortive. Changes in the skin occasionally allow

FIGURE 21

Diagrammatic representation of the (a) 'ganglion trigger' theory, (b) 'skin trigger theory' and (c) 'ganglion and skin trigger theory of virus reactivation during latency as proposed by Hill and Blyth (1976)


GANGLION TRIGGER
SKIN TRIGGER
GANGLION AND SKIN TRIGGER THEORIES
a
b
c
STIMULUS
LATENT INFECTION
LATENT INFECTION
LATENT INFECTION
RELEASE OF VIRUS
AXONAL FLOW
FREQUENT RELEASE OF VIRUS
AXONAL FLOW
? FREQUENT RELEASE
INCREASED RELEASE OF VIRUS
SKIN
STIMULUS
STIMULUS
CLINICAL LESION
SUBCLINICAL MICROFOCI OF INFECTION
INCREASED SUSCEPTIBILITY OF SKIN TO INCOMING VIRUS
SUBCLINICAL MICROFOCI OF INFECTION
INCREASED SUSCEPTIBILITY OF SKIN TO INCOMING VIRUS
ELIMINATED
CLINICAL LESION
ELIMINATED
CLINICAL LESION

these microfoci to grow into visible lesions either by stimulating virus replication or by temporary suppression of local defence (Hill and Blyth, 1976). The theory explains that inducing agents like U.V. light and trauma do not act directly upon latent virus in the ganglion, instead the stimuli induce some physiological changes (inflammation) in the skin which provoke the development of lesions. Among the local changes that may be induced in the skin after exposure to U.V. light is the release of prostaglandin (Eaglstein and Weinstein, 1975) particularly prostaglandin E2 (PGE2). PGE2 has been shown to reactivate latent virus in mice after 2 to 3 days of intradermal administration (Blyth et al., 1976) and to increase the size of the plaque but not the virus yield in Vero cells (Harbour et al., 1978) probably by enhancing the cell to cell spread of virus. However, the skin trigger theory fails to explain the mechanism of action of some inducing agents which do not act on skin e.g. stress, anxiety, depression, fatigue (Klein, 1982). A further possibility is that effective inducers of recurrent disease may produce changes both in the skin and ganglia (Figure 21c). This hypothesis was supported by the observation of Hill (1981) who demonstrated that infectious virus can be found in cervical ganglia of 10% of latently infected mice 1 to 4 days after ear stripping with sellophane tape.

1.14.f. IMMUNOLOGY OF LATENT HERPES SIMPLEX VIRUS INFECTION

The precise role of the immune response in latent HSV infection is not known. Several elements of the immune system have been shown to be involved in the response to herpes infection including: macrophages (Zisman et al., 1970), cytotoxic T cells (Nash et al., 1980), natural killer cells (NK) (Ching and Lopez, 1979), suppressor T and suppressor B cells (Nash and Gell, 1980; Nash et al., 1981), interferon (Gresser et al., 1976), antibody and complement (Burns etal., 1975; Oldstone

and Lampert, 1979). Humoral immunity alone, has been found of limited importance both in eliminating acute infection at least in nude mice (Openshaw et al., 1979a) and in maintaining latency (Sekizawa et al., 1980). On the other hand cell-mediated immunity (CMI) has been shown as an important factor for host protection against herpes infection (Lodmell et al., 1973; Oakes, 1975; Rager-Zisman and Allison, 1976) and in recovery from HSV-1 infection in mice (Nagafuchi et al., 1979).

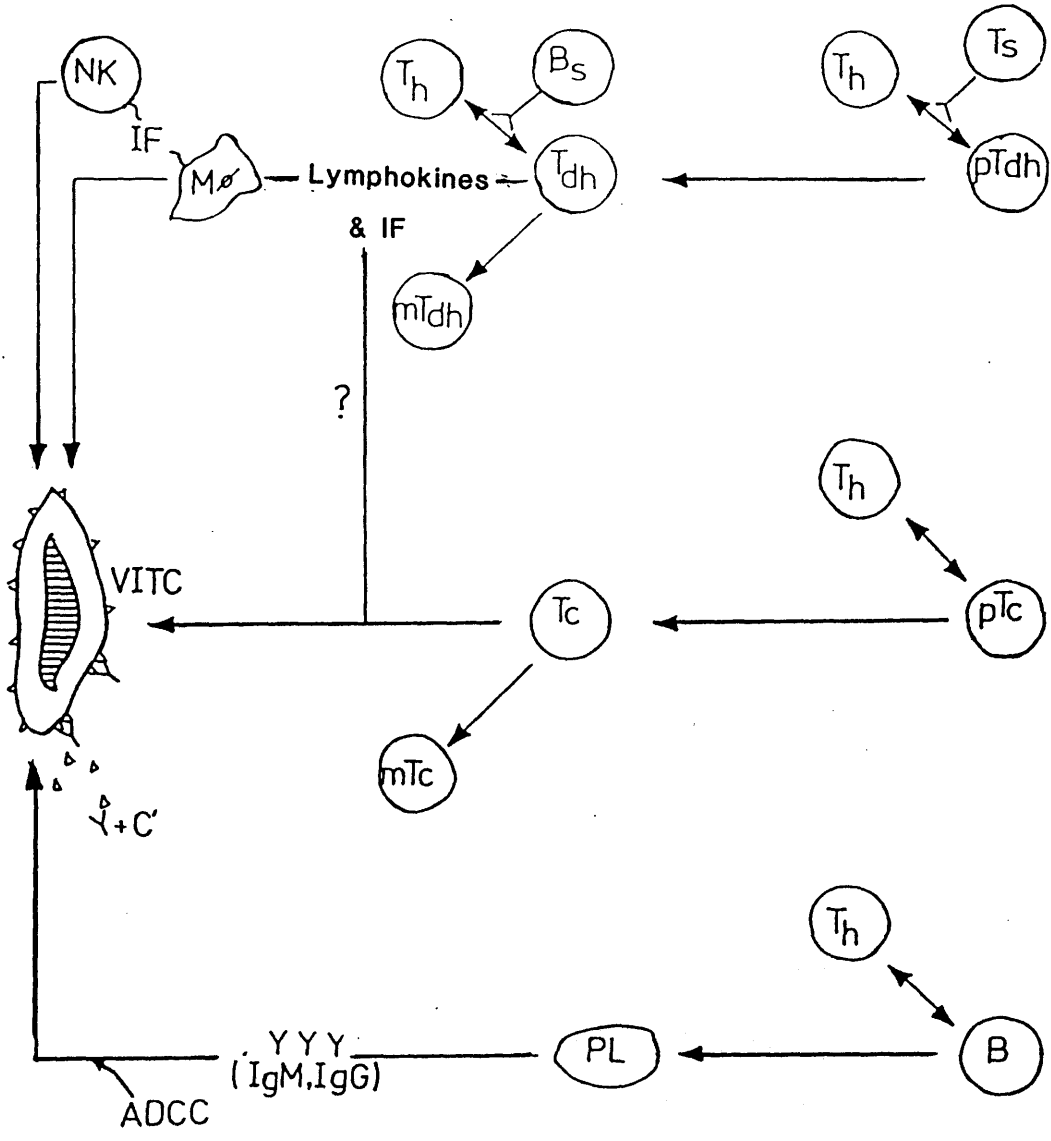
The fact that T cells (a component of CMI) play a role in the maintenance of latency was demonstrated by Kastrukoff et al., 1981). They showed that in C3H mice where CMI is acutely suppressed (i.e. mice were thymectomized one month after infecting with HSV and later irradiated, then reconstituted with anti-Thy 1.2 hybridoma antibody plus complement treated bone-marrow, and subsequently given antilymphocyte serum) a severe illness developed and the virus was observed in trigeminal ganglia, lip and CNS at a time when anti HSV antibody was present. Control (uninfected) or chronically infected but sham treated mice remained well. However, some mice in the experimental group did recover, possibly due to the action of circulating antibody.

Wildy et al (1982), based on the analysis of the results in the mouse ear model (Nash and Gell, 1980; Nash et al., 1980, 1981), have proposed that three immune mechanisms are initiated following herpes virus infection: (i) delayed type hyper sensitivity (DTH), (ii) cytotoxic T cell responses and (iii) neutralizing antibody. All the three immune responses are believed to be active during the primary, recurrence and recrudescence stages of infection, and that T helper cells (Figure 22) play a key role essential for the induction of all three responses.

FIGURE 22

A schematic representation of the proposed T cell mechanisms involved in the induction of protective immune responses, as defined using the mouse ear model. Adapted from Wildy et al (1982).

T_S, suppressor T cells; Th, helpter T cells; Tdh, delayed hypersensitivity T cells; Tc, cytotoxic T cells; pT, precursor T cells; mT, memory T cells; Bs, suppressor B cells; B, activated B cells; pL, plasma cells, MØ, activated macrophage; NK, natural killer cell; ADCC, antibody-dependent cell cytotoxicity; IF, interferon, C', complement; VITC, virus infected target cell (neurone or epidermal skin cell); Δ, herpes virus antigens; $\dashv\!\!<$, suppression of immune cell functions.


NK
IF
Mø
Lymphokines
& IF
Th
Bs
Tdh
mTdh
?
Th
Ts
pTdh
VITC
Y+C'
Tc
mTc
Th
pTc
Th
B
PL
Y Y Y
(IgM,IgG)
ADCC

1.14.g. VIRUS AND HOST FACTORS IN HERPES SIMPLEX VIRUS LATENCY

(i) Viruses

Temperature sensitive mutants of both HSV-1 and HSV-2 have been shown to vary in their ability to establish a latent infection in animal models (Lefgren et al., 1977; Watson et al., 1980; Al-Saadi et al., 1983) indicating a role of virus factors in latency. Moreover, in latently infected tree-shrew, HSV-2 ts mutants can be recovered from the spleen and not from other tissues (Darai et al., 1980).

Virus genes required for the establishment of latent infections have not been defined. The role of the thymidine kinase (tk) gene has long been speculated upon in the establishment and maintenance of latency. Thymidine kinase negative mutants of HSV-1 which have been found to be extremely avirulent and multiply poorly in vivo, were found to induce latency in trigeminal ganglia of mice (Field and Wildy, 1978; Tenser and Dunstan, 1979; Field and Darby, 1980) and guinea pigs (Tenser et al., 1979) after corneal inoculation but with more difficulty (approximately 10^6 to 10^8 pfu of input virus) than the wild-type viruses and their multiplication is often restricted in the nervous system. In many instances, a great difference is noticed between the wild-type and tk negative mutant latency capacity (Tenser and Jones, 1982). It is interesting that tk negative mutants of HSV-1 replicate efficiently in actively growing cells but fail to replicate in non-multiplying serum starved cells (Jamieson et al., 1974), an observation which suggests that HSV specified deoxypyrimidine kinase activity is essential for HSV replication under conditions where the host cells are not making thymidilate. Since neurones are non-dividing cells, it is hypothesized that HSV tk expression is important in the pathogenesis of HSV infection and necessary for the establishment of sensory ganglion neurone infection (Tenser and Dunstan, 1979).

A number of deletion mutants in viral genes including the α 22 gene (Sears et al., 1985) and HSV-1 Ori_L (Polvino-Bodnar et al., 1987) have been shown to establish latent infections of sensory neurones in mice.

Studies with ts mutants of HSV-1 (Watson et al., 1980; Stevens, 1981; Clements and Subak-Sharpe, 1983) and of HSV-2 (Al-Saadi et al., 1983) revealed differences in the capacity of the mutants to establish latent infections in the mouse brain and ganglia. Comparison of latency phenotype (i.e. latency positive and latency negative) with the properties of the mutants expressed under restrictive conditions (Brown et al., 1973; Subak-Sharpe et al., 1974; Marsden et al., 1976; Stow et al., 1978; Gerdes et al., 1979) led to the following conclusions: (i) viral DNA synthesis (HSV-1 and HSV-2) may be irrelevant to the establishment of a latent infection, as some DNA positive and some DNA negative mutants were equally able to establish latent infections, while other DNA positive and DNA negative mutants were latency negative (Watson et al., 1980; Stevens, 1981; Al-Saadi et al., 1983); (ii) there is a loose correlation between absence of morphologically identifiable type I viral products in infected cells (neuroblastoma cells in culture; neurones in brain) and the capacity to establish latent infection.

(ii) Host factors

Studies in mice have revealed that inbred strains of mice differ in their ability to resist infection in that susceptible mice develop paralysis and death (Lopez, 1975) and mouse strains have been categorized as resistant (C57BL/6) moderately susceptible (Balb/c) or very susceptible (A/J). Similar observations of strain susceptibility in mice are made by Kirchner et al (1978). Lopez (1980) reported that differences in strain susceptibility to HSV in mice are genetically

determined and appear to be mediated by natural killer cells. Similarly intraperitoneal inoculation of HSV-2 (Mogensen, 1976) in a series of inbred strains of mice revealed that some strains of mice were resistant while others were susceptible to focal necrotic hepatitis caused by the HSV-2. Resistance to HSV-2 seems to be sex linked (i.e. the resistance gene is located on the X-chromosome) and it is involved in macrophage function (Mogensen, 1980).

In the guinea pig model, much more frequent (45 to 90% occurrence of HSV recrudescence has been observed than that in mice (Scriba, 1975; Donnenberg et al., 1980), indicating the importance of host factors.

In man, when the histocompatibility antigen types of 260 patients (who had a clear history of frequent circumoral herpes infection) were compared with 606 normal (control) subjects, a positive association between the HLA antigen A1 and the incidence of recrudescent herpes was observed (Russell and Schlaut, 1977). A suggestive association with antigen A29 and B8 was also noticed. However, this association has not been explained and it is not known whether such individuals are susceptible to infection with the virus. Nevertheless, the observation is, that one factor that increases susceptibility to this condition in man (recrudescence) may be inherited (Russell and Schlaut, 1977).

Section II

1.14.h. IN VITRO MODELS OF LATENCY

While animal models have been used to mimic in vivo latency in humans, in vitro systems using cell cultures are being developed to gain insight into the molecular processes involved in the establishment and maintenance of HSV latency (Wigdahl et al., 1983; Nilheden et al., 1985; Russell and Preston, 1986; Cook and Brown, 1986; Yura et

al., 1987).

To establish *in vitro* latency systems, virus replication has been suppressed by (i) treatment of the infected cells with inhibitors of virus replication such as bromo-vinyl deoxyuridine, in conjunction with interferon (Wigdahl *et al.*, 1982a,b), (ii) infecting the cells at a low multiplicity of infection (0.003 pfu/cell) and incubating the infected cells at the elevated temperature of 42°C (Russell and Preston, 1986; Cook and Brown, 1987) or (iii) infecting cells in the presence of anti HSV antibodies for the first one or two passages (Nilheden *et al.*, 1985). Reactivation of the virus is achieved by down shift to a permissive temperature, or by superinfection with (i) homotypic HSV (Cook and Brown, 1987), (ii) human cytomegalovirus (Wigdahl *et al.*, 1982a,b), (iii) intertypic HSV (Nilheden *et al.*, 1985) or (iv) intertypic *ts* mutants of HSV (Russell and Preston, 1986).

The *in vitro* models of latency have yielded useful information towards an understanding of the molecular biology of latent HSV infections.

HSV-1 was shown to grow lytically in all the three cell types namely epithelial, keratocytes and endothelial cells of rabbit cornea at 37°, however at supra optimal temperatures of 42°C, the 3 cell types were found to support a non-productive infection with HSV (Cook and Brown, 1986) as no infectious virus was observed for up to 28 days post-infection. However, on reducing the incubation temperature to 37°C infectious virus was again obtained from all the cell types. In further studies with rabbit corneal cell cultures, Cook and Brown (1987) found that incubation of the infected cells in the presence of the viral inhibitor acycloguanosine during the last five days of a 14 day incubation at 42°C, did not reduce the frequency of viral shedding following transfer to 37°C, indicating that the rabbit corneal cells

support a latent as opposed to persistent infection. Further, superinfection of cells (which failed up to 29 days post infection to shed virus spontaneously) with an XbaI site deletion mutant of HSV-1 at 37°C, yielded both the initial infecting virus and recombinants between the parental and superinfecting genomes.

HSV-2 has been shown to produce a latent infection of human foetal lung cells incubated at supra optimal temperature of 42°C (Russell and Preston, 1986). Transfer of the infected cultures from 42°C to 37°C did not yield any infectious virus for at least 6 days. Reactivation of the latent virus was generally achieved by intertypic superinfection at 38.5°C with ts mutants of HSV-1, or with human cytomegalovirus but not with adeno virus type 2 or 5. In further studies in the in vitro system, Russell et al (1987) demonstrated that latent HSV-2 could be reactivated by superinfection with HSV-1 tsk (a ts mutant in the IE-3 gene of HSV-1) but not with dl 1403 (a deletion mutant in the IE-1 gene, Stow and Stow, 1986), indicating that the IE-1 gene product, Vmw 110 is required for reactivation of latent HSV in the in vitro system. Further, tsk and dl 1403 were able to establish a latent infection of human foetal lung cells with an efficiency similar to the wild-type, indicating that, at most, only limited gene expression is necessary for the establishment of latency.

Using an in vitro system of HSV latency in neurones isolated from dissociated rat foetus sensory ganglia or of human foetal lung (HFL) fibroblasts, Wigdahl et al (1984) investigated the state of the latent viral DNA by the Southern hybridization techniques. No detectable alterations in the size or molarity of the HSV-1 terminal or junction genomic DNA fragments obtained by HindIII, XbaI or BamHI digestion of the latently infected neuronal or HFL cells DNA, as compared with digestion of a reconstruction mixture of purified HSV-1 virion and HFL

cell DNA, were observed. Interpretation of the data was that the predominant form of the HSV-1 genome in latently infected cell populations is nonintegrated, linear and nonconcatameric.

All the in vitro models of HSV latency have been criticised on the basis that the treatments used including elevated temperature and drugs probably block virus replication rather than establish a latent infection (Price, 1982). Caution must, therefore, be exercised in extrapolating in vitro results to in vivo experiments or to the natural host i.e. humans. For example, a nonintegrated, linear, nonconcatameric form of the HSV genome in latently infected cells reported by Wigdahl et al (1984a), is in contrast to the findings of an endless configuration of the HSV genome in human brains (Efstanthiou et al., 1986) and in mouse brain (Rock and Fraser, 1983, 1985).

1.5. EYE INFECTIONS WITH HERPES SIMPLEX VIRUS

Herpes simplex virus infection is endemic, with 60 to 70% of children aged 5 and 90% of adults having neutralizing antibodies against HSV (Leopold and Sery, 1963; Smith et al., 1967). However, only 20 to 30% are estimated to ever manifest clinical disease and less than 1% exhibit ocular disease (Kaufman et al., 1983). HSV is the most common infectious cause of central corneal ulcers and corneal blindness in developed countries (Foster and Duncan, 1981). HSV keratitis most frequently occurs between ages 30 to 70 (Leopold and Sery, 1963). Approximately 98% of non-neonatal ocular infections are from HSV-1 (Neumann-Haefelin et al., 1978); 70% to 80% of ocular herpes presenting in the first month of life is from HSV-2 (Nahmias et al 1970). Clinically the two types of HSV disease cannot be differentiated. Depending upon the circumstances of infecting virus HSV ocular diseases are divided into (1) primary and (2) secondary infections and are outlined in brief, as follows:

1.15.a. PRIMARY OCULAR HSV INFECTION

Humans who have no previous immunity to HSV, on first exposure to the virus, may develop primary eye disease. It can be present in various forms and affects different components of the eye. The incubation period ranges from 2 to 12 days and the infection occurs in both sexes. Depending upon the part of the eye involved, and the strain of the infecting virus, the clinical manifestations of the primary disease vary in severity. Some of the important clinical manifestations are described in the following sections.

(i) Eye lids: Primary ocular HSV infection of eye lids is manifested by vesicle eruptions on the lids and lid margins. After 1 to 2 weeks vesicles may ulcerate, followed by crusting, desiccation and healing within 4 weeks. In the absence of secondary bacterial infections, the disease is usually self limiting.

(ii) Conjunctiva: The most constant finding in primary ocular HSV infection is acute follicular conjunctivitis with or without involvement of eye lids (blepharoconjunctivitis). The primary conjunctivitis is self limiting and lasts for 2 to 3 weeks.

(iii) Cornea: Epithelial keratitis usually accompanies or closely follows the onset of follicular conjunctivitis, but can occur late in primary disease. Initially groups of superficial cells are infected but as the disease progresses, the infected cells may desiccate and punctate keratitis resolves, or the lesions may extend to form a coarse punctate, stellate or dendritic keratitis. A basic feature of all these forms of herpetic epithelial keratitis is the poor adherence of infected cells to each other and to the basement membrane (Coster et al., 1977).

Epithelial keratitis in primary disease is self limiting and

resolves usually without scarring within 4 weeks (Thygeson, 1967). Damage to the ocular tissues is mainly due to replication of the virus in the epithelial cells.

1.15.b. PATHOGENESIS OF OCULAR HERPES SIMPLEX VIRUS

Much of the information on the pathogenesis of ocular herpes has been obtained from studies in experimental animals including rabbits (Irvine and Kimura, 1967; Nesburn et al., 1967, 1972; Wander et al., 1980; Green et al., 1984), mice (Hill et al., 1975; Lopez, 1975; Stulting et al., 1985) and guinea pigs (Tabbara et al., 1974; Wander et al., 1987).

Following intra corneal inoculation of the virus in rabbits and mice, the pattern and course of the clinical disease simulates that of humans (Williams et al., 1965; Metcalf et al., 1976; Hill, 1987). In rabbits the clinical symptoms of stromal keratitis include development of epithelial lesions reaching a peak in severity in 5 to 7 days post infection, followed by disciform oedema lasting for several weeks. Infiltration with lymphocytes, macrophages and polymorphonuclear cells renders the stromal layer opaque, followed by vascularization of the cornea and tissue necrosis (Irvine and Kimura, 1967). Tissue destruction is mainly due to the host immune response to antigens present in the stroma (reviewed by Easty, 1985).

Loss of corneal sensitivity is a common feature of HSV keratitis in humans (Easty, 1985) and a similar loss of sensation has been reported in mice following infection of the cornea after scarification (Tullo et al., 1983) and in rabbits after intrastromal inoculation (Metcalf, 1982). The precise mechanisms of such disfunction are not clearly known but are believed to be due to direct or indirect effects of the virus on the sensory nerve (Hill, 1987).

In mice, following direct corneal inoculation of HSV, often with

scarification (though this mode of virus entry into the eye is rare in humans), virus can be isolated for about 9 to 10 days and clinical disease rapidly ensues (Tullo et al., 1983). For example, severe keratitis develops by the 4th to 5th day post inoculation. Peripheral replication of the virus is associated with rapid invasion of virus into the nervous system.

Before describing the mechanisms of spread of the virus in the nervous system, it would be appropriate to describe some of the salient features of the innervation of the anterior portion of the eye. The cornea and iris receive their sensory innervation from the trigeminal ganglion. The sympathetic nerve supply is from the superior cervical ganglion (SCG) and the parasympathetic nerve fibres are derived from the ciliary ganglion. The ciliary ganglion is linked to the nasociliary branch of the ophthalmic division of the trigeminal nerve, whose fibres traverse the ganglion and run through the short ciliary nerves to the eye where they subserve sensation in the cornea, iris and choroid. The trigeminal ganglion (TG), unlike other sensory ganglia, may best be considered as a fusion of 3 ganglia (ophthalmic, maxillary and mandibular divisions; see Figure 23).

(i) Spread of the virus after corneal inoculation in mice

Tullo et al (1982a) observed that after inoculation of the mouse cornea, virus was isolated on day 2 from the ophthalmic part of the TG and the ipsilateral brain stem. Two days later virus was found in the maxillary part of the ganglion and after a further day, in the mandibular branch. These observations of spread of virus in the TG were interpreted as demonstrating that after ocular infection, the non-ophthalmic parts of the TG may become infected via the "back door route".

The course of events in the spread of virus within the nervous

FIGURE 23

Diagrammatic representation of the three branches of the human trigeminal ganglion (TG). Adapted from Al Saadi, 1984.

A. Lateral view

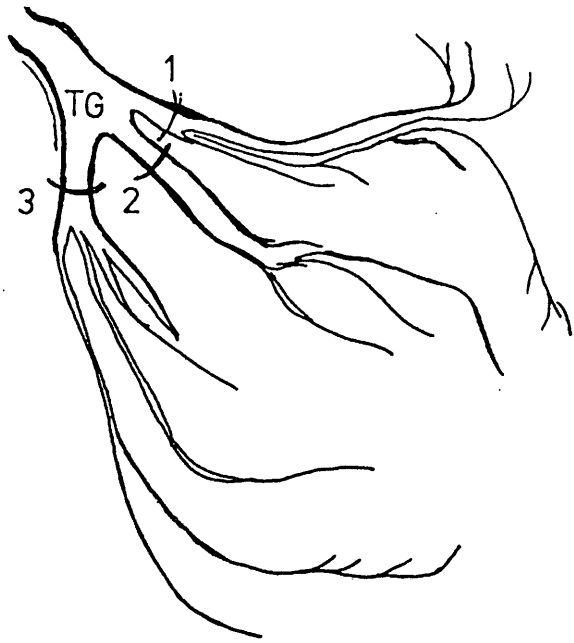
B. Superior view

1. Ophthalmic branch

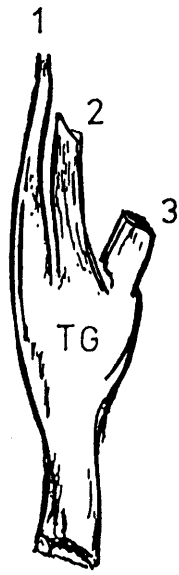
2. Maxillary branch

3. Mandibular branch

A



B



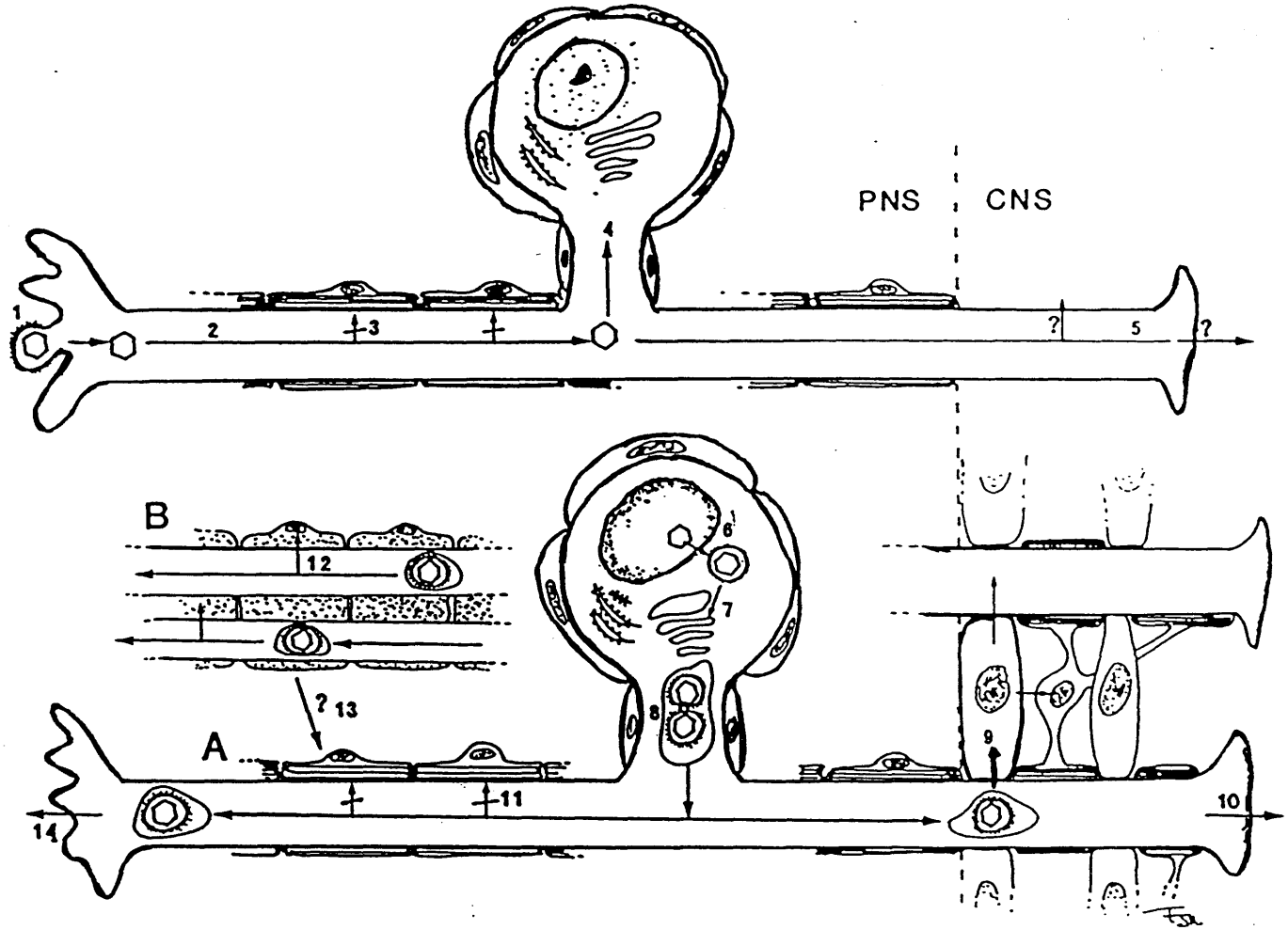
system following ocular infection of HSV have recently been reviewed by Hill (1987). Briefly, as shown in Figure 24, (i) Penetration of HSV in the nervous tissue occurs by the fusion of the virus envelope with the cell membrane as evidenced by the presence of particles resembling naked viral nucleocapsids, on electromicroscopic examination of neurites soon after inoculation of HSV in cultures of sensory neurones (Lycke et al., 1984). (ii) The viral nucleocapsid is transported within the axon by retrograte axonal flow towards the CNS. (iii) The loss of envelope renders the virus non-infectious, thereby damaging its ability to leave the axon. The inability to isolate infectious virus from nerves (Klein, 1985) supports the speculation of the non-infectious nature of the virus. (iv) The nucleocapsids transported to the body of the neurone may interact with its nucleus resulting in either a latent infection or productive replication (Hill, 1985). (v) It is possible that some of the naked nucleocapsids may be transported beyond the ganglion into the CNS. (vi) Productive infection of neurones results in release of enveloped particles from the neuronal nucleus within 24 hr of infection. (vii) On electronmicroscopic examination of neurones at this stage of infection, a number of cytoplasmid vesicles containing groups of enveloped particles are seen (Hill and Field, 1973). (viii) Enveloped virus particles, as revealed by electromicroscopic examination of neurones infected with HSV and PRV (Hill et al., 1972; Field and Hill, 1974) are transported intra-axonally either towards the periphery or towards the central nervous system. (ix) Virus transported centrally reaches the nerve root and by virtue of the envelope it may leave the axon to infect astrocytes and oligodendroglial cells (Hill, 1983). (x) Within the CNS the virus may infect other neurones by crossing synapses (Bak et al., 1977). (xi) Virions transported centrifugally by virtue of their envelope, have

FIGURE 24

Diagrammatic representation of the main events in the spread of HSV in a sensory nerve.

The upper part of the diagram (events 1-5) refers to the original input virus and the lower part (events 6-14) to stages that may occur after replication of virus in the neurone.

A, is a myelinated fibre and B non-myelinated.


PNS
CNS
1
2
3
4
?
5
?
B
12
?
13
A
6
7
8
9
10
11
14

the ability to leave axons, but in myelinated fibres (A in Figure 24), the myelin and the complex interdigitation between Schwann cells may act as a physical barrier. (xii) In non-myelinated fibres there is no such barrier and Schwann cells which enwrap several axons in their cytoplasm may become infected 'from within'. (xiii) Virions produced from Schwann cells because of their ability to support an abortive infection (Hill, 1985) in vitro may be sufficient to infect cells of adjacent myelinated or non-myelinated axons. (xiv) The enveloped virus particles are transported centrifugally to the site of primary infection and the infectious virus may be isolated from 2 to 3 days after inoculation at the peripheral site (Klein, 1985).

Depending upon the severity of and amount of virus replication in primary disease, virus may reach the contralateral eye through the parasympathetic and optic nerve and clinical disease may follow. In fact in rabbits, the development of herpetic keratitis followed by retinitis was first seen in the infected eye within a few days and in the contralateral eye 8 to 12 days later (Goodpasture and Teague, 1923; Kimura, 1962; Kaufman, 1982). Pettit et al (1968) using fluorescent antibody techniques investigated the route of virus spread in ocular herpes in rabbits and demonstrated virus specific antibody fluorescence along the ciliary nerve neurones in a centripetal fashion to the optic nerve, brain, optic chiasma and over to the other eye. However, the spread of virus by the haematogenous route in these diseases was not investigated.

(ii) Role of virus factors in ocular disease pathogenesis

Both host and virus factors are believed to modulate the outcome of ocular infections with HSV. Besides host factors including the host immune response which plays a significant role, the genetic properties of the infecting virus strain have also been shown to modulate the disease pattern. Specifically (i) Wander et al (1980) from their

studies on the pathogenesis of ocular herpes in rabbits, using various strains of HSV, concluded that the type of ocular disease produced in rabbit eyes is an inherent property of the infecting strain and is independent of the titer of the infecting virus. Strains that produce epithelial disease fail to produce stromal disease regardless of the inoculum size. (ii) Clinical isolates from human epithelial HSV disease failed to produce stromal disease when tested in animals, whereas known stromal disease producing strains regularly reproduce this disease pattern even after serial propagation in cell culture (Irvine and Kimura, 1967; Metcalf et al., 1976). The stromal or epithelial disease producing property of the virus was mapped between 0.70 and 0.83 m.u. by analysis of recombinant virus isolated from stromal and epithelial disease producing parents.

Day et al (1987) observed that after corneal infection in mice, the ability of the virus to spread from the cornea to the CNS is determined by the nucleotide sequences within a 6 Kb viral genome fragment. Analysis of recombinants isolated from the encephalitis producing virus (HSV-2 strain 186) parents, demonstrated that the 6 Kb virus DNA sequences include the HSV-1 (17) Ori_L, the HSV-1 (17) gene for DNA polymerase and portion of the HSV-1 (17) gene coding for the major DNA binding protein.

1.15.c. LATENCY FOLLOWING HERPES SIMPLEX VIRUS OCULAR DISEASE

The various processes underlying the establishment maintenance and control of HSV latency have been discussed in section 1.14, and the same are relevant to the establishment of latency ensuing HSV ocular disease. However, some relevant observations on ocular disease latency are discussed.

As shown in Figure 25, following ocular infection, HSV establishes a latent infection in sensory (trigeminal) ganglia as well

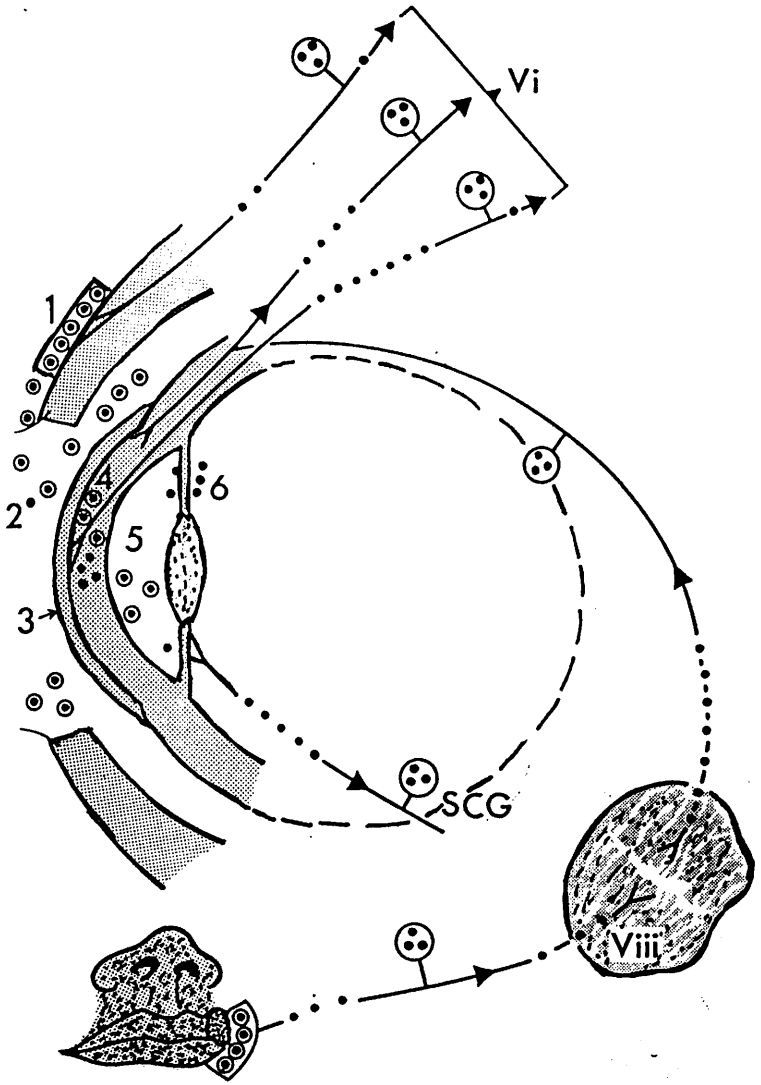
FIGURE 25

Schematic representation of the spread of virus to the ganglia in ocular infection with HSV.

1. Infection with HSV in the region of the eyelids
2. Virus may enter the tears
3. Invasion of corneal epithelium by the virus
4. Spread of virus via the sensory neurones to become latent in the posterior root ganglion

5,6. Virus may penetrate the deeper tissues of the eye.

Virus from orolabial lesions may also spread to the brain and then to the ipsilateral and contralateral eyes.


Vi
1
2
3
4
5
6
SCG
Viii

as in the superior cervical (SCG) and ciliary ganglia (Martin et al., 1977; Tullo et al., 1982a). Infection of the trigeminal ganglia may also occur from the mandibular or maxillary divisions (Tullo et al., 1982a). Virus may spread to the central nervous system and may pass across the synapses and enter the sensory neurones of the other dermatomes (Tullo et al., 1983). It is likely that the number of neurones involved by latent virus infection would be less in the other dermatomes.

Recovery of latent virus from the TG is achieved by in vitro growth after explantation or by cocultivation of the ganglion tissues with cells permissive for HSV.

HSV has also been shown to establish latent infections in the corneas of man and experimental animals. Human corneal explants removed during the course of treatment for chronic stromal keratitis have shed virus 5 to 11 days post explanation (Shimeld et al., 1982; Tullo et al., 1985; Easty et al., 1987; S.D. Cook, personal communication). Similar to the findings of latent virus in human corneas, HSV has been recovered from the corneas of latently infected rabbits (Cook et al., 1987 and this thesis) and mice (Easty et al., 1987). Cook and Brown (1986) observed that HSV-1 can grow in all the three cell types namely epithelial, keratocytes and endothelial cells of rabbit corneal cultures. However, growth of the virus was fastest in epithelial cells compared to keratocytes or endothelial cells. Epithelial and keratocyte cultures were shown to support a latent as compared to a persistent infection with HSV-1 (Cook and Brown, 1987; also see section 1.14).

1.15.d. REACTIVATION OF HERPES SIMPLEX VIRUS FROM LATENCY

The mechanisms of HSV reactivation from latency are not well understood. Probably both host and viral factors are involved. As

shown in Figure 26, virus reactivated from the sites of latency (TG and SCG) travels down the neurones and frequently results in either symptomless shedding of virus in the tearfilm or in an evident recrudescent disease (Stevens et al., 1972; Asbell et al., 1984). Periodic recurrence with shedding of virus in the tear film has been observed both in humans (Easty, 1985) and latently infected rabbits (Stevens et al., 1972).

The recrudescent disease of the eye due to reactivated HSV may be more severe than the primary disease. The most common target of recurrent HSV ocular disease is the cornea, with the conjunctiva usually spared. Whereas in primary disease keratitis is generally confined to the epithelium, it is self limiting and heals without scarring. In recurrent disease keratitis may extend to the stroma and endothelium, persists for months and results in permanent visual loss (Blodi, 1985). The common pathways in the clinical course of HSV keratitis are displayed in Figure 27.

Epithelial keratitis is best characterized by the presence of active virus. Clinical manifestations of epithelial keratitis in recurrent disease are similar to those in primary disease, but tend to be associated with greater underlying stromal inflammation. Dendrites may occur at the primary sites or at any other corneal location. Patients with recurrent epithelial keratitis have a 33 to 68% chance of having another episode within 2 years (Shuster et al., 1981).

Metaherpetic ulcers are sterile, de-epithelialized lesions that follow epithelial or stromal disease in which the basement membrane has been disrupted and most often arise in a quiet eye after active keratitis has subsided (Blodi, 1984). These ulcers may persist for weeks to months. A major complication of the ulcers is stromal melting due to collagenase production (Kaufman, 1964).

FIGURE 26

Schematic diagram showing routes of entry of virus into the tissues of the eye.

Vi refers to the three divisions of the trigeminal ganglion, SCG refers to superior cervical ganglion, ⊙ indicates virus particles.

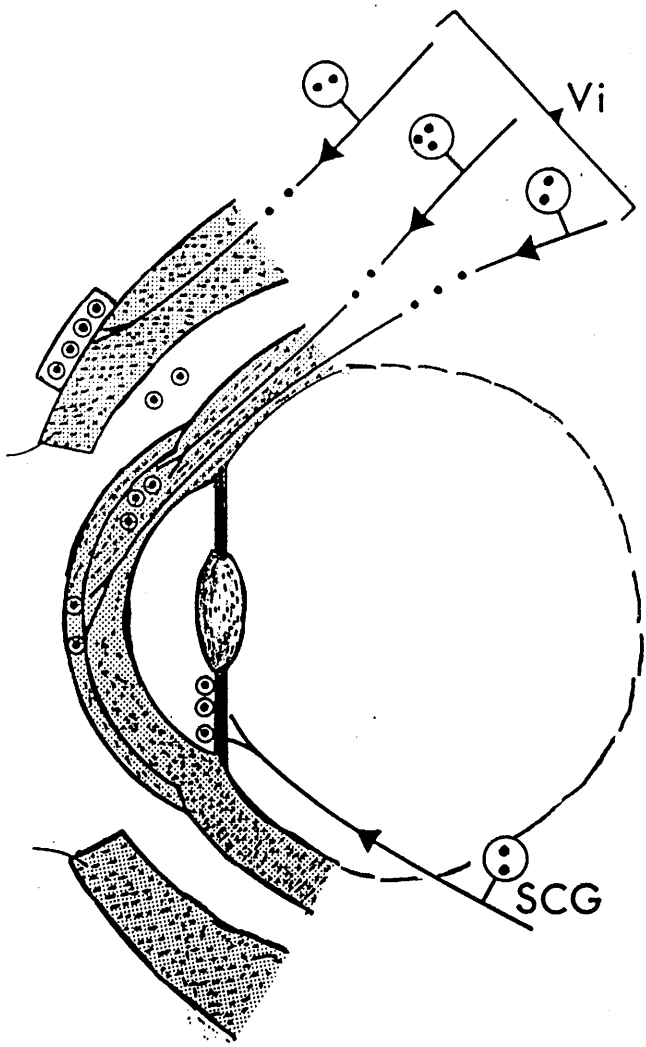
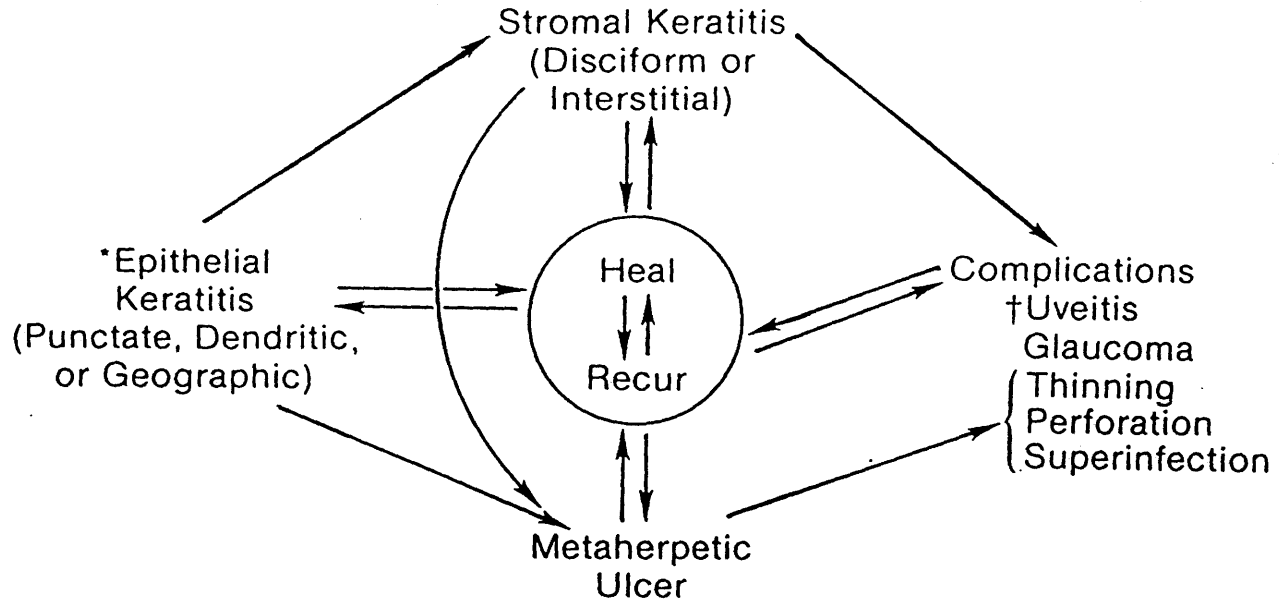

Vi
SCG

FIGURE 27

Common pathways of herpes simplex virus keratitis.

* HSV recoverable, † HSV recoverable in some cases


Stromal Keratitis
(Disciform or
Interstitial)
*Epithelial
Keratitis
(Punctate, Dendritic,
or Geographic)
Heal
Recur
Complications
†Uveitis
Glaucoma
Thinning
Perforation
Superinfection
Metaherpetic
Ulcer

Stromal keratites due to recurrent HSV are of two major types and both are thought to be predominantly immunologic in pathogenesis (Meyers and Chitjian, 1976). (1) The disciform subtype of stromal keratitis is postulated to result from delayed hypersensitivity (CMI) whereas (2) the non-disciform or interstitial subtype results from immune complex hyper sensitivity (antigen-antibody-complement-mediated).

Besides keratitis the other infrequent ocular diseases due to recurrent HSV include (1) uveitis associated with epithelial keratitis. The severe form of uveitis results from the presence of viral replication in aqueous and uveal tissue. (2) HSV retinitis associated with HSV encephalitis.

(i) The genome of reactivated HSV

The genotypes of virus isolates obtained from recurrent herpes keratitis in humans (Asbell et al., 1984; Easty et al., 1987) or in mice or rabbits (Gerdes and Smith, 1983; Easty et al., 1987) have been found to be stable over a period of time in that the viral restriction endonuclease profiles from different episodes of recurrent keratitis in one individual are identical.

Gerdes and Smith (1983) and Hill et al (1987) in rabbit experiments observed that different strains of HSV despite establishing latent infections of TG with equal frequency were heterogenous in their spontaneous or epinephrine induced shedding in the tearfilm, and concluded that reactivation potential, both spontaneous and induced, is determined by the genotype of the strain. Gerdes and Smith (1983) also observed that establishment of latency by a superinfecting strain was inhibited in ganglia previously latently infected with HSV. However, an acute infection of a latently infected ganglion could be established by superinfection with a heterologous

strain.

(ii) Experimental models for reactivation of HSV from latency

Reactivation of the latent virus has been reported in response to diverse stimuli (see section 1.14). In latently infected animals, ocular shedding of virus can be achieved experimentally by (1) electrical or mechanical stimulation of the TG (Green et al., 1979). (2) manipulation of the corneal epithelium to induce Arthus type hypersensitivity (Anderson et al., 1961) or (3) topical or intramuscular administration of epinephrine (adrenalin) (Laibson and Kibrick, 1966, 1969). Epinephrine (0.01%) iontophoresis (0.8 mAmps for 8 min) for 3 consecutive days (Kwon et al., 1982) facilitates the uniform application of epinephrine to the cornea and causes reactivation of virus in 100% of rabbits latently infected with the HSV-1 strain McKrae (Hill et al., 1983, 1987; Dunkel and Pavan-Langston, 1987).

The precise mechanism of action of epinephrine induced reactivation of latent virus from the trigeminal ganglia is not fully understood. Following iontophoresis of latently infected rabbit eyes, epinephrine has been shown to localise in the epithelium and stromal layers of the cornea as detected by the histochemical adrenochrome oxidation method (Dunkel and Pavan-Langston, 1987) and the induction of ocular and ganglionic HSV reactivation from latently infected rabbit eyes was postulated to be due to the synergistic action of epinephrine on the corneal epithelium and of epinephrine metabolites at the ganglionic level.

During investigation of the kinetics of virus replication in neural tissues of rabbits, Hill et al (1983) observed that epinephrine iontophoresis to the eyes of latently infected rabbits resulted in reactivation of HSV-1 in both the trigeminal and superior cervical

ganglia. The TG is sensory while the SCG is autonomic. The iris contains primarily sympathetic receptors, the ciliary body contains parasympathetic and the cornea contains mostly sensory fibres and some sympathetic fibres. The epinephrine activated sympathetic nerve fibres in the cornea may act to stimulate sensory fibres, reactivating HSV-1 in both the TG and SCG and ultimately leading to HSV shedding into the tearfilm. However, the precise mechanism of action of epinephrine in reactivation of the latent virus is unknown.

1.16. HERPES SIMPLEX VIRUS VIRULENCE

Besides causing pathology at the site of infection, significant acute replication in the peripheral nervous system after a primary infection can result in infection of the central nervous system. The disease thus produced ranges in severity from transverse myelitis to fatal encephalitis, indicating the neuro invasive and neurovirulent nature of HSV (Fenner et al., 1974).

The molecular mechanisms underlying the neurovirulence of HSV are largely unknown. Pathogenesis studies of HSV in experimental animals have shown that different strains of HSV are heterogenous in their virulence. Isolates of HSV-2 are found to be normally more neurovirulent than HSV-1 when inoculated by various routes into experimental animals such as rabbit (Plummer et al., 1968; Oh et al., 1972, 1973), guinea pig (Scriba and Tatzar, 1981) and mouse (Nahmias et al., 1969; McKendall, 1980; Thomson et al., 1986; Halliburton et al., 1987). Within one serotype different strains also differ in their neurovirulence (Dix et al., 1983; Sedarati and Stevens, 1987).

Studies aiming to identify the viral gene(s) controlling pathogenicity of the virus in laboratory animals have been inconclusive. The viral thymidine kinase gene (tk) had been shown to be essential for the display of neurovirulence in guinea pigs

following intra vaginal inoculation. No clinical signs were observed on infection with tk deficient strains of HSV-2, though virus growth was similar to highly pathogenic tk producing strains (Stanberry et al., 1985). Studies in mice with various HSV-1 strains have revealed that tk though inessential for virus growth (replication) in actively growing tissue culture cells, its failure to be expressed in vivo results in a marked reduction in neurovirulence (Field and Wildy, 1978; Field and Darby, 1980; Tenser et al., 1981b). However in subsequent studies in mice on the pathogenesis of various HSV strains or intertypic recombinants, no correlation was observed between tk activity and virulence of the virus (Sedarati and Stevens, 1987; Halliburton et al., 1987).

Low neurovirulence of HSV-1 DNA polymerase mutants has been observed following intracerebral but not on peripheral inoculation of mice (Field and Coen, 1986). Larder et al (1986) were able to restore the wild type pathogenicity of the DNA polymerase attenuated strain RSC-26 by marker rescuing the mutation with the genetic information of the virulent parental strain - SC16. Studies in mice with a glycoprotein C (gC-2) deletion mutant of HSV-2 strain 333 have excluded from having a role in neurovirulence as the virulence (indicated by the mortality rate) of the deletion mutants was similar to that of the wild type strain (Johnson et al., 1986).

Recently various studies have been carried out to localise the region of the virus genome involved in neuropathogenicity. Thomson et al (1986) have demonstrated that in mice the avirulent phenotype of the HSV-1 strain KOS could be attributed to 0.25 to 0.53 m.u. of the genome as the substitution of this region from the virulent HSV-1 strain 17 syn^+ in a KOS x 17 syn^+ recombinant produced enhanced virulence. Genes encoded between 0.25 to 0.53 m.u. include those for tk, DNA polymerase and the major DNA binding protein (reviewed by

Wagner, 1985).

Studies with the HSV-1 strain HFEM in the tree shrew system (Darai and Scholz, 1984) indicated that the genes controlling virulence lie between 0.73 and 0.82 m.u. A similar region of the genome (0.79 to 0.83 m.u.) has also been implicated in virulence in the mouse model system using the avirulent intertypic recombinant RE_6 (Javier et al., 1987). However, studies of Halliburton et al (1987) involving 31 HSV-1 x HSV-2 intertypic recombinants in mice have demonstrated that the recombinants were attenuated independently of the virulence of the parent virus. The recombinants were derived at different times from diverse HSV-1 and HSV-2 strains having variable neurovirulence for mice and had crossovers throughout the genome. These results indicated that virulence of HSV is not confined to a single region but is controlled multigenically. Similar findings have been reported by Sedarati and Stevens (1987) in their studies with three different HSV-1 strains in mice. It was demonstrated that lesions for neurovirulence in HSV-1 strains F, HF and HFEM were located in different regions of the genome as complementation of neurovirulence of strains HF, HFEM by the strain F was observed and all three strains were able to complement the non-neuroinvasive strain KOS.

1.17. AIMS OF THE PROJECT

Herpes simplex virus infections are important not only because of their primary disease pathology but also because of the ability of the virus to establish latent infections in the neurons of the ganglia of the peripheral nervous system and to a lesser extent at peripheral sites. The ability of the virus to go latent results in recurrences throughout life caused by a variety of stimuli.

Herpes virus keratitis in man is widespread and is a common cause

of non-traumatic blindness. Following primary infection of the eye, the virus efficiently establishes a latent infection in the sensory neurones of the trigeminal ganglia and probably less efficiently in a small proportion of the cells of the cornea. Recurrent episodes of keratitis due to reactivation of endogenous virus frequently result in corneal ulcers which can progress to impair vision and eventually result in blindness.

The rabbit eye has provided an efficient model system in which to study herpes virus keratitis and the various factors affecting the establishment, maintenance and reactivation of latent HSV.

The work described in this thesis has addressed itself to (i) the analysis of virus genes controlling HSV reactivation potential and (ii) the analysis of virus genes determining virulence of HSV.

Previous work using the rabbit eye model has shown that different strains of HSV although establishing latent infections of trigeminal ganglia with equal frequency, varied considerably in their ability to reactivate spontaneously from latency. This led to the conclusion that some insight into the viral genes controlling reactivation could be gained by the construction of recombinants between HSV strains of low and high recurrence phenotypes and determining variation of reactivation potential correlated to the DNA structure of the recombinants.

This thesis describes (1) the construction of recombinants between HSV-1 strain McKrae and HSV-2 strain HG52, (ii) the analysis of the genome structure of the recombinants by restriction endonuclease digestion, (iii) the reactivation potential and latency potential of the recombinants compared to the parental viruses in the rabbit eye model, (iv) the virulence of the recombinants compared to the parental viruses after corneal infection, and (v) the ability of

HSV to go latent in rabbit corneal cells.

In addition, the construction and genome analysis of HSV-1, McKrae X HSV-2, HG52 recombinants has led to an important and significant observation which may provide some insight into the relationship between HSV replication and recombination. Unselected recombinants isolated after cotransfection of intact genomes of one parent with the total products from restriction endonuclease cleavage of the DNA of the other parent have demonstrated, on genome analysis, preferential recombination between sequences containing an origin of replication. All the recombinants isolated though predominantly of one parental type contained at least one heterologous origin of replication. The implications of this finding are discussed.

Chapter 2

MATERIALS AND METHODS

TISSUE CULTURE CELLS

The baby hamster kidney fibroblastic cell line (BHK 21/C13) established by Macpherson and Stoker (1962) was used throughout this study.

VIRUSES

The viruses used in this study were:

1) HSV-1 strain McKrae (Williams et al., 1965) which forms non-syncytial (syn^+) plaques on BHK 21/C13 cells.
2) HSV-2 strain HG52 (Timbury, 1971) and its temperature sensitive mutants ts 1, ts 5, ts 9 and ts 13 derived by 5-bromodeoxyuridine mutagenesis (Halliburton and Timbury, 1973, 1976). The type 2 viruses form mixed morphology (syn/syn^+) plaques on BHK 21/C13 cells.
3) HSV-1 strain 17 syn^+ (Brown et al., 1973) and its variant X2 (Brown et al., 1984) and a temperature sensitive (ts) mutant ts 1201 (Preston et al., 1983) which form non syncytial (syn^+) plaques on BHK 21/C13 cells.

TISSUE CULTURE MEDIA

Growth media

Glasgow modified Eagle's medium (Busby et al., 1964) was supplied as a 10x concentrate by Gibco Limited, U.K. BHK 21/C13 cells were grown in IX medium supplemented with 100 units/ml penicillin, 100 ug/ml streptomycin, 10% tryptose phosphate broth (TPB) and 10% calf serum (ETC_{10}). The serum concentration was reduced to 5% (ETC_5) for maintenance of cells. Calf serum was replaced with 5% pooled human serum (EH_5) to overlay virus titrations.

Other media included:

EFn .. Eagle's medium containing n% foetal calf serum

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

L-broth .. 170 mM NaCl, 1% (w/v) Difco bactotryptone, 0.5% (w/v) yeast extract, 100 ug/ml ampicillin or 10-15 ug/ml tetracycline.

EC_{10} 1/5 met. EC_{10} medium containing 1/5 of the normal concentration of methionine.

BUFFERS AND SOLUTIONS

Denhardt's buffer (DB) - 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinyl-pyrollidone, 0.1% (w/v) BSA.

Electrophoresis buffer (E.buffer) - 36 mM Tris-HCl pH 7.8, 30 mM $NaH_2Po_4.H_2O$, 1 mM EDTA

HEPES buffered saline (HEBS) - 130 mM NaCl, 4.9 mM KCl, 1.6 mM Na_2HPO_4, 5.5 mM dextrose, 21 mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanosulphonic acid) pH 7.05

Hybridization buffer - 10 mM Tris-HCl pH 7.5, 3xSSC, 5xDB containing 100 ug/ml of salmon sperm DNA

Nick translation buffer (NTB) - 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$ 50 ug/ml BSA, 1 mM DTT

Phosphate buffered-saline (PBS) - 170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4 pH 7.2 (Dulbecco and Vogt, 1954)

Reticulocyte suspension buffer (RSB) - 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5

SDS Polyacrylamide gel buffer (1x) (tank buffer) - 53 mM Tris, 53 mM glycine 1% (w/v) SDS

Resolving gel buffer - 1.5 mM Tris-HCl pH 8.9, 0.4% (w/v) SDS

Stacking gel buffer - 0.49 M Tris-HCl pH 6.7, 0.4% (w/v) SDS

Polyacrylamide gel fix/stain - A mixture of methanol, water and acetic acid in the ratio of 50/40/7 (v/v) containing 0.2% (w/v) Coomassie brilliant blue

Polyacrylamide gel destain - A mixture of methanol, water and acetic acid in the ratio of 50/880/70

Acrylamide - 30% Acrylamide solution (+5% linker) - 28.5 gms acrylamide and 1.5 gm N-N'-Methylenebisacrylamide, used for all gradient gels; made up to 100 ml and filtered through a Whatman number 2 filter prior to use

Boiling mix - 1 ml stacking gel buffer, 0.8 ml 25% SDS, 0.5ml 2-B mercapto ethanol, 1 ml glycerol and 0.025 ml of a saturated solution of Bromophenol blue. Diluted 1 in 3 parts with distilled water, and if necessary, the pH adjusted by the addition of NaOH to give a blue colour

Restriction enzyme buffers (RE) - These were prepared as 10x stock according to BRL or Maniatis et al (1982)

10 x for BamHI - 200 mM Tris-HCl pH 8.0, 1 M NaCl, 70 mM $MgCl_2$ 20 mM 2-Mercapto ethanol and 1 mg/ml BSA

10 x for KpnI - 100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM DTT and 1 mg/ml BSA

10 x for XbaI, HpaI, HindIII and Eco RI - 200 mM Tris-HCl pH 8.0, 1 M NaCl, 70 mM $MgCl_2$ and 1 mg/ml BSA

Wash buffer - 10 x SSC, 0.1% SDS and 4.7N sodium phosphate pH 7.0

PBSCa - PBS containing 2% calf serum

T.E. - 1 mM EDTA, 10 mM Tris-HCl pH 7.4

NTE - 10 mM Tris-HCl pH 7.5, 10 mM NaCl and 1 mM EDTA

SSC - 150 mM NaCl, 15 mM Tri sodium citrate

TBE - 89 mM Tris, 89 mM Borate, 3 mM EDTA

Gel Soak I - 200 mM NaOH, 600 mM NaCl

Gel Soak II - 1 M Tris-HCl pH 8.0, 0.59 M NaCl

S.O.C. - 2% Bactopeptone, 0.5% yeast extract, 10 mM NaCl 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose, D.W. to make 100 ml

Restriction enzyme stop mixture (RE stop) - 10% Ficoll, 100 mM EDTA in 5XE buffer containing bromophenol blue to colour

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

Trypsin - 0.25% (w/v) Trypsin powder (Difco Limited) in tris saline

Versene - 0.6 mM EDTA in PBS containing 0.002% (w/v) phenol red

Trypsin-versene - 1 volume of trypsin mixed with 4 volumes of versene

Alkaline lysis

solution I - 50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA, 5 mg/ml lysozyme added just prior to use

solution II - 0.2 M NaOH, and 1% SDS

solution III - 5 M Potassium acetate pH 4.8

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

Phenol - Phenol for extraction of DNA refers to phenol (BDH) melted at 60°C by heating in a water bath and saturated with an equal volume of NTE buffer

Chloroform - Chloroform for extraction of DNA refers to 24 volumes of analar grade chloroform (BDH) mixed with 1 volume of isoamyl alcohol

PLASMIDS

The following recombinant plasmids carrying HSV fragments ligated into pAT 153 (Twigg & Sherratt, 1980), were transfected into the host Escherichia coli K_{12} strain HB101 (Boyer & Roulland-Dussoix, 1969) and propagated in L-broth containing 100 ug/ml ampicillin for BamHI and HindIII clones or 10-15 ug/ml tetracycline for KpnI clones. The plasmids had previously been constructed in the Institute of Virology, Glasgow, by a number of people and were made available by Dr V.G. Preston. The map location of the HSV inserts is given below:

Plasmid clone	HSV-1 (17 syn^+) insert	Map location (m.u.)
pGX 121	KpnI b	0.024 - 0.108
pGX 122	KpnI c	0.434 - 0.518
pGX 1233	KpnI d	0.59 - 0.672
pGX 125	KpnI f	0.120 - 0.19
pGX 126	KpnI g	0.73 - .80
pGX 128	KpnI i	0.224 - 0.282
pGX 146	KpnI x	0.42 - 0.434
pGX 148	KpnI z	0.672 - 0.682

pGX 92	BamHI v	0.39	-	0.41
pGX 2	BamHI k	0.81	-	0.852
pGX 34	BamHI x	0.95	-	0.964
pGX 60	BamHI y	0.964	-	0.978
pGX 40	BamHI z	0.938	-	0.950
	HSV-2 (HG52) insert			
pGZ 26	Hind III a	0.524	-	0.736
pGZ 11	Hind III b	0.170	-	0.264
pGZ 15	Hind III h	0.282	-	0.402

Plasmid pS1 - containing a 535 bp Sau 3A subfragment of Bam HI x of HSV-1 strain 17 cloned into pAT 153 at the Bam HI site was kindly supplied by Dr. Nigel D. Stow.

RADIOCHEMICALS

All radiolabelled compounds were obtained from Amersham International plc., Amersham.

CHEMICALS

Analytical grade chemicals were used where available. These were obtained from five companies including Sigma Chemical Company; BDH Chemicals; Koch Light Laboratories; May & Baker Limited; and James Burroughs Limited. Vetalar (Ketamine hydrochloride, 100 mg/ml) was supplied by Parke-Davis, U.K. while Rompun (Xylazine, 500 mg/vial) was supplied by Bayers Limited, U.K.

MISCELLANEOUS

Plastic petri dishes for tissue culture were supplied by Nunclon Limited.

Nitrocellulose membranes (0.45 um) were purchased from Schleicher and Schull, West Germany.

Photographic film was obtained from Kodak Limited, London.

Disposable ECG electrodes type VL-00-S were supplied by Medicotest, Denmark.

EXPERIMENTAL ANIMALS

New Zealand White rabbits (1.5-2 kg) were obtained from a commercial breeder - Hyline Rabbit Farms, U.K.

2.1. GROWTH OF CELLS

BHK21/C13 cells were grown in rotating 80 oz glass culture bottles containing 100 ml ETC10 in an atmosphere of 5% CO_2, 95% air. Confluent bottles of cultured cells (aproximately 3 x 10^8 cells) were harvested by washing the monolayer twice with 25 ml trypsin-versene and resuspending the cells in 20 ml ETC10. Cells from one bottle were sufficient to seed 10 new bottles. To obtain confluent monolayers in 24 hr at 37°C. 50 mm and 35 mm plastic petri dishes were seeded at a density of 2 x 10^6 and 1 x 10^6 cells per dish respectively while 24 well Linbro-trays and 96 well microtiter trays were seeded at 5 x 10^5 and 5 x 10^4 cells/well respectively.

2.2. GROWTH OF VIRUS

2.2.a. Production of virus stocks

Growth medium from almost confluent BHK 21/C13 monolayers in 80 oz roller bottles was replaced with 25 ml of EC5 containing 0.003 pfu per cell of virus. The infected monolayers were incubated at 31°C until extensive cytopathic effect had developed. Infected cells were shaken into the medium and harvested by centrifugation at 1500 x g for 10 min at 4°C. Cell released virus from the supernatant medium was concentrated by centrifugation at 25000 x g for 2 hr at 4°C. The virus pellet was resuspended in a small volume of supernatant medium, sonicated until homogenous and stored at -70°c after checking sterility. Cell associated virus was prepared by sonicating the cell pellet from the low speed spin in a small volume of EC_5 and clarified by centrifugation at 1500 x g for 10 min at 4°C. Sonication and centrifugation of the cell pellet was repeated if necessary. The supernatants, containing cell associated virions were pooled, checked for sterility, aliquoted in 1 ml volumes and stored at -70°C.

2.2.b. Sterility checks on virus stocks

Virus stocks and cells were checked for sterility by streaking a loopful of virus or cell suspension on blood agar plates. The plates were incubated at 37°C and at room temperature (RT) and checked for bacterial or fungal contamination after 1 week. Cells were also checked for PPLO contamination by the Cytology section of the Institute.

2.2.c. Titration of stock virus

Serial 10 fold dilutions of virus stocks were made in PBSCa. 100 ul aliquots of each dilution were inoculated in duplicate onto semiconfluent monolayers of BHK 21/C13 cells in 50 mm petri dishes from which the medium had already been removed. Following absorption for 45 min at 37°C, monolayers were overlaid with 4 ml of EH_5 to neutralize unadsorbed virus and to prevent secondary plaque formation. After 2 days at 37°C or 3 days at 31°C the monolayers were stained with Giemsa for 30 min at RT, washed with tap water and plaques counted using a dissection microscope. The virus titers were expressed as $\log_{10}$ pfu/ml.

2.2.d. Plaque purification of virus

The human serum containing medium was drained from BHK21/C13 plates containing very few plaques (5 to 20) and the monolayers washed twice with PBSCa. Using a dissecting microscope, the virus infected cells from individual, well seperated plaques were taken up with narrow bore tipped 50 ml eppendorf pipettes. These cells were transferred to glass vials containing 0.5 ml PBSCa and sonicated. After titration of the original plaques the procedure was repeated twice to obtain stable, purified virus isolates.

2.3. VIRUS DNA TECHNIQUES

2.3.a. Preparation of HSV DNA

The methods of Wilkie (1973) and Stow and Wilkie (1976) were followed. In brief, nearly 80% confluent monolayers of BHK 21/C13 cells in 10, 80 oz bottles were infected with HSV at a moi of 0.003 pfu/cell. When extensive cpe had developed, cells were shaken into the medium and pelleted by low-speed centrifugation. Cytoplasmic extracts were prepared from the pellet by treating with 0.5% NP_{40} (v/v) in 10 ml of RSB. After 10 min incubation on ice, samples were centrifuged at 700 x g for 3 min to remove nuclei and cell debris. The supernatant was pooled with the clarified virus-infected cell medium while the pellet was resuspended in RSB/NP_{40} and extracted once more. Virus from the pooled supernatant was pelleted by centrifugation at 250 x g for 2 hr at 4°C; the virus pellet was resuspended in 8 ml of NTE by sonication and lysed with SDS (2%, v/v) in the presence of 10 mM EDTA for 10 min on ice. The virus DNA was extracted sequentially, once with an equal volume of phenol and once with chloroform; precipitated with ethanol; lyophilized and redissolved in a small quantity of distilled water.

In some cases the DNA was further purified by caesium chloride density gradient centrifugation by adjusting the refractive index of the DNA solution to 1.041 by addition of heat inactivated powdered caesium chloride. After centrifugation at 150,000 x g (42,000 rpm) for 18-20 hr at 15°C in a Beckman TV865B rotor, the gradient was dripped through a 20 gauge needle, into 12 to 15 fractions. Fractions containing DNA detected by ethidium bromide gel electrophoresis and UV illumination were pooled, dialysed against several changes of TE for 16-20 hr, ethanol precipitated, redissolved in distilled water and stored at -20°C.

2.3.b. Estimation of DNA concentration

Estimations of DNA concentration were made by comparison of different volumes of a DNA solution with standard DNA of known concentration after electrophoresis through an ethidium bromide stained agarose gel and visualisation by long wave length (365 nm) UV illumination. Bacteriophage lambda DNA or HSV DNA of known concentration were used as standards.

2.3.c. In vivo labelling of viral DNA with [^{32}] orthophosphate

Radio-labelling of viral DNA with [^{32}P]orthophosphate was carried out using the Linbro well technique of Lonsdale (1979) as modified by Brown et al, (1984). Linbrowells seeded 24 hr previously with 5 x 10^5 BHK 21/C13 cells in PIC medium were infected with 10 pfu per cell of pretitrated virus stocks. Following absorption for 1 hour at 37^{o}C infected monolayers were washed twice with PIC and overlaid with 450 ul of PIC. After a further 2 hr at 31^{o}C, 50 ul of PIC containing 50 uCi of [^{32}P]orthophosphate was added to each well and incubation continued for 48 hr at 31^{o}C. Radio-active [^{32}P]-labelled viral DNA was obtained by lysis of the infected cells with 2.5% (w/v) SDS at RT and sequential extraction with phenol and chloroform and ethanol precipitation. Aliquots of the DNA dissolved in distilled water were digested with a range of restriction endonucleases at concentrations sufficient to give complete digestion in 4 hr at 37^{o}C. Digests were analysed by electrophoresis on agarose gels.

2.3.d. Restriction enzyme digestion of viral DNA

Viral DNA (1-2 ug/sample) to be digested with restriction endonucleases was mixed with 3 ul of an appropriate 10 x RE buffer containing 2-3 units of endonuclease per ug of DNA in a total volume of 30 ul in distilled water. After 4 hr at 37^{o}C enzymatic digestion was terminated by adding one-sixth volume of RE stop solution.

2.3.e. Agarose gel electrophoresis of viral DNA

Agarose gels (0.5-1.2%) were made by dissolving an appropriate amount of agarose in 250 ml of E buffer. The agarose solutions were poured into 260 mm x 165 mm glass plate whose edges had been sealed with teflon tape and in which a 12 or 15 tooth comb had been inserted to form wells. When the gel had set the tape and comb were removed and the gel plate placed on a horizontal platform in a tank containing enough E buffer to form a shallow layer of liquid on top of the agarose. DNA samples (36 ul) containing one sixth volume of RE stop were electrophoresed at $2V/cm^2$ for 16-20 hr following which gels were dried at $80^{o}C$ and autoradiographed by exposing to Kodak X-Omat S film.

2.3.f. Purification of DNA fragments from agarose gels

After electrophoresis and visualisation under U.V. illumination, gel slices containg DNA fragments were excised and the DNA eluted from the agrose by high voltage electrophoresis in TBE. DNA was further purified by treatment with phenol and chloroform (1:1, v/v) and precipitated with ethanol.

2.4. TRANSFECTION OF CULTURED CELLS

2.4.a. Cotransfection experiments to construct HSV-1 X HSV-2 RECOMBINANTS

For transfection of BHK21/C13 cells with HSV DNA, the modified calcium phosphate technique of Stow and Wilkie (1976) was followed. Semi confluent monolayers of BHK 21/C13 cells (4×10^6 cells) in 50 mm dishes from which growth medium had been drained were overlaid with 400 ml of HEBS containing 10 ug/ml calf thymus DNA, 0.1 to 0.2 ug of intact HSV DNA, 1 to 2 ug of the total fragments from HpaI or XbaI cleaved McKrae or HG52 DNA (reciprocal to the intact genomes), and 130 mM calcium chloride.

In cotransfections involving individual HG52 DNA fragments, a 10 to 15-fold molar excess of the HG52 DNA fragment either purified from the gel or plasmid borne was cotransfected with intact McKrae DNA (0.1 to 0.2 ug per 50 mm dish). The concentration of calf thymus DNA was either reduced or omitted from the transfection solution to avoid saturation of cells with exogenous DNA.

45 min post infection at 37°C dishes were overlaid with 4 ml of EC_5. Four to 6 hr post infection at 31°C, EC_5 was replaced with 1 ml of 25% dimethylsulfoxide (DMSO) in HEBS for 4 min at RT. Residual DMSO was removed from the monolayers by gently washing twice with EC_5. Four ml of EC_5 was added to each dish and incubation continued for a further 3 to 5 days at 31°C. Well isolated individual plaques were picked into 0.5 ml PBSCa, sonicated, plaque purified and grown into 50mm plate stocks.

2.5. PLASMID DNA TECHNIQUES

2.5.a. Large scale isolation of plasmid DNA

Large scale isolation of plasmid DNA was achieved by the alkaline lysis method of Maniatis et al, (1982). Escherichia coli bacteria carrying plasmids containing HSV DNA inserts in pAT were grown in 5 ml L-broth culture containing the appropriate antibiotic by inoculating 25 ul of a glycerol stock of bacteria into the medium and incubating at 37°C for 18 hr. This culture was transferred to 500 ml of antibiotic containing L-broth and incubated for a further 18 hr at 37°C in a Gallenkemp orbital incubator. The bacteria were pelleted at 8000 x g for 5 min at 4°C and resuspended by vortexing in 14 ml of alkaline lysis solution I. Incubation on ice was continued and 20 ml of freshly made solution II and 15 ml of an ice cold solution III were sequentially added at 10 min intervals by vortexing. Ten min later plasmid DNA was recovered in the supernatant after centrifugation at

4°C for 30 min at 45,000 x g in a SS34 rotor. DNA was sequentially extracted with phenol and chloroform and precipitated with 2 x volumes of ethanol at RT and pelleted at 40,000 x g for 30 min in a SS34 rotor. The DNA pellet was washed with 70% ethanol, lyophilized and redissolved at 4°C overnight in 8 ml of TE buffer containing 10 ug per ml RNAase I. The DNA was further purified by CsCl gradient equilibrium centrifugation.

2.5.b. Caesium chloride gradient purification of plasmid DNA

Plasmid DNA in a solution containing caesium chloride and ethidium bromide at final concentrations of 1 g/ml and 600 ug/ml respectively (final density 1.55 g/ml), was centrifuged at 130,000 x g (40,000 rpm) for 36 hr at 20°C in a Beckman Type-50 or Type-65 rotor (Maniatis et al, 1982). Plasmid DNA bands were visualised by long-wave UV (365 nm) irradiation and the lower band, containing supercoiled plasmid DNA, was collected by side puncture of the the tube with a needle and syringe. Following removal of ethidium bromide from the solution by sequential extractions with isoamyl alcohol saturated with caesium chloride, the DNA was dialysed overnight against TE. DNA was finally precipitated with ethanol, pelleted by centrifugation and redissolved in distilled water and quantified.

2.6. DNA BLOT HYBRIDIZATION

2.6.a. Transfer of viral DNA fragments to nitrocellulose membranes

The procedure followed was essentially that of Southern (1975) in which single stranded DNA is immobilized on a nitrocellulose membrane, The agarose gel containing the separated DNA fragments was treated with gel soak I for 1 hr to denature the DNA and neutralized in gel soak II for 1 hr. The gel was then transferred onto two sheets of Whatman 3 mm filter paper which were in contact with, but not covered,

by 10 x SSC buffer. A sheet of nitrocellulose moistened with SSC was placed on top of the gel, followed by four sheets of 3 mm filter paper cut 2 mm smaller than the gel size. Finally, a weighted stock of cut paper towels was laid on the Whatman paper. The following day, the nitrocellulose sheet was removed, air dried and baked in a vacuum oven at 80°C for 2 hr. Efficient transfer of DNA fragments from the gel was monitored by visualizing the blotted gel under UV light.

2.6.b. In vitro [^{32}P]-labelling of viral DNA by nick translation

Viral DNA fragments were labelled with [^{32}P] essentially as described by Rigby et al, (1977). 0.5ug DNA was labelled with 2 uCi [^{32}P] dCTP using 1 unit of DNA polymerase in a 30 ul reaction mixture containing 3 ul 10 x nick translation buffer, 2.25 ml each of 0.2 mM dATP, dGTP, dTTP; 1 ul each of 1% (w/v) BSA and 1 x 10^{-6} mg/ml DNAase. Following a 2 hr incubation at 15°C the DNA was precipitated on dry ice for 15 min with 0.6 volume of isopropanol and 0.1 volume of 3M sodium acetate. The DNA pellet, recovered by microfugation for 5 min was resuspended in 50 ul distilled water and reprecipitated with isopropanol and 3M sodium acetate. The labelled DNA, redissolved by boiling for 5 min in 100 ul of 80% formaldehyde was used as a probe for hybridization.

2.6.c. DNA Blot hybridization procedure

DNA hybridization in aqueous solution, under high stringency conditions was carried out according to the procedures of Southern (1975) and Denhardt (1966). Nitrocellulose membranes containing DNA fragments were prehybridized in 10 ml of hybridization buffer in sealed plastic bags submerged in a shaking water bath at 75°C. After a minimum of 2 hr, the buffer was replaced with 10 ml fresh hybridization buffer containing the denatured [^{32}P]-labelled nick translated DNA probe. Hybridization at 75°C was terminated after 48 hr

by washing for 1 hr each at 60°C with 4 x 1 litre volumes of wash buffer. Blots were air dried and placed in contact with Kodak X-Omat S films for 16-24 hr at RT or fluorographed in conjunction with a DuPont phosphotungstate intensifying screen at -70°C for 3-4 days.

2.7. ANALYSIS OF VIRUS INDUCED POLYPEPTIDES

2.7.a. Infection of cells and labelling with [^{35}S]-methionine

80% confluent BHK21/C13 monolayers in 30mm petri dishes containing 1 x 10^6 cells were infected with virus at a moi of 20 pfu per cell in a 0.1 ml inoculum. After 1 hr adsorption at 37°C, the cells were gently washed twice with 1 ml of EC_{10} 1/5 met. medium preheated to 37°C to remove unabsorbed virus and were finally overlaid with 0.5 ml of the same medium. The infected monolayers were incubated at 37°C and at 3 hrs post absorption, 50 uCi of [^{35}S] methionine was added to each infected dish.

2.7.b. Sample preparation

Essentially the method of Marsden et al (1976) was followed. After 18-24 hr incubation at 37°C, the radioactive medium from the infected dishes was discarded, the cells were washed twice with PBS, and solubilized by incubation for 10 min at 60°C in boiling mix diluted 1 in 3 with distilled water. The samples, transferred to glass vials were heated to 100°C for 2 min and a 100 ul spotted onto a Whatman number 1 filter disc, which was air dried. Following sequential washing of the discs twice in ice cold trichloroacetic acid (5% w/v) and once with absolute alcohol, the amount of radioactivity was measured in each disc in the usual way. The radioactive contents in each sample were standardized, using boiling mix as a diluent,so that each contained the same amount of radioactivity per unit volume.

2.7.c. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Polypeptides were separated on discontinuous slab gels prepared according to the method of Studier (1973) as modified by Marsden et al (1976). Gradient gels composed of a 5-12% linear gradient of acrylamide cross linked with 1 part in 20 (w/v) N-N'-methylenebisacrylamide in resolving buffer were formed using a Technicon fractionating pump operating at 1.76mls/min. Polymerization was achieved by the addition of ammonium persulphate (0.006% w/v) and TEMED (0.004% v/v) to the gel solution just before pouring into the gel sandwich, held in a tank containing cold water in order to disperse heat during polymerization. The gel was overlaid carefully with either resolving gel buffer (1/4 strength) or butan-2-ol, to ensure a smooth interface for polymerization. Stacking gel was prepared shortly before sample loading and contained 5% acrylamide cross linked with 1 part in 30 (w/w) N-N'-methylenebisacrylamide in stacking gel buffer. Wells were formed with teflon combs. 0.01ml samples of each infected cell extract were loaded into wells and electrophoresed in a freshly prepared tank buffer at 40 mAmps for 3 to 4 hr or at 10 mAmps for 18 hr or until the Bromophenol blue dye front emerged from the end of the gel. At the end of electrophoresis the gel was removed and fixed/stained for 1 hr at room temperature, destained and rehydrated by addition of several changes of destain for 2 hr. The gel was dried onto paper using a Bio Rad gel slab drier, operating under a vacuum of 30 inches of mercury, and exposed to Kodak X-Omat, X-ray films for various lengths of time and developed in a X-Omat film processor.

2.8. STUDIES WITH RECOMBINANT VIRUSES IN RABBITS

2.8.a. Inoculation of rabbit eyes

Individual New Zealand white rabbits were inoculated with 50ul of

PBS containing 10^5 to 10^7 pfu of either HSV-1 McKrae or HSV-2, HG52 or McKrae X HG52 recombinants into the lower cul-de-sacs of their left eyes. Closed eye lids were gently massaged against the unscarified cornea for 1 min. Only the left eye of each rabbit was inoculated to abide by the Animal Experimentation Act regulations. Primary ocular infections including severe eye inflammation, dendritic or geographic ulcerations of the corneal epithelium and stromal involvement were confirmed by examination with a strong light in the presence of fluorescein stain from days 2 to 7 post infection. The exact titer of the virus inoculum was determined by titration of the virus on the same day as infection of the rabbit eyes.

2.8.b. Determination of virus shedding from the rabbit eyes

Eye swabs were collected in 0.5ml of PBS containing 1% (v/v) bovine foetal calf serum after gently rotating a sterile cotton tipped applicator in the upper cul-de-sac then across the cornea and into the lower cul-de-sac of the left eye of each rabbit. Swabs were vortexed for 1 min and the cotton tipped applicator sequeezed out into the PBS. A 150-200ul aliquot of the PBSFc was inoculated onto each of two semi-confluent BHK21/C13 50mm monolayers. Following absorption for 45 min the dishes were overlaid with EC_5 or EH_5 and incubated at 31°C for one week. Monolayers showing extensive cpe were harvested by scraping the infected cells into the medium. Virus was released by sonication at -70°C. A representative number of individual plaques from the dishes overlaid with EH_5 were picked into PBSca, grown to plate stocks and stored at -70°C till further analysis.

2.8.c. Iontophoresis of epinephrine (adrenalin) to induce shedding of virus in the rabbit eyes

Rabbit eyes were iontophoresed with l-epinephrine (adrenalin) essentially as described by Kwon et al (1981). An eye cup was inserted

with its periphery within the limits of the corneal limbus of the left eyes of rabbits pre-anaesthetised with xylazine (4mg/kg, intramuscular) and ketamine (20mg/kg, intramuscular). A 0.01% l-epinephrine (w/v, in water) solution was added into the eye cup. The cathode was attached to a shaved area of the rabbit trunk through an ECG electrode and the anode made a wet contact with the ocular solution through a paper wick. A direct current (0.8 mAmp; 7 volts) was applied for 7 min. Iontophoresis was performed once a day for 3 consecutive days. Eye swabs were collected each day for a minimum of one week and screened for shedding of infectious virus in the tear film by inoculation of control BHK21/C13 cells.

2.8.d. Explantation of trigeminal ganglia and corneas

Rabbits failing to shed virus in the tear film for one week, 3-4 weeks post iontophoresis, were sacrificed and both left and right eye balls and the trigeminal ganglia were dissected out in EF_{25} and transferred under aseptic conditions to the laboratory. Tissues were washed 3 times in cold EF_{25}. Corneal discs were dissected out from the rest of the eye ball, washed again with EF_{25}, divided into 6 segments and explanted into 200-250 ul EF_{25} in wells of microtiter plates. Each trigeminal ganglion was similarly divided into six segments and seeded in EF_{25} in microtiter plates. The latter were incubated at 31°C. Supernatant medium (150 to 200ul) from each well explant was added to semiconfluent BHK21/C13 cell monolayers grown in 96 well microtiter plates and screened for virus daily for the subsequent 4 weeks. Infected cells from these wells in which the supernatant produced cpe on BHK21/C13 cells within 4 days, were harvested, the virus released by sonication and stored at -70°C till further analysis.

2.8.e. Identification of viral isolates

Viral isolates from ocular swab cultures and tissue explants were analysed by restriction endonuclease digestion of [^{32}P] *in vivo* labelled DNA by the method of Lonsdale (1979) as described previously.

2.9. DNA CLONING

2.9.a. Sub-cloning of HSV fragments

The linearised plasmid pS1 containing HSV-1 Ori_S was treated with calf intestinal phosphatase at a concentration of 5 units per ug of plasmid DNA. After incubation at 37°C for 1 hr, the DNA was extracted once with phenol: chloroform (1:1 v/v), once with chloroform, and precipitated with ethanol. A 3x molar excess of a purified HSV2 DNA fragment (HindIII *h*) relative to the phosphatase treated plasmid (10ng) was incubated overnight at 15°C in 20ul of ligation buffer containing 1 unit of T_4 DNA ligase. DNA was sequentially extracted once with phenol:chloroform and once with chloroform, ethanol precipitated and redissolved in a small volume of ligation buffer.

2.9.b. Transformation of E.coli

One ul (10-20ng) of ligated DNA (HSV-1 Ori_S plus HG52 HindIII *h*) was added to 20ul of frozen *E.coli* DH_5 ligation competent cells (BRL) in chilled sterile Eppendorf tubes. This suspension was incubated on ice for 30 min followed by a 40 seconds pulse in a 42°C water bath. The tubes were then transferred to ice, 80ul S.O.C. added to each and shaken at 225 rpm at 37°C for 1 hr before being plated on L-broth agar containing ampicillin. After 16-18 hr incubation at 37°C, individual colonies were picked, grown in 5ml of L-broth as overnight suspension cultures and the plasmid DNA analysed for the presence of the HSV fragment by digestion with restriction endonucleases. As controls, DH5 cells were similarly transformed with an equal amount of the HSV fragment DNA alone, linearised plasmid pS1 DNA, and plasmid pS1 after

ligation with T_4 ligase. Colonies containing an HSV insert were further plaque purified before a large scale plasmid DNA stock was prepared.

CHAPTER 3

RESULTS

3.1. INTRODUCTION

The aim of the work presented in this thesis was to determine the viral gene(s) controlling reactivation of HSV from latency in the rabbit eye model. On the basis of spontaneous virus shedding, different strains of HSV have been classified as high frequency recurrence (HFRc) phenotype or low frequency recurrence (LFRc) phenotype, in the rabbit eye model (Gerdes and Smith, 1983). HSV-1 strain McKrae and HSV-2 strain HG52 were designated as HFRc and LFRc respectively. As both strains were able to go latent in trigeminal ganglia (TG) with equal frequencies, it was suggested that the viral genes involved in reactivation could be identified by the construction of intertypic recombinants between HFRc and LFRc parents. To this end a series of McKrae X HG52 recombinants have been constructed and their behaviour in vivo compared to the parental McKrae and HG52 strains using the rabbit eye model of latency.

3.2. REACTIVATION POTENTIAL OF WILD TYPE HSV-2 (HG52) AND HSV-1 (McKRAE) IN THE RABBIT EYE MODEL OF LATENCY

To confirm the findings of Gerdes and Smith (1983) and to determine whether the recurrence phenotype holds true for induced reactivation, out bred New Zealand White rabbits were inoculated in the left eyes only with 50 ul of virus suspension containing $5x10^5$, $5x10^6$ or $5x10^7$ pfu of either HSV-1 strain McKrae or HSV-2 strain HG52. Rabbits inoculated with $5x10^6$ and $5x10^7$ pfu of McKrae either died soon after the initial infection or had to be sacrificed within a few days post infection because of severe distress including encephalitis. Rabbits infected with $5x10^6$ or $5x10^7$ pfu of HSV-2 strain HG52 could tolerate the infecting dose without any adverse effects. Spontaneous shedding of virus was detected by screening tear film swabs from the infected eyes on control BHK21/C13 cells. Two to three weeks post

infection swabs were collected and screened daily for 7 days. Reactivation of latent virus was induced by iontophoresis of epinephrine (adrenalin) daily for 3 consecutive days between 6-8 weeks post infection. Reactivated virus was detected by inoculation, onto BHK21/C13 cells, of tear film swabs from the infected eyes for 7 days post iontophoresis. Before epinephrine iontophoresis rabbit eyes were briefly screened for spontaneous shedding. Results of spontaeous and epinephrine induced virus shedding are presented in Table 2. Of the 11 rabbits infected with $5x10^5$ pfu of McKrae, all shed virus both spontaneously and on induction with epinephrine iontophoresis. Spontaneous shedding of virus was detected between day 21 and 34 post infection. As the eyes were only monitored once (for 7 days) for spontaneous shedding (apart from immediately prior to epinephrine iontophoresis), it is possible that virus was being shed in the tear films on days when the eyes were not being monitored. Following epinephrine iontophoresis, reactivated virus was first detected as early as 24 hr post iontophoresis and continued to be shed in some of the rabbits up to 8 days post iontophoresis. None of the rabbits were shedding spontaneously immediately prior to epinephrine iontophoresis.

Of 154 left eye tear film swabs screened from 22 rabbits infected with different doses of HG52, no spontaneous shedding was detected from any of the eyes during the period of observation. None of the 9 rabbits inoculated with either $5x10^5$ or $5x10^6$ pfu of HG52 demonstrated any evidence of virus reactivation upon iontophoresis of epinephrine. However, 2 of the 13 animals each inoculated with $5x10^7$ pfu of HG52 shed virus in their tear films after iontophoresis of epinephrine. Reactivated virus was first detected on day 3 and continued to be shed up to 5 days post iontophoresis.

Two to three weeks after iontophoresis and when the tear films

TABLE 2

Spontaneous and epinephrine iontophoresis induced reactivation of HSV1 (McKrae) and HSV2 (HG52) from rabbit eyes

Virus (dose/rabbit)	Rabbit No.	Left eye spontaneous shedding (days post infection)	Left eye epinephrine induced shedding (days post iontophoresis
McKrae	1	27-34	3-5
$5x10^5$ pfu	2	27-34	2-6
	3	27-34	3-6
	4	27-34	3-6
	5	27-34	3-5
	6	21-25	1-5
	7	21-25	1-5
	8	21-25	1-5
	9	21-25	2-8
	10	21-25	2-3
	11	21-25	2-5
HG52			
$5x\ 0^5$ pfu	1	-	-
	2	-	-
	3	-	-
$5x10^6$ pfu	1	-	-
	2	-	-
	3	-	-
	4	-	-
	5	-	-
	6	-	-
$5x10^7$ pfu	1	-	-
	2	-	3-5
	3	-	3-5
	4	-	-
	5	-	-
	6	-	-
	7	-	-
	8	-	-
	9	-	-
	10	-	-
	11	-	-
	12	-	-
	13	-	-

were negative for spontaneous shedding of virus, rabbits were sacrificed. Both left and right corneas and trigeminal ganglia (TG) were explanted *in vitro* and screened for 6-8 weeks for the release of latent virus. Results of shedding of latent virus from the explanted ganglia and corneas are presented in Table 3. From 11 animals infected with $5x10^5$ pfu of McKrae, infectious virus was recovered from all the explanted LTG between 5 and 20 days post explantation. Virus was also recovered from the RTG of 4 animals between 5 and 20 days post explantation. Release of infectious virus was detected between 5 and 30 days post explantation from all the explanted LTG from 8 rabbits infected with either $5x10^6$ or $5x10^7$ pfu of HG52. The RTG from one of the rabbits inoculated with $5x10^7$ pfu of HG52 released virus between 13 and 20 days post explanation.

Left corneal explants from one out of eleven rabbits infected with $5x10^5$ pfu of McKrae released virus between 18 and 21 days post explantation. Neither the corneal explants from the rabbits infected with HG52 nor the right corneas from the rabbits infected with McKrae were positive for release of latent virus.

The results presented in Tables 2 and 3 substantiate the spontaneous shedding findings of Gerdes and Smith (1983) and indicate that the high frequency recurrence phenotype (HFRc) of HSV-1 strain McKrae and low frequency recurrence phenotype (LFRc) of HSV-2 strain HG52 are due, at least in part, to virus encoded functions.

3.3. RESTRICTION ENDONUCLEASE MAPPING OF THE McKRAE GENOME

Before constructing recombinants between HSV-1 strain McKrae and HSV-2 strain HG52, it was necessary to determine the restriction endonuclease profiles of the McKrae virus genome. The restriction patterns of the McKrae DNA were compared with those of HSV-1 strain 17 *syn*$^+$ on digestion with various endonucleases. The restriction maps of

TABLE 3

Release of virus from explanted trigeminal ganglia (TG) and corneas

Virus (Dose/rabbit)	Rabbit No.	Left TG (Days post explantation)	Right TG	Left Cornea	Right Cornea
McKrae	1	7-8	-	-	-
$5x10^5$ pfu	2	6-17	-	-	-
	3	6-17	7-17	-	-
	4	5-17	-	-	-
	5	5-16	5-16	-	-
	6	12-20	14-20	18-21	-
	7	8-20	15-20	-	-
	8	13-20	-	-	-
	9	9-13	-	-	-
	10	9-13	-	-	-
	11	7-15	-	-	-
HG52					
i) $5x10^6$ pfu	1	8-20	-	-	-
	2	11-20	-	-	-
	3	18-20	-	-	-
ii) $5x10^7$ pfu	1	8-20	-	-	-
	2	8-20	-	-	-
	3	5-20	-	-	-
	4	7-20	13-20	-	-
	5	22-30	-	-	-

HSV-1 strain 17 of Wilkie (1976) and Davison (1981) are shown in Figure 28 together with those of HSV-2 strain HG52 (Cortini and Wilkie, 1978).

3.3.a. HindIII profile

Comparison of the HindIII profile of McKrae DNA with that of strain 17 revealed the absence of the 0.5 molar (M) m fragment; the 1M n fragment and the two 0.25 M joint fragments f (i+m) and c (d+m) (Figure 29A). Two novel high molecular weight bands can be seen: one comigrating with fragments a, b, and the second running slightly below fragments d, e. A novel band running slightly above fragments h, i is also observed in the McKrae profile. The data is consistent with the absence of the HindIII m/n site in McKrae giving rise to a fragment of 7.4×10^6 mw (m+n) which is migrating slightly above h, i (7×10^6 m.w.). The size of the novel joints (m+n+i) 14.4×10^6 mw i.e. f' and (m+n+d) 25.4×10^6 mw i.e. c' is consistent with the positions of the bands running below d, e (17.5×10^6 mw) and with a, b (26×10^6 mw) respectively.

3.3.b. EcoRI profile

The EcoRI restriction profile of the Mckrae genome revealed the same number of restriction sites as strain 17 (Figure 29B). The mobilities of three bands were different from those of strain 17. Fragment h was slightly larger in size and fragments k and l were slightly smaller.

3.3.c. BglII profile

Comparison of the BglII digests of McKrae DNA with that of strain 17 indicated equal numbers of restriction fragments in each digest. No differences in mobilities of the various fragments were observed (Figure 30A).

FUGURE 28

Physical maps of HSV-1 strain 17 and HSV-2 strain HG52 DNAs for the restriction endonucleases XbaI, HindIII, EcoRI, HpaI, BglII, KpnI and BamHI (Wilkie, 1976; Cortini and Wilkie, 1978; Davison, 1981). The restriction sites in HSV-1 are shown above, and those in HSV-2 are shown below the prototype genome model of HSV. The origin of the joint fragments is as follows:

Strain 17: XbaI, a = d+[s]; b = g+[s].

HindIII, b = d+g; c = d+m; e = i+g; f = i+m.

EcoRI, b = e+k; c = j+k.

HpaI, a = c+m; d = g+m.

Bgl II, a = f+h; b = j+h; c = f+l; e = j+l.

KpnI, a = r+j; e = r+k.

BamHI, k = s+q.

Strain HG52: XbaI, a = h+c, b = i+c, e = h+g; f = i+g.

HindIII, c = k+i, d = k+j; f = m+i; g = m+j.

EcoRI, b = k+f; c = k+h; d = m+f, e = m+h.

HpaI, b = f+[s]; c = g+[s].

BglII, a = k+d; b = m+d; e = k+h; f = m+h.

Kpn I, b = c+r; e = f+r.

BamHI, g = v + u.

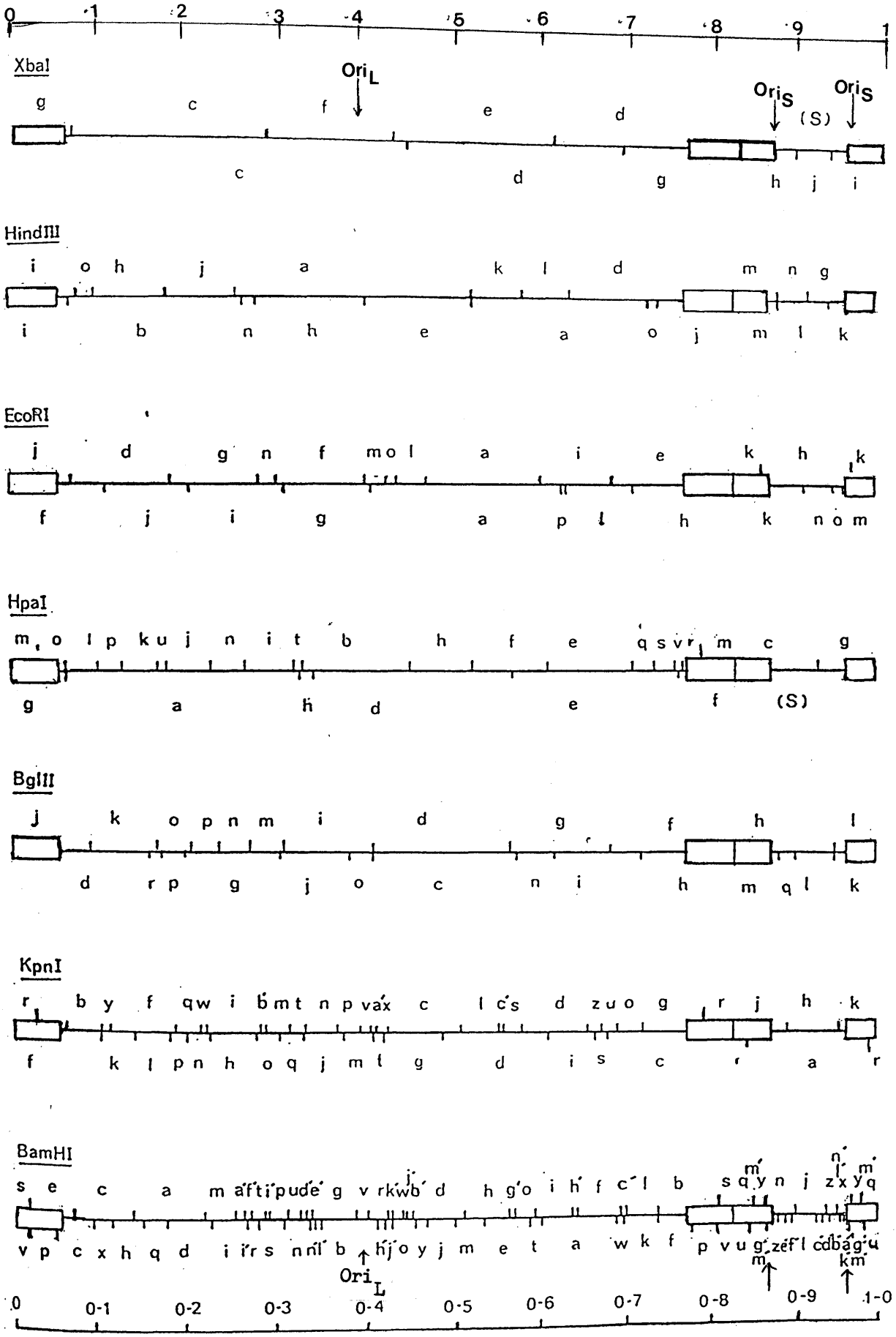

0 1 2 3 4 5 6 7 8 9 1
XbaI
Ori L
Ori S
(S)
Ori S
g c f e d
c d g h j i
HindIII
i o h j a k l d m n g
i b n h e a o j m l k
EcoRI
j d g n f m o l a i e k h k
f j i g a p l h k n o m
HpaI
m o l p k u j n i t b h f e q s v r m c g
g a h d e f (S)
BglII
j k o p n m i d g f h l
d r p g j o c n i h m q l k
KpnI
r b y f q w i b m t n p v a x c l c s d z u o g r j h k
f k l p n h o q j m l g d i s c a r
BamHI
s e c a m a f t i p u d e g v r k w b d h g o i h f c l b s q y n j z x y q
v p c x h q d i i r s n 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
Ori L
0 0-1 0-2 0-3 0-4 0-5 0-6 0-7 0-8 0-9 1-0

FIGURE 29

Autoradiographs of HindIII (A) and EcoRI (B) restriction digests of HSV-1 strain 17 and HSV-1 strain McKrae DNA. [^{32}P]-labelled virus DNA was digested with the enzymes under standard conditions and electrophoresed on 0.6% agarose gels. Letters refer to specific HSV-1 fragments (Figure 28). (◁) denotes missing fragments.

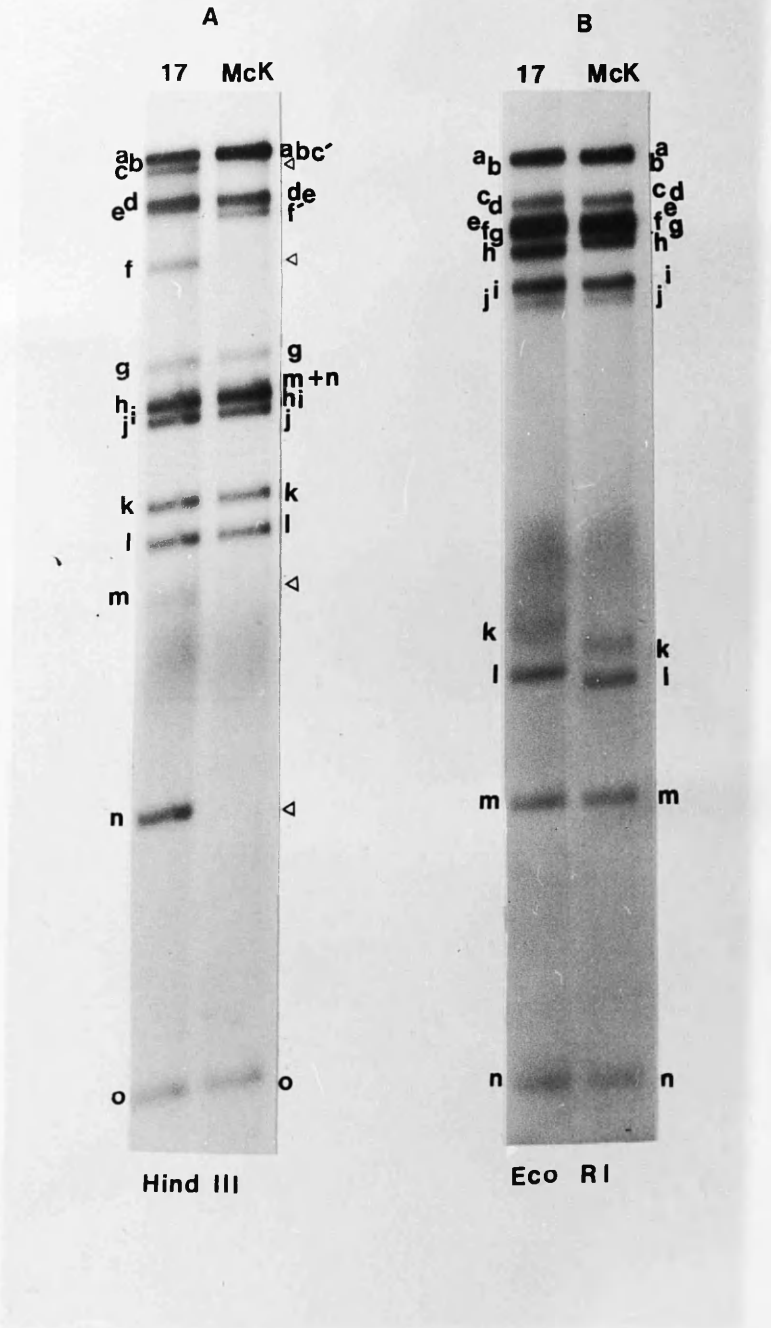
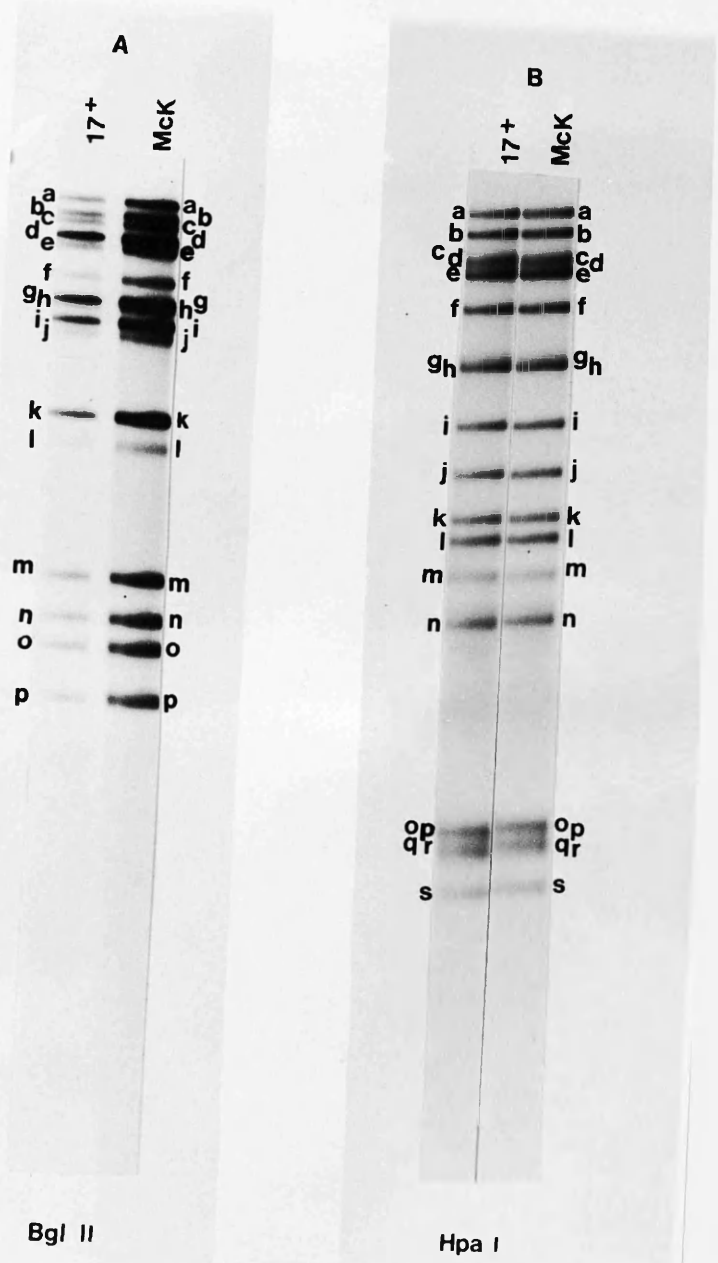

A
17
McK
abc'
de
f'
g
m+n
hi
j
k
l
m
n
o
Hind III
B
17
McK
a
b
c
d
e
f
g
h
i
j
k
l
m
n
Eco RI

FIGURE 30

Autoradiographs of BglII (A) and HpaI (B) digests of HSV-1 strains 17 and McKrae DNAs. [^{32}P]-labelled virus DNA was digested with the enzyme under standard conditions and electrophoresed on 0.6% (BglII) and 0.8% (HpaI) agarose gels. Letters refer to the specific HSV-1 fragments (Figure 28).



Bgl II

Hpa I

3.3.d. HpaI profile

No differences were observed in the number or sizes of the various fragments obtained from a HpaI digest of Mckrae compared to that of strain 17 (Figure 30B).

3.3.e. BamHI restriction profile

The BamHI profile of the McKrae genome showed a number of differences when compared with that of strain 17 (Figure 31A). The 1M a band is smaller by approximately $1x10^6$ mw and, therefore, migrates closer to the b band. The molar j and n bands are larger by $0.1x10^6$ mw and are migrating closer to i and m respectively.

Other differences were investigated by Southern blot analysis. As shown in Figure 31B, Southern blotting of BamHI digests of McKrae DNA with the Kpnl x fragment of strain 17 cloned in plasmid pGX146 revealed hybridization with BamHI r and with a band of approximately $1.7x10^6$ mw running below the s t fragments (B ii). With strain 17 there was normal hybridization to fragments r and w indicating that w in McKrae is larger by $0.3x10^6$ mw. Probing of the BamHI digests of the two viruses with the Kpnl i fragment (cloned in plasmid pGX128) of strain 17 gave, as expected, hybridization with the BamHI m, t, a' and f' fragments which were colinear in both digests (B, iii). Similarly probing with the BamHI V fragment (cloned in plasmid pGX92) of strain 17 revealed the colinearity of fragment v in both strains (B, iv). Hydbridization of BamHI digests of both viruses with the BamHI z fragment (cloned in plasmid pGX40) of strain 17 showed that the z fragment in McKrae is larger by $1.4x10^6$ mw than that of strain 17 (B, v).

3.3.f. Kpnl profile

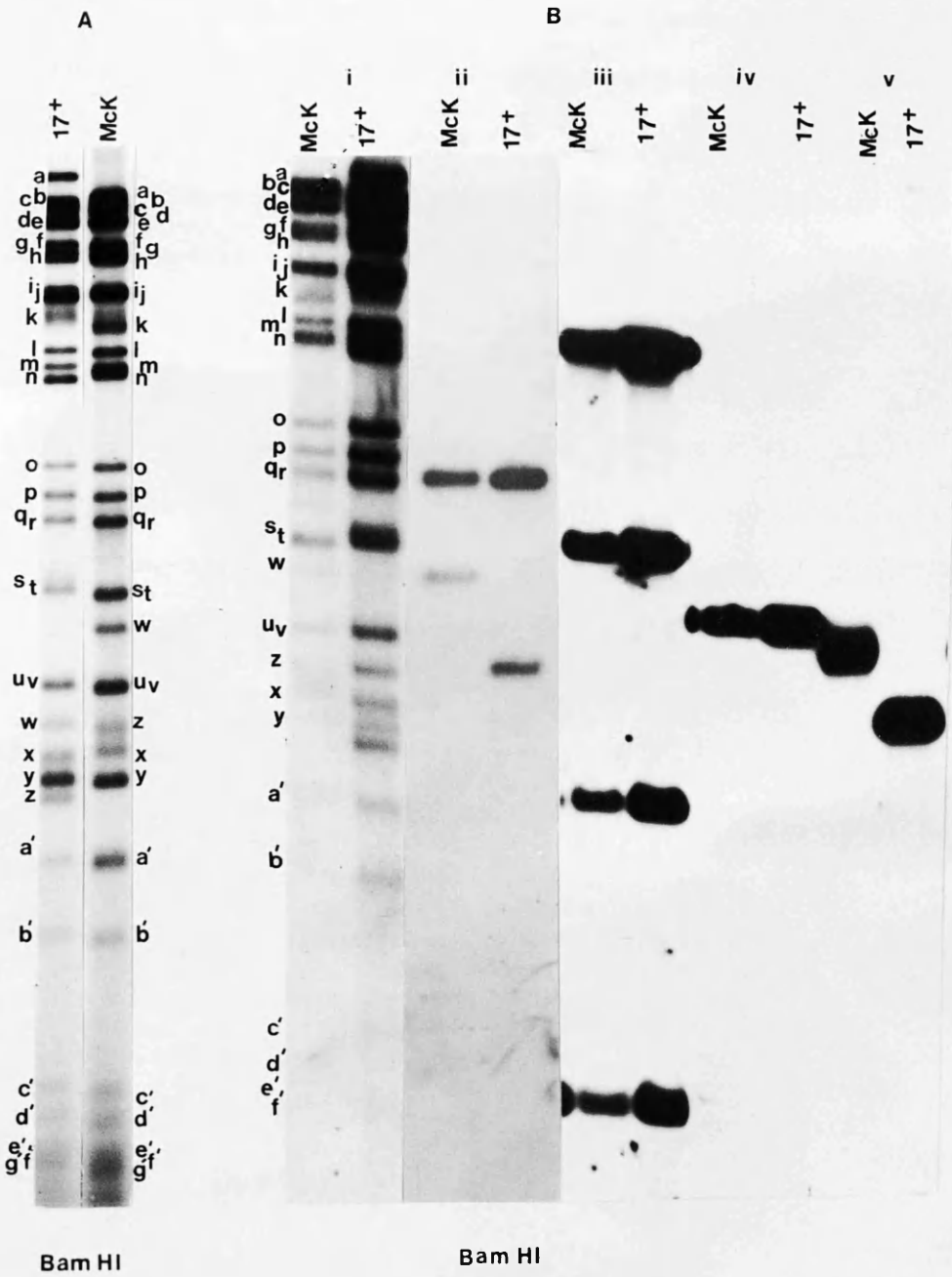
The Kpnl digest of the McKrae genome exhibited most variation

FIGURE 31

Mapping of BamHI restriction sites in the McKrae genome relative to strain 17.

A. Autoradiographs of [^{32}P]-labelled BamHI digests of strain 17 and McKrae DNA. Letters refer to specific HSV-1 BamHI fragments (Davison, 1981).

B. Autoradiographs of nitrocellulose blot strips containing BamHI restriction fragments of HSV-1 strain McKrae (left track of each pair) and HSV-1 strain 17 (right track of each pair) to which nick translated DNA probes have been hybridized. The probes were HSV-1 strain 17 DNA (i); the recombinant plasmid pGX146 (KpnI x) DNA (ii); the recombinant plasmid pGX128 (KpnI i) DNA (iii); the recombinant plasmid pGX92 (BamHI v) DNA (iv); and the recombinant plasmid pGX40 (BamHI z) DNA (v). Letters denote specific BamHI fragments (Figure 28).


A
17+
McK
Bam HI
B
i
ii
iii
iv
v
McK
17+
Bam HI

when compared with that of strain 17 (Figure 32A). Three of the fragments in the group a b c and d showed variation in mobilities and at least two appeared to be larger. In addition the mobilities of the g, h and r fragments also appeared to be altered.

Southern blot hybridization of KpnI digests of McKrae with KpnI cloned fragments of strain 17 (Figure 32B) showed that KpnI c (contained in plasmid pGX122) hybridized to one of the large fragments at the top of the gel (B, iii). In addition KpnI x (contained in plasmid pGX146) also hybridized to this band (B, vii) indicating that the KpnI c/x site in McKrae has been deleted. Similarly hybridization of KpnI d (contained in plasmid pGX123) to the other large fragment (B, iv) indicated an increase in the size of d possibly due to removal of the d/z site. When KpnI b and KpnI g were used as probes, (Bii, Bvi) there was hybridization to two fragments near the top of the gel and to a third fragment running near u. This is interpreted as indicating the presence of an additional KpnI site in both copies of R_L such that the b and g fragments are reduced in size by approximately 2×10^6 mw. The observed difference in the mobilities of the g' b' fragments in tracts in which the BamHI g and BamHI b fragments of strain 17 have been used as probes is due to these tracts arising from two independent blots of McKrae DNA digested with KpnI and electrophoresed at two different times. Probing with KpnI f of strain 17 (contained in plasmid pGX125) indicated the colinearity of this fragment in both McKrae and 17 (B, v).

The major alterations in the restriction sites of McKrae relative to strain 17 are depicted in Figure 33.

3.4. CONSTRUCTION OF McKRAE X HG52 RECOMBINANTS

3.4.a. Introduction

As the purpose of this study was to map the viral genes

FIGURE 32

Mapping of Kpnl restriction enzyme sites in the McKrae genome relative to strain 17.

A. Autoradiographs of [^{32}P]-labelled Kpnl digests of McKrae and strain 17 virus DNA. Letters refer to specific HSV-1 Kpnl fragments (Figure 28).

B. Autoradiographs of nitrocellulose blot strips containing Kpnl restriction fragments of HSV-1 McKrae (ii to vii and left track in i) and HSV-1 strain 17 (right track in i) to which nick translated DNA probes have been hybridized. The probes were HSV-1 strain 17 DNA (i); the recombinant plasmid pGX121 (Kpnl b) DNA (ii); the recombinant plasmid pGX122 (Kpnl c) DNA (iii); the recombinant plasmid pGX123 (Kpnl d) DNA (iv); the recombinant plasmid pGX125 (Kpnl f) DNA (v); the recombinant plasmid pGX126 (Kpnl g) DNA (vi) and the recombinant plasmid pGX146 (Kpnl x) DNA (vii). Letters refer to specific Kpnl fragments (Figure 28).

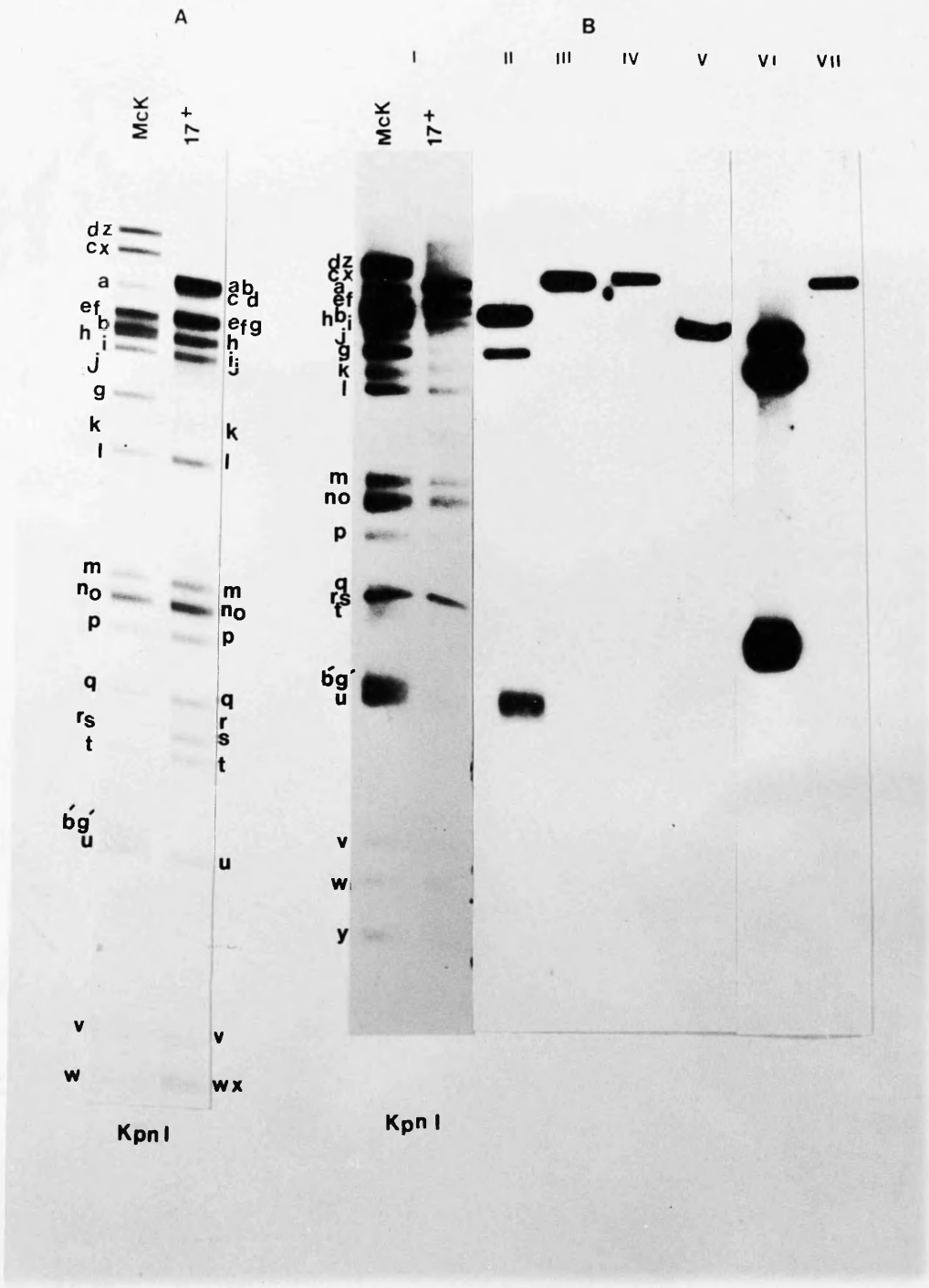

A
McK
17+
dz
cx
a
ef
b
h
i
j
g
k
l
m
no
p
q
rs
t
bg
u
v
w
ab
c d
efg
h
i j
k
l
m
no
p
q
r
s
t
u
v
wx
Kpn I
B
I
II
III
IV
V
VI
VII
McK
17+
dz
cx
a
ef
b i
h
j
g
k
l
m
no
p
q
rs
t
bg
u
v
w
y
Kpn I

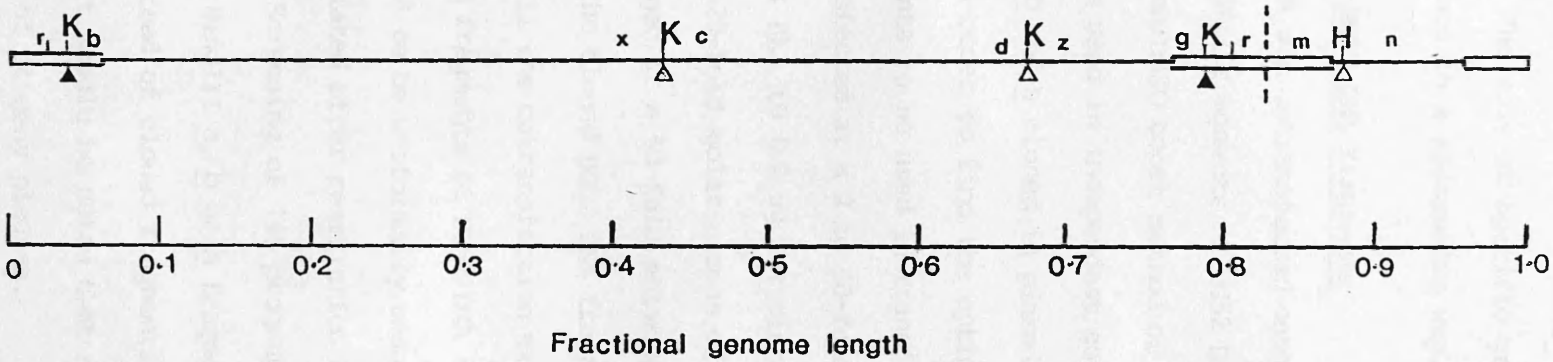
FIGURE 33

Diagrammatic representation of major alterations in restriction endonuclease sites in the McKrae genome relative to strain 17.

H - HindIII, K - Kpnl site

Δ- site deleted, ▲ extra site

Letters refer to specific KpnI and HindIII fragments.


r1
Kb
x
Kc
d
Kz
g
Kr
m
H
n
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Fractional genome length

controlling reactivation of HSV from latency by making recombinants between HSV-1 strain McKrae and HSV-2 strain HG52, the logical approach was to insert specific cloned fragments of HG52 into the intact McKrae genome. The role of specific groups of genes could, therefore, be determined in a systematic way.

3.4.b. Use of cloned HG52 DNA fragments

Intact McKrae DNA was cotransfected onto BHK21/C13 cells with cloned individual HindIII fragments of HG52 DNA by the calcium phosphate precipitation/DMSO boost method of Stow and Wilkie (1976). The HG52 DNA fragments used in independent cotransfection experiments included Hind III a, b and h cloned in plasmids pGZ26, pGZ11, and pGZ15 respectively. In order to find the optimum concentration of DNA of the various fragments to be used in transfection experiments, the fragments were cotransfected at a 2 to 50-fold molar excess over the intact McKrae genomes (0.1 to 0.2 ug per dish). Progeny plaques were obtained when up to a 20-fold molar excess of the fragment was used. No plaques were obtained at a 50-fold molar excess. A 10-fold to 15-fold molar excess of the cloned HG52 DNA fragment was selected as the maximum and used in all the cotransfection experiments.

The use of cloned fragments of HG52 DNA in making McKrae X HG52 recombinants was found to be uniformally unsuccessful in that no recombinants were isolated after restriction endonuclease screening of 1138 progeny plaques. Screening of 748 progeny plaques from cotransfections using HindIII a, b or h fragments excised and purified from agarose gels instead of cloned fragments also did not yield any recombinant viruses. It should be noted that no selection pressure was used in the isolation of progeny plaques.

3.4.c. Marker transfer technique

The inability to isolate any recombinants from the experiments

outlined above could have been due, at least in part, to an inherent failure in the system e.g. the BHK21/C13 cells being used may not have been efficient for transfection or poor DNA preparation or a poor technique. On the other hand it may merely reflect the inability to detect recombinants because of lack of a selectable marker.

The technique of marker transfer of ts mutations was used to make recombinants between McKrae and HG52. Intact McKrae genomes were cotransfected with HG52 ts mutant DNA (ts13 or ts1 or ts12). The ts mutant genomes were digested with appropriate restriction enzyme, electrophoresed on agarose gels and the DNA fragments containing the ts mutations were excised from the gels. The DNA was electroeluted and ethanol precipitated before being used in cotransfection experiments. The growth of the progeny plaques was compared at the permissive temperature (PT)-31^{o}C and at the nonpermissive temperature (NPT)-38.5^{o}C. Of 927 plaques analysed (345 for ts13, 275 for ts1 and 307 for ts12), none demonstrated temperature sensitivity indicating that the HG52 fragments containing the ts mutations had not been recombined into the McKrae genomes.

3.4.d. Random genome recombination using intact virion infections

Isolation of intertypic recombinants between different strains of HSV-1 and HSV-2 by co-infection of cells with ts mutants of both strains and selection of wild-type recombinant viruses at the NPT has been reported (Timbury and Subak-sharpe, 1973; Esparza et al, 1974; Morse et al, 1977).

To obtain intertypic recombinants between HSV-1 strain McKrae and HSV-2 strain HG52, BHK21/C13 cells were co-infected at 37^{o}C with 5 pfu per cell of each virus. Twenty-four hr after infection at 37^{o}C, the cells were scraped into the medium, sonicated and the progeny virus titrated under an EH_5 overlay. Of nearly 500 progeny plaques analysed

by restriction endonuclease digestion of [^{32}P]-orthophosphate labelled viral DNA no recombinants were detected.

3.4.e. Intratypic marker rescue of ts mutations

In order to rule out the possibilty that the inability to isolate any McKrae X HG52 recombinants from the experiments outlined above was due to an inherent failure in the system e.g. the BHK21/C13 cells used, being inefficient for transfection; poor DNA preparation; poor technique etc., intratypic marker rescue of ts mutations was carried out.

0.2 ug of HG52 ts5 DNA was cotransfected at 31^{o}C with 2 ug of HG52 ts9 DNA by the calcium phosphate technique as described in Methods section 2.4. On development of extensive cpe, the transfected cells were scraped into the medium and sonicated to release progeny virus. Virus yields at the PT (31^{o}C) and NPT (38.5^{o}C) were compared. In control experiments the DNA of one of the ts mutants was substituted with an equal quantity of calf thymus DNA. The virus yields at the PT and NPT expressed in pfu/ml are presented in Table 4.

As expected the individual ts mutant DNAs did not yield progeny virus which grew at 38.5^{o}C while a progeny virus titer of 4x10^{5} pfu/ml and 6x10^{6} pfu/ml was obtained at 31^{o}C from ts5 and ts9 DNA transfections respectively. When the cells were cotransfected with the DNA of both mutants a progeny yield of 7x10^{4} pfu/ml was obtained at 38.5^{o}C and 6x10^{6} pfu/ml at 31^{o}C. The results show that the DNAs of ts5 and ts9 had transfected efficiently and had recombined to yield wild type progeny virus capable of growth at 38.5^{o}C. The results also show that the BHK21/C13 cells used throughout this study were capable of being transfected and of supporting HSV recombination and that the inability to obtain recombinants between McKrae and HG52 was not due to the transfection procedure or due to an inherent property of the

TABLE 4

Intratypic marker rescue of HG52 (ts 5 x ts 9) ts mutants

Mutant DNA	Progeny virus yield pfu/ml at PT (31°C)	NPT (38.5°C)
ts 5	4×10^5	$< 10^1$
ts 9	6×10^6	$< 10^1$
ts 5 x ts 9	6×10^6	7×10^4

cells being used.

3.4.f. Restriction endonuclease cleaved genomes

BHK21/C13 cell monolayers ($4x10^6$ cells) in 50 mm dishes were cotransfected with intact McKrae genomes (0.2 ug) and a 10-fold molar excess of the total products of either Hpal or Xbal cleaved HG52 genomes (2 ug per dish). Progeny plaques were picked, their DNA labelled in vivo with (^{32}P) orthophosphate and analysed by digestion with various restriction endonucleases.

Of 405 plaques analysed from cotransfection experiments using Hpal digested HG52 genomes, 8 recombinants were identified (a recombination frequency of 1.97%). Of 236 progeny plaques screened from cotransfections using Xbal cleaved HG52 genomes, 3 recombinants were identified (a recombination frquency of 1.27%). The overall frequency of recombination was 1.72% (11 out of 641).

3.5. CHARACTERIZATION OF THE McKRAE X HG52 RECOMBINANTS

3.5.a. Nomenclature of the recombinant viruses

All the recombinants were plaque purified three times before raising virus stocks. The recombinants isolated from transfections using Hpal cleaved HG52 genomes were designated R10/3/1, R10/3/4/6, R10/4/1, R20/4/1, R30/3, R40/2/2, R40/2/4 and R43/2/2. Recombinants isolated from transfections involving Xbal cleaved HG52 genomes were designated R5/26/1, R47/27/1 and R7/3/3.

3.5.b. Clonal relationship of the recombinant viruses

Recombinants R10/3/1 and R10/3/4/6 were isolated as separate, individual plaques from a single transfection experiment. Similarly recombinants R40/2/2 and R40/2/4 although from separate plaques were from a single transfection. The two recombinants from each transfection could therefore be clonally related. The other

recombinants were isolated from independent transfections and were, therefore, clonally unrelated.

3.5.c. Genome structure of the recombinant viruses

The genome structure of the recombinants was deduced from restriction endonuclease analyses of recombinant virus DNA labelled in vivo with [^{32}P] orthophosphate and digested with a range of restriction enzymes. The boundaries of the heterologous insert sequences were mapped by comparing the presence or absence of restriction sites in the recombinant genomes corresponding to those of the type I or type II parent.

(i) Genome analysis of recombinant R10/3/1

The genome structure of R10/3/1 was deduced from the analyses of HpaI and BamHI restriction endonuclease profiles.

In the HpaI digest the 1M HSV-1 b and h bands are absent (Figure 34A) and a novel high molecular weight molar fragment approximately $18x10^6$ mw) is migrating at the top of the gel (■). The absence of HSV-1 b and h and not t and f indicates that the HpaI t/b and h/f sites of type I are present while the b/h site is deleted. The data suggest the replacement of b and h of type I with a portion of HpaI d of HG52 extending from the h/d site of type II to h/f site of HSV-1. The size of the fragment at the top of the gel would be consistent with this hypothesis. However, the straight removal of the type I b/h site without insertion of type II sequences would also give a band of similar size.

A BamHI digest (Figure 34B) of the R10/3/1 DNA showed that the HSV-1 bands g, b', v, r, w and d were missing (▷). Molar bands corresponding to b, h', j', o, y and j of type II are present (◀). The presence of type II j' and o from the area of the genome normally

FIGURE 34

Autoradiographs of HpaI (A) and BamHI (B) restriction digests of viral DNA [^{32}P]-labelled *in vivo*. Recombinant R10/3/1 (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 DNA fragments, the physical map locations of which are shown in Figure 28. ▷ represents missing fragments; ■ represents novel fragments compared to the McKrae genome; ◀ represents fragments derived from the HG52 DNA.

A
McK
R10/3/1
HG52
Hpa I
B
McK
R10/3/1
HG52
Bam HI

occupied by k' and j' of HSV-1 suggests the absence of the latter fragments from the recombinant genome.

The presence of type II j and not m indicates the presence of the HSV-2 BamHI j/m site. Absence of type I d and presence of h indicates the retention of the type-I d/h site. The data indicates that the right hand end of the HG52 insert extends to the 0.51 m.u. BamHI j/m site. The small fragment of approximately 0.7×10^6 mw from the right hand end of the HSV-1 BamHI d fragment extending between 0.51 m.u. j/m site of type II and 0.536 m.u. d/h site of type-I is now migrating at the position of HSV-1 BamHI b' (■). The latter being absent from the recombinant genome due to the presence of the HSV-2 o and y fragments.

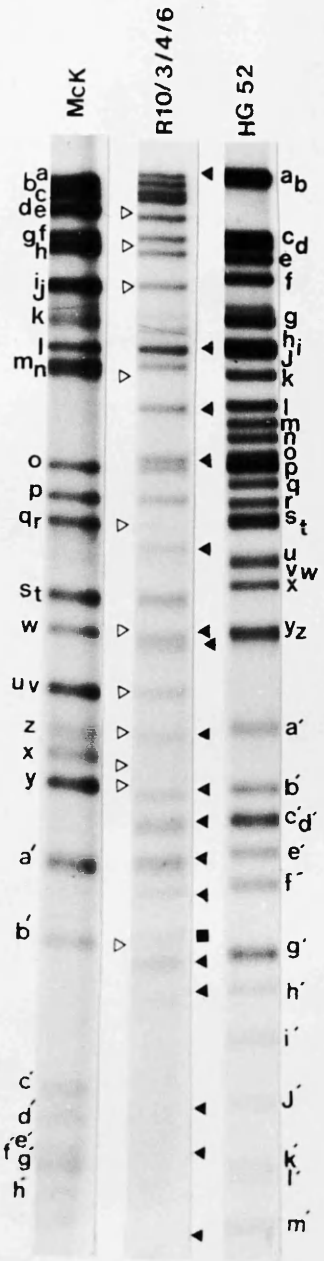
On the left hand side absence of HSV-1 g and not e' and presence of HSV-2 b and hence the l/b site suggests that the crossover must have taken place between the HSV-1 e'/g site and the HSV-2 l'/b site. Taken together the restriction endonuclease digests of R10/3/1 indicate the presence of an insert of HG52 sequences into the McKrae genome between 0.35 and 0.51 m.u. Recombination, therefore, must have taken place between the intact McKrae genome and HSV-2 sequences originating from the Hpal d fragment.

(ii) Genome analysis of recombinant R10/3/4/6

When the BamHI profile of R10/3/4/6 was compared with that of McKrae and HG52, inserts of HG52 sequences were present both in the long unique (U_L) and short region [S] of the genome (Figure 35). In the U_L, HSV-1 bands g, v, r, w, b' and d are absent (▷). Molar bands corresponding to HG52 BamHI b, h', o and y are present (◀). The HG52 j band is comigrating with l of type I. The presence of a novel 0.7×10^6 mw fragment (■) migrating at the position of HSV-1 b' delimits the right hand boundary of the HSV-2 insert to 0.51 m.u. as in R10/3/1. The presence of the HSV-2 b and hence l'/b site suggests a

FIGURE 35

Autoradiographs of BamHI restriction digests of viral DNA [^{32}P]-labelled in vivo. Recombinant R10/3/4/6 DNA (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 DNA fragments, the physical map locations of which are shown in Figure 28. ▷ represents missing fragments; ■ represents novel fragments as compared to the McKrae genome. ◀ represents fragments originating from the HG52 genome.


McK
R10/3/4/6
HG 52
Bam HI

cross over between the e'/g of type I and l'/b site of type II as observed in R10/3/1.

In the short region of the genome HSV-1 BamHI bands q, y, n, j, z and x have been replaced with u, g', z, e', f', l, c', d', b', k', m', and a' of HG52 (◀). These bands constitute the whole of the short regions of the genomes. The joint fragment k in the recombinant genome is slightly smaller than its counterpart in McKrae because of substitution of type I q with type II u. The absence of type II v and the presence of type I s and type II u bands in the recombinant indicates the cross over at the [illegible] region of the genome.

Restriction endonuclease analysis of R10/3/4/6 demonstrates that the genome consists of HSV-1 sequences between 0.0 to 0.35 m.u. and 0.51 to 0.82 m.u. and HSV-2 (HG52) sequences from 0.35 to 0.51 m.u. and 0.82 to 1.0 m.u. Recombination must have taken place between the intact McKrae genome and part of HpaI d plus the intact [S] region of HG52.

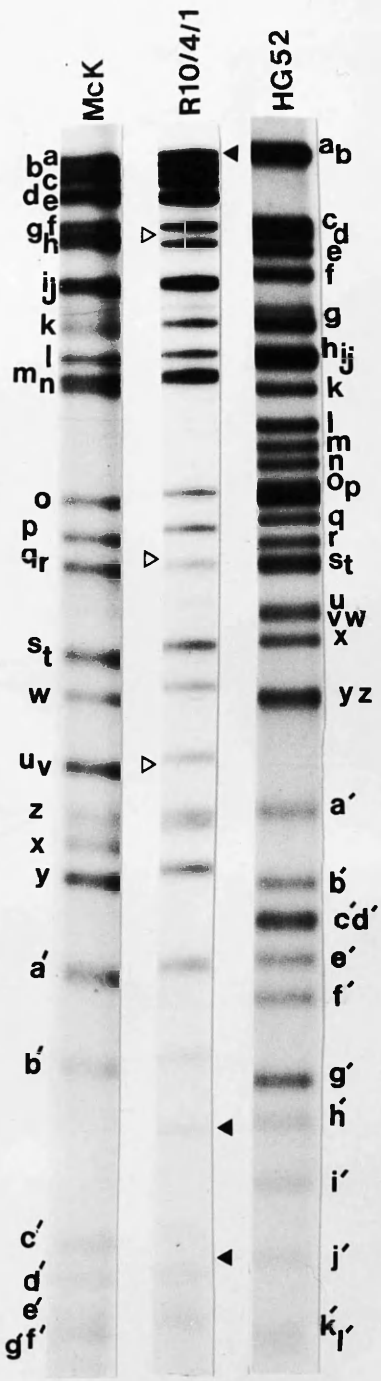
(iii) Genome analysis of recombinant R10/4/1

BamHI analysis of R10/4/1 (Figure 36) demonstrated that HSV-1 bands g, v and r are absent (▷) while the HSV-2 fragments b, h' and j' are present (◀). This indicates loss of the HSV-1 g/v, v/r and r/k' sites and the presence of HSV-2 b/h' and h'/j' sites. The presence of e' and, therefore, the e'/g site of type I and of b and, therefore, the l'/b site of type II indicates that the cross over must have taken place in the region of the genome delimited by these two sites. On the right hand side the presence of HSV-1 w and therefore, the k'/w site delimits the HG52 sequences up to 0.43 m.u.

Recombinant R10/4/1 thus contains an insert of HG52 DNA sequences between m.u. 0.35 and 0.43. Recombination must have taken place between the intact McKrae genome and part of the HpaI d fragment (0.35

FIGURE 36

Autoradiographs of BamHI restriction digests of viral DNA [^{32}P]-labelled *in vivo*. Recombinant R10/4/1 DNA (central lane); McKrae DNA (left lane); and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 DNA fragments the physical map locations of which are shown in Figure 28. ▷ represents missing fragments; represents novel fragments as compared to the McKrae genome. ◀ represents fragments originated from the HG52 genome.


McK
R10/4/1
HG52
Bam HI

- 0.576 m.u.) of HG52.

(iv) Genome analysis of recombinant R20/4/1

Analysis of a HpaI digest of recombinant R20/4/1 (Figure 37A) indicates that the HSV-1 b, f and h fragments are absent (▷) and a novel high molecular weight (approximately $26x10^6$ mw) fragment is present at the top of the gel (■). The absence of the HSV-1 b, h and f fragments and hence the HpaI b/h and h/f sites indicates the replacement of the McKrae DNA sequences with that of HG52 in this region of the genome. Absence of the HpaI e fragment of HG52 limits the HG52 insert to the HpaI d/e site (0.576 m.u.). Since the size of the novel fragment (■) (approximately $26x10^6$ mw) is larger than that of HG52 d (approximately $22x10^6$ mw) and the HSV-1 f fragment is also absent, the data are consistent with the fusion between the HpaI d fragment of HG52 and part of the HSV-1 f fragment extending between the d/e site of type II and the h/f site of type I. The cross over has therefore, occurred between the h/f site (0.52 m.u.) of McKrae and the d/e site (0.576 m.u.) of HG52. On the left hand side absence of the HpaI h fragment of HG52 delimits the insert up to the HSV-2 h/d site (0.35 m.u.). The cross over must have occurred between the HSV-1 t/b site and the HSV-2 h/d site.

Fine mapping of the HG52 insert was obtained from a BamHI digest of R20/4/1 DNA (Figure 37B). HSV-1 M bands d, g, r, w, v and b' are absent (▷) and have been replaced by HSV-2 bands b, j, o, y, h' and j' (◀). The HSV-1 k' and j' bands cannot be seen on this gel but their absence from the recombinant genome is deduced by the presence of the HSV-2 j' and o fragments spanning the corresponding region of the genome. The presence of the HSV-2 b fragment and hence the l'/b site (0.35 m.u.) delimits the left hand boundary of the HG52 DNA insert. The presence of HSV-1 e' and absence of g indicates that the

FIGURE 37

Autoradiographs of HpaI (A) and BamHI (B) restriction digests of viral DNA [^{32}P]-labelled *in vivo*. Recombinant R20/4/1 DNA (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 DNA fragments, the physical map locations of which are shown in Figure 28. ▷ represents missing fragments; ■ represents novel fragments compared to the McKrae genome; ◀ represents fragments derived from the HG52 genome.

A
McK
R10/3/1
HG52
Hpa I
B
McK
R10/3/1
HG52
Bam HI

cross over has occurred between the e'/g site of type I and the l'/b site of type II. On the right hand side the presence of HSV-2 j and not m indicates that the HSV-2 j/m site (0.51 m.u.) has been retained. The HSV-1 h fragment is larger in size (■) and is now migrating between the e and f fragments. This increase in the size of h would be consistent with the absence of the d/h site of HSV-1 generating a larger h fragment running from the j/m site of type II to the h/g' site of type I. The crossover has occurred between the d/h site of type I and m/e site of type II.

The absence of the HpaI h/f site (0.53 m.u.) of type I (Figure 37A) together with the absence of the HSV-2 BamHI m fragment and hence the m/e site (0.54 m.u.) in R20/4/1 DNA, indicates that the crossover has taken place between 0.53 and 0.54 m.u. Recombination, therefore, must have taken place between the intact McKrae genome and part (0.35 m.u. to 0.53 m.u.) of the HpaI d fragment of HG52.

(v) Genome analysis of recombinant R30/3

In a HpaI digest of R30/3 DNA (Figure 38A) the HSV-1 molar bands a, c, d and the 0.5M g band are absent (▷). A novel high mw band (■) migrating at the top of the gel can be seen. The digest indicates that the HSV-1 c and g fragments have been replaced with HSV-2 [S]. The novel joint fragment consisting of HSV-1 m and HSV-2 [S] is now running at the top of the gel (■). The extra band (●) seen running above the m fragment is due to additional a sequences on one of the m fragments. The same explanation holds true for the high mw fragment (●) seen migrating slightly above the novel joint fragment (■). Absence of HSV-2 f and presence of HSV-1 m indicates that the crossover has taken place at the joint region of the genome.

Analysis of the BamHI digest (Figure 38B) indicates that the HSV-1 g, j, n, q, r, v, z, y and x fragments are absent (▷) and have

FIGURE 38

Autoradiographs of HpaI (A) and BamHI (B) restriction digests of viral DNA [^{32}P]-labelled *in vivo*. Recombinant R30/3 DNA (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 DNA fragments, the physical map locations of which are shown in Figure 28. ▷ represents missing fragments; ■ represents novel fragments compared to the McKrae genome; ◀ represents fragments derived from the HG52 genome; ● represents additional 'a' sequences on the end and joint fragments.

A
McK
R 30/3
HG 52
Hpa I
B
McK
R 30/3
HG 52
Bam HI

been replaced with the HSV-2 b, h', u, g', e', f', l, y, z, b', c', d', a', k' and m' fragments (◀). This confirms the presence of HG52 DNA sequences in the U_L and short regions of the genome. In U_L, the left hand boundary of the HG52 DNA insert is mapped at approximately 0.35 m.u. by the presence of HSV-2 BamHI b and the absence of HSV-1 g. On the right hand side the presence of HSV-2 j and not o delimits the HG52 insert at 0.43 m.u. In the short region of the genome the HSV-1 BamHI g, y, n, j, z and x fragments are absent (▷) and have been replaced by The HSV-2 u, g', m', z, e', f', l, c', d', k' and a', b' fragments (◀). the HSV-1 fragments m', n' and l' have run off the gel but the presence of g' and d of HSV-2 indicates their absence. The data are consistent with the replacement of the short region of HSV-1 with that of HSV-2.

R30/3, therefore, contains inserts of HG52 DNA between 0.35 and 0.43 m.u. in U_L and between 0.82 and 1.00 m.u. in the short region. Recombination, therefore, has taken place between the intact McKrae genome and an intact HpaI [S] plus part of HpaI d of HG52.

(vi) Genome analysis of recombinant R40/2/2

Precise analysis of the genome structure of R40/2/2 was obtained from the Kpnl (Figure 39A), HpaI (Figure 39B), and BamHI (Figure 40) digest patterns.

The Kpnl digest (Figure 39A) indicates that the HSV-1 cx, l, p, v and c' fragments are absent (▷) and have been replaced with HSV-2 g and m and a novel fragment (■) running just above HSV-1 f. As HSV-2 t and HSV-1 a' comigrate, they are not easily distinguishable. However, the presence of HSV-2 m and g at each side of t demonstrates that the fragment is type II t and not type I a'.

The presence of HSV-2 m and not j and the simultaneous absence of p and not n of type I delimits the HG52 insert to the j/m site of type

FIGURE 39

Autoradiographs of KpnI (A) and HpaI (B) restriction digests of viral DNA [^{32}P]-labelled *in vivo*. Recombinant R40/2/2 DNA (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 DNA fragments, the physical map locations of which are given in Figure 28. ▷ represents missing McKrae fragments; ■ represents novel fragments as compared to the McKrae genome; ◀ represents fragments derived from HG52 DNA.


A
McK
R401212
HG52
dz
cx
a
e f
hb
ji
g
k
l
m
no
p
q
rs
t
b' g'
u
v
w
y
a'
b'
c'
a b
c d
e
f
g h
i
j
k
m l
n
o
pq
rs
t
Kpn 1
B
McK
R401212
HG52
a
d c
e
f
g
i
j
l
m
n
o p
q
r s
t
u
v
a b
c d
e
f g
h
Hpa 1

II. As HSV-1 n and o comigrate, they are not readily distinguishable. On the right hand side of the HG52 insert, the absence of c' and not s (it is not obvious from the gel that s is present because it comigrates with r) of HSV-1 delimits the boundary of the insert to the c' s site of type I, thus generating a novel fragment of approximately $8x10^6$ mw running from the g/d site of type II to the c'/s site of type I. The size of the novel fragment seen on the gel (■) is consistent with this analysis.

In the HpaI digest (Figure 39B) the HSV-1 fragments b, f and g are missing (▷) and have been replaced by fragment d (◀) of HSV-2. The presence of the total HSV-2 d fragment indicates the retention of the 0.35 m.u. h/d and 0.576 m.u. d/e sites of HG52 and thus the limits of the HG52 insert.

Confirmation of the crossover positions was obtained from the BamHI digest of R40/2/2 DNA (Figure 40). The HSV-1 d, g, h, r, w, v and b' fragments (▷) have been replaced with HSV-2 b, j, m, o, y (◀) plus a novel fragment e' (■). The presence of e' ($2.8x10^6$ mw) migrating just below HSV-2 BamHI m confirms a crossover within the HSV-2 e fragment between the m/e site of type II and g', o site of type I. The presence of the b fragment of type II and hence the l', b site and the absence of g and not e of type I indicates that the crossover has taken place between the e', g site of type I and the l', b site of type II.

R40/2/2 contains HG52 DNA between 0.35 and 0.576 m.u. and has been generated by recombination of an intact HSV-2 HpaI d fragment with an inact McKrae genome.

(vii) Genome analysis of recombinant R40/2/4

A Bam HI digest of recombinant R40/2/4 DNA (Figure 41) revealed absence of the HSV-1 M bands g, v, r, w, b' and d (▷). HSV-2 fragments

FIGURE 40

Autoradiographs of BamHI restriction digests of viral DNA [^{32}P]-labelled in vivo. Recombinant R40/2/2 DNA (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 DNA fragments, the physical map locations of which are given in Figure 28. ▷ represents missing McKrae fragments; ■ novel fragments present as compared to the McKrae genome; ◀ represents fragments originating from the HG52 genome.

McK
R40/2/2
HG52
Bam HI

FIGURE 41

Autoradiographs of BamHI restriction digests of viral DNA [^{32}P]-labelled *in vivo* R40/2/4 (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 fragments, the physical map locations of which are given in Figure 28. ▷ refers to missing fragments; ■ refers to novel fragments as compared to the McKrae genome and ◀ refers to fragments derived from HG52 DNA.

McK
R40/2/4
HG52
Bam HI

FIGURE 42

Autoradiographs of BamHI restriction digests of viral DNA [^{32}P]-labelled *in vivo*. R43/2/2 (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 fragments the physical map locations of which are given in Figure 28. ▷ represents missing fragments compared to the McKrae genome; ◀ represents fragments derived from HG52 DNA.

McK
R43/2/2
HG52
Bam HI

present from the corresponding region of the genome include b, h, j, o and y (◀). The absence of g and presence of e of type I in conjunction with the presence of b and absence of e of type II indicates that the left hand boundary of the HG52 insert is formed by crossing over between the e'/g site of type I and the l'/b site of type II. As type II j comigrates with h and i, its presence can not be determined from the gel. However, the absence of d of type I and j of type II in conjunction with the presence of HSV-2 y indicates retention of the HSV-2 Bam HI y/j site. A cross over between this site of HSV-2 and within fragment d of HSV-1, would generate a smaller fragment d' (■) of approximately $4x10^6$ m w. This fragment appears to be comigrating with HSV-1 i and j. The structure of R40/2/4 is therefore McKrae except for an insert of HG52 sequences between 0.35 and 0.475 m.u. and has been generated by recombination of the intact McKrae genome with part of the HSV-2 Hpa I d fragment.

(viii) Genome analysis of recombinant R43/2/2

A Bam HI digest of R43/2/2 is shown in Figure 42. HSV-1 bands q, y, n, j, z and x (▷) have been replaced by the HSV-2 fragments u, g' z, m', e', f', l, c', d', b', k' and a' (◀). The data indicates that the whole of the short region of HSV-1 has been replaced by the corresponding region of type II. The presence of u and not v of HSV-2 indicates that the crossover has taken place around the joint region of the genome. Slight reduction in the size of the HSV-1 joint fragment k(s+v) indicates the replacement of HSV-1 q by HSV-2 u. The genome of R43/2/2 is therefore, type I in the long region and entirely typeII in the short region. R43/2/2 has been generated by recombination between an intact McKrae genome and a HG52 HpaI [S] fragment.

FIGURE 43

Autoradiographs of BamHI restriction digests of viral DNA [^{32}P]-labelled *in vivo*. R5/26/1 (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 fragments, the physical map locations of which are given in Figure 28. ▷ represents missing fragments compared to the McKrae genome. ◀ represents fragments derived from HG52 DNA.

McK
R5i26i1
HG 52
Bam HI

(ix) Genome analysis of recombinant R5/26/1

A Bam HI profile of R5/26/1 is shown in Figure 43. The HSV-1 g/v and v/r cleavage sites are absent in this recombinant as demonstrated by the absence of the g, v and r fragments from the digest (▷). The presence of HSV-2 fragments b, h' and j' (◀) from the corresponding region of the genome indicates that the cross over must have taken place between the HSV-1 e'/g site and the HSV-2 l'/b site to form the left hand boundary of the HG52 insert. At the right hand end of the HG52 insert, the presence of HSV-1 w and hence the k'/w site and the presence of j'and hence the j'/o site of type II indicates that the cross over has taken place between these two sites, to give an insert of HG52 sequences between 0.35 and 0.43 m.u.

Sine R5/26/1 was isolated from a co-transfection of intact McKrae genomes and Xbal cleaved HG52 genomes, recombination must have taken place between an intact McKrae genome and the right hand sequences of HG52 Xbal c (0.0 to 0.45 m.u.).

(x) Genome analysis of recombinant R47/27/1

A BamHI digest of R47/27/1 is shown in Figure 44. The HSV-1 fragments g, v and r (▷) have been replaced with the HSV-2 b, h' and j' fragments (◀). The presence of HSV-2 b and absence of HSV-1 g indicates that the cross over on the left hand side must have taken place between the HSV-1 e'/g and HSV-2 l'/b sites. Though it is not possible to see HSV-1 k' on this gel, the presence of j' of HSV-2 from the corresponding region of the genome indicates the absence of the k' fragment. The presence of w of type I and absence of o of type II indicates that the cross over on the right hand side must have occurred between the j/o site of type II and the k'/w site of type I. The structure of R47/27/1 is therefore, of McKrae except for HG52 sequences between 0.35 and 0.43 m.u. The recombination leading to a

FIGURE 44

Autoradioragraphs of BamHI restriction digests of viral DNA [^{32}P]-labelled *in vivo*. R47/27/I (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 fragments, the physical map locations of which are given in Figure 28. ▷ represents missing fragments compared to the McKrae genome; ◀ represents fragments derived from HG52 DNA.

McK
R47/27/1
HG52
Bam HI

generation of R47/27/1 must have taken place between an intact McKrae genome and the right hand sequences of HG52 Xbal c (0.00 to 0.45 m.u.)

(xi) Genome analysis of recombinant R7/3/3

Comparison of a Bam HI digest of R7/3/3 with that of McKrae and HG52 is shown in Figure 45. The HSV-1 g, v, and r fragments are absent (▷) and the HSV-2 b and h' fragments are present (◀). The presence of HSV-2 h' and not j' indicates a crossover between the HSV-2 Bam HI h'/j' site and r/k' site of HSV-1. The small fragment (approximately 0.2×10^6 mw) thus generated is not distinguishable on the gel. The presence of b and hence the l'/b site of type II and of e' and hence the e'/g site of type I indicates that a cross over has taken place between these two sites.

Recombinant R7/3/3, therefore, contains HG52 sequences between 0.35 and 0.43 m.u. and like R47/27/1 has been generated by recombination between HG52 Xbal c and an intact McKrae genome.

The structures of the eleven McKrae x HG52 recombinants in relation to HSV-1 and HSV-2 BamHI restriction endonuclease sites are shown in Figure 46.

3.6. ANALYSIS OF RECOMBINANT VIRUS INDUCED INFECTED CELL POLYPEPTIDES

The SDS-PAGE technique has been extensively used in the study of HSV infected cell polypeptides. The majority of HSV-1 and HSV-2 ts mutants have been examined by this method and characteristic polypeptide profiles have been recognised for most of the ts mutants (Marsden et al., 1976). Analysis of polypeptides induced by intertypic recombinants of HSV and correlation of the data with the crossover points in the recombinant DNAs has been used to map the genetic loci specifying these polypeptides (Marsden et al., 1978; Ruyechan et al., 1979). Therefore the infected cell polypeptide profiles induced by the McKrae X HG52 recombinants with respect to both type I and type II

FIGURE 45

Autoradiographs of BamHI restriction digests of viral DNA [^{32}P]-labelled *in vivo*. R7/3/3 (central lane); McKrae DNA (left lane) and HG52 DNA (right lane). Letters refer to specific HSV-1 or HSV-2 fragments the physical map locations of which are shown in Figure 28.

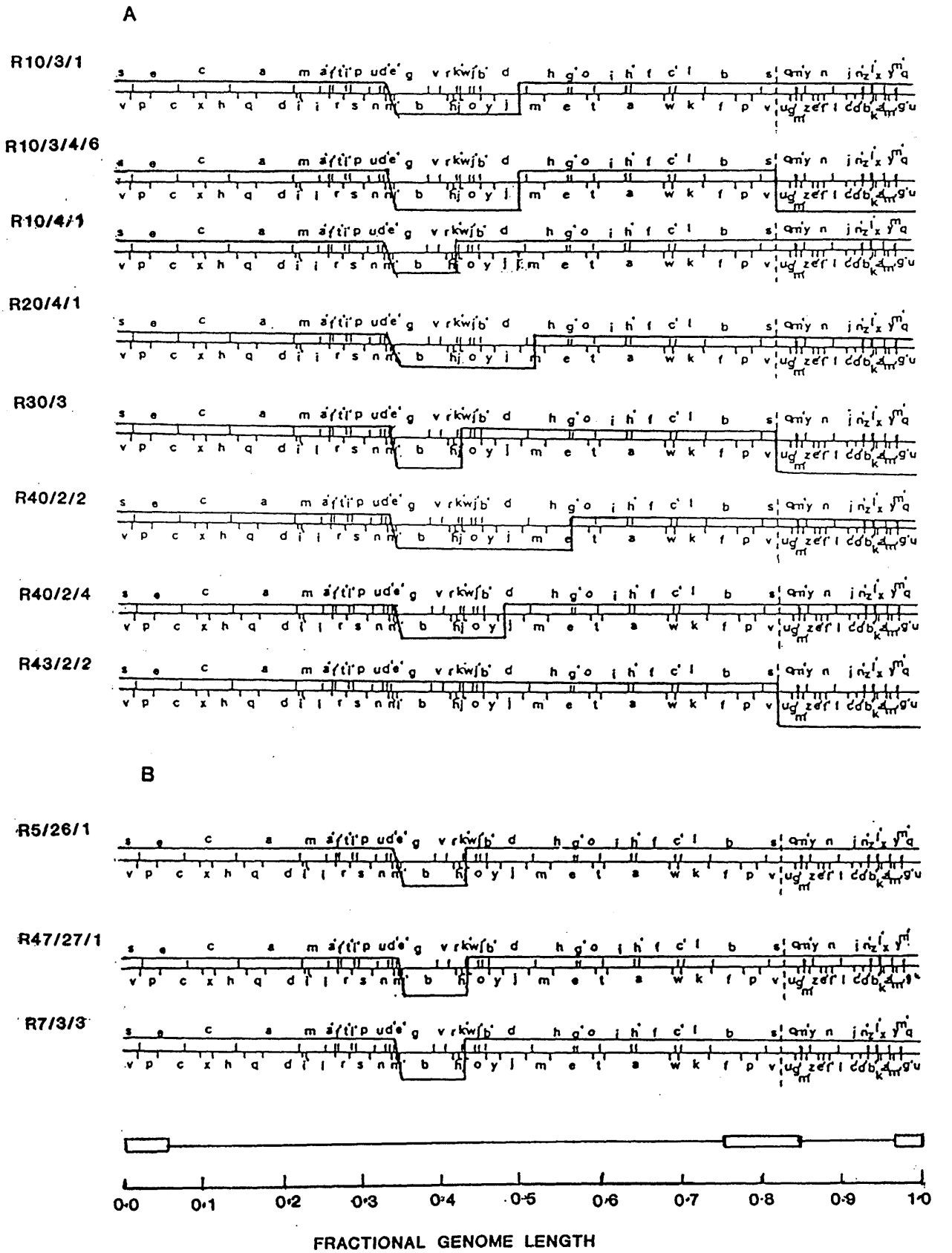
▷ represents missing fragments compared to the McKrae genome;

▶ reprsents fragments derived from HG52 DNA.

McK
R7/3/3
HG52
Bam HI

FIGURE 46

Diagrammatic representation of the crossover sites in BamHI digests of eleven McKrae x HG52 recombinant viruses. Locations of the BamHI sites in HSV-1 (above the line) and HSV-2 (below the line) are indicated by the small vertical lines. Solid horizontal lines represent the genome structure of the recombinant viruses. Letters refer to specific BamHI fragments, the physical map locations of which are given in Figure 28.


A
R10/3/1
R10/3/4/6
R10/4/1
R20/4/1
R30/3
R40/2/2
R40/2/4
R43/2/2
B
R5/26/1
R47/27/1
R7/3/3
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
FRACTIONAL GENOME LENGTH

parents, may provide information on the genetic loci encoding a particular polypeptide.

To analyse polypeptides induced by the intertypic recombinants, the recombinant virus and parental type I and type II virus infections were carried out simultaneously using a single batch of BHK 21/C13 cells and labelled with [^{35}S]-methionine as described in Materials and Methods (Section 2.7). Infected cell polypeptides induced by HSV-1 strain 17 were included in each experiment as controls. The polypeptides were resolved on 5 to 12% SDS-polyacrylamide gradient gell.

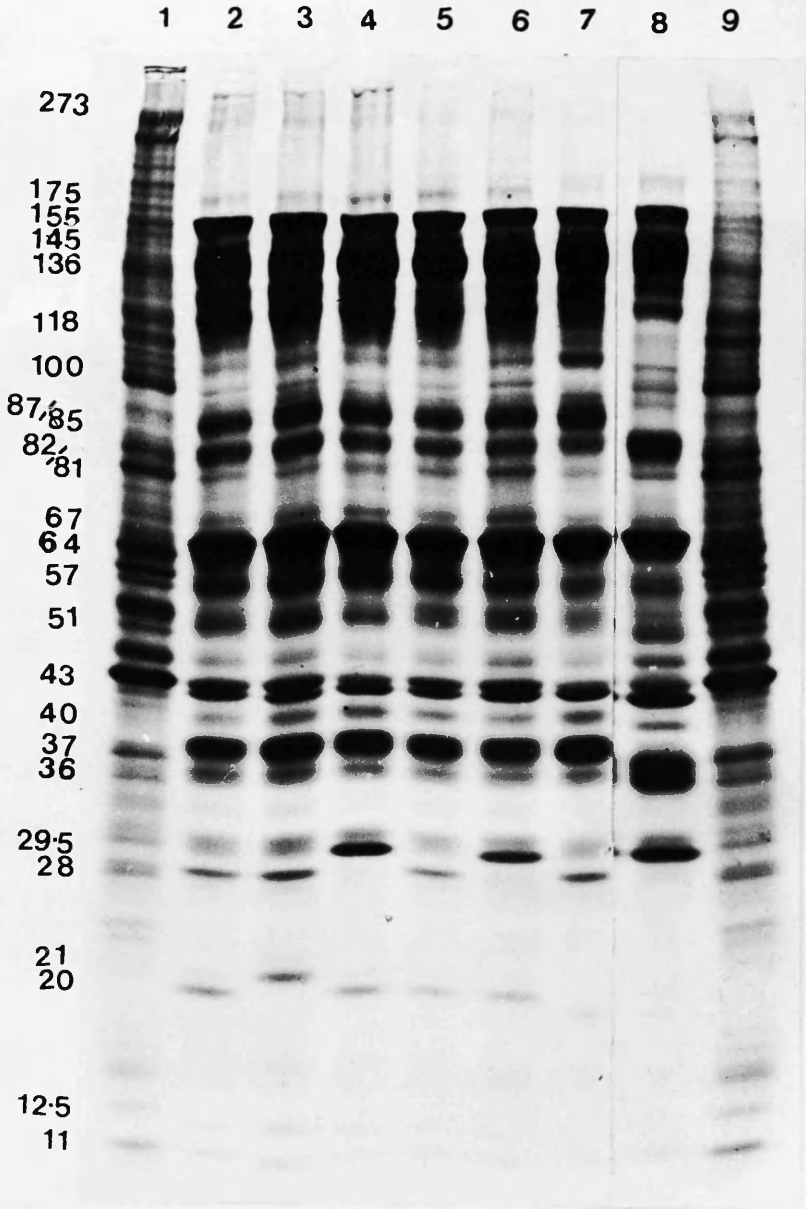
An autoradiograph of infected cell polypeptide profiles obtained following SDS-PAGE of BHK 21/C13 cells infected with HSV-1 strain McKrae, HSV-1 strain 17 syn^{+}, recombinants R10/3/1, R10/4/1, R20/4/1, R30/3 and HSV-2 strain HG52 is shown in Figure 47. The infected cell polypeptide profile obtained from a McKrae infection is in agreement with that of strain 17 (Marsden et al., 1976). The infected cell polypeptide profiles of R10/4/1 and R30/3 infections are indistinguishable from that of a HSV-1 McKrae infection. In R10/3/1 and R20/4/1 the infected cell polypeptide of HSV-1, Vmw 28K has been replaced with Vmw 29.5K of HG52. The HG52 DNA insert in R10/3/1 and R20/4/1 extends to 0.51 and 0.53 m.u. respectively. In R10/4/1 and R30/3, which induce Vmw 28K, the HG52 insert in U_L extends to 0.43 m.u. These data indicate that Vmw 29.5K of HSV-2 is encoded by the genes located between 0.43 and 0.51 m.u. Vmw 29.5K had already been mapped to a similar region of the genome (0.43 to 0.48 m.u.) by Marsden et al (1978).

The infected cell polypeptides specified by the other recombinants did not provide any new information on the location of particular polypeptides.

FIGURE 47

Autoradiographs of a 5 to 12.5% acrylamide gradient gel, showing the infected cell polypeptides induced by recombinants R10/3/1, R10/4/1, R20/4/1, R30/3, HSV-1 strain 17, HSV-1 strain McKrae, HSV-2 strain HG52 and in mock infected BHK-21/C13 cells at 37°C. The gel tracks are, from the left, as follows:

1. Mock infected
2. HSV-1 strain McKrae
3. HSV-1 strain 17
4. R10/3/1
5. R10/4/1
6. R20/4/1
7. R30/3
8. HSV-2 strain HG52
9. Mock infected


1 2 3 4 5 6 7 8 9
273
175
155
145
136
118
100
87/85
82/81
67
64
57
51
43
40
37
36
29·5
28
21
20
12·5
11

3.7. LATENCY REACTIVATION POTENTIAL OF THE McKRAE X HG52 RECOMBINANTS IN RABBITS

3.7.a. Recombinant viruses studied in the rabbit eye model

Four of the recombinant viruses were studied for their ability to go latent following infections of rabbit eyes and to reactivate both spontaneously and on induction with epinephrine iontophoresis. As all the recombinants were not isolated at the same time, individual recombinants were tested as they became available. The four recombinants R10/3/1, R10/3/4/6, R40/2/2 and R43/2/2 chosen for rabbit eye infections had genomes with the widest limits of the HG52 inserts. The genomes of the other recombinants all had HG52 inserts within the limits covered by the four chosen recombinants.

(i) HSV-1 McKrae

Rabbits were inoculated in the left eyes only with $5x10^5$ pfu of McKrae as positive controls for recombinant infections. The data presented in Table 5 are from two groups of rabbits consisting of 5 rabbits in one group and 3 in the second, which were infected at two separate times.

Similar to the results described in Tables 2 and 3 all the animals had a latent infection as demonstrated by release of virus from explanted LTG and in some cases from RTG. In addition, all animals shed virus post epinephrine iontophoresis.

However, in the second group of rabbits (6 to 8) no spontaneous shedding of virus was detected which may merely indicate that virus was not being shed during the period of screening (one week). It should also be noted that one out of six segments of the left corneal explants from 2 animals, released virus between 28 to 31 and 15 to 18 days post explantation respectively.

TABLE 5

Time (days) of release of infectious virus

Virus dose	Rabbit No.	Spontaneous shedding	Post ionto-phoresis	Left T.G.	No. of segments positive	Right T.G.	No. of segments positive	Left cornea	No. of segments positive
McKrae 5×10^5 pfu	1	27-34	3-5	7-18	2	-	-	-	-
	2	27-34	2-6	6-17	2	-	-	-	-
	3	27-34	3-6	6-17	4	7-17	1	-	-
	4	27-34	3-6	5-17	3	-		-	-
	5	27-34	3-5	5-16	5	5-16	1	-	-
	6	-	1-6	12-20	3	14-20	1	-	-
	7	-	1-6	8-20	3	15-20	1	28-31	1
	8	-	1-6	12-19	3	-	-	15-18	1

(ii) Recombinant R10/3/1

The left eye of each of 5 rabbits was infected with $2x10^6$ pfu of recombinant R10/3/1 containing a HG52 insert between 0.35 and 0.51 m.u. All the rabbits developed keratitis leading to corneal ulcers within 7 days post infection and could tolerate the infecting dose of virus without showing any signs of distress or encephalitis. Results of virus shedding following epinephrine iontophoresis induction and from the TGs upon explantation are shown in Table 6.

No spontaneous shedding of virus was detected on daily screening of the tear films from the infected eyes between 30 and 35 days post infection. Epinephrine iontophoresis performed when the eyes were negative for spontaneous shedding, induced reactivation of latent virus which was detected in the tear films of the left eyes of each rabbit. Virus was first detected on day 2 or 3 post iontophoresis and continued to be shed for 1 to 3 days. Sixty-one days post infection the left trigeminal ganglia were explanted, subdivided into 6 segments and cultured *in vitro*. Some of the ganglion segments from each rabbit released infectious virus between days 21 and 33 post explantation. No infectious virus was obtained from the RTG and corneal explants up to 5 weeks of *in vitro* culture and screening of the explants. BamHI digests of the viral DNA of the isolates obtained after epinephrine induction and from the explanted trigeminal ganglia showed profiles identical to the infecting virus.

Since infectious virus was recovered from the left eyes of all five rabbits after induction with epinephrine iontophoresis, it appears that recombinant R10/3/1 has the HFRc phenotype of its McKrae parent and not the LFRc phenotype of the HG52 parent. The genetic information between 0.35 to 0.51 m.u. derived from the LFRc parent, therefore does not determine the reactivation differential between McKrae and HG52.

TABLE 6

Time (days) of release of infectious virus

Virus dose	Rabbit No.	Spontaneous shedding	Post ionto-phoresis	Left T.G.	No. of segments positive	Right T.G.	No. of segments positive	Left cornea	No. of segments positive
R10/3/1	1	-	2-5	26-31	2	-	-	-	-
2×10^6 pfu	2	-	3-5	25-32	2	-	-	-	-
HG 52 insert	3	-	3-4	22-27	3	-	-	-	-
0.35-0.51 m.u.	4	-	2-5	22-27	2	-	-	-	-
	5	-	2-5	21-33	3	-	-	-	-

(iii) Recombinant R10/3/4/6

As indicated in Table 7, in this recombinant the genetic information between 0.35 to 0.51 and 0.82 to 1.00 m.u. of R10/3/4/6 is derived from HG52. Each of the 6 rabbits inoculated in the left eyes with $1.5x10^6$ pfu of R10/3/4/6 tolerated the infecting dose without any signs of distress and exhibited characteristic symptoms of herpes virus keratitis within 7 days post infection. Screening of tearfilms from the infected eyes daily between 30 to 35 days post infection showed no spontaneous virus shedding. Reactivated virus could be detected in the tear films taken from the infected eyes as early as one day after epinephrine iontophoresis performed when the rabbits were not shedding any spontaneous virus in their tear films. The reactivated virus could be detected in the tear film till 7 days post iontophoresis.

Explanted left trigeminal ganglia released infectious virus between day 16 and day 29 post explantation. Infectious virus was also recovered from the right trigeminal ganglion explants from one of the animals between 28 and 30 days post explantation. The number of ganglionic segments releasing virus varied between 1 and 5. The BamHI DNA profiles of reactivated virus isolates and isolates from the explanted ganglia were identical to that of input virus.

Since R10/3/4/6 was capable of being reactivated with 100% frequency, it was designated to have a HFRc phenotype. The sequences between 0.82 and 1.00 m.u. i.e. the whole of the short region, either alone or in conjunction with the sequences between 0.35 and 0.51 m.u. therefore do not determine reactivation differential between McKrae and HG52.

(iv) Recombinant R40/2/2

The left eyes of each of 4 rabbits were inoculated with $2x10^7$ pfu

TABLE 7

Time (days) of release of infectious virus

Virus dose	Rabbit No.	Spontaneous shedding	Post iontophoresis	Left T.G.	No. of segments positive	Right T.G.	No. of segments positive	Left cornea	No. of segments positive
R10/3/4/6	1	-	3-4	16-29	3	-	-	-	-
1.5 x 10^6 pfu	2	-	1-4	16-25	4	28-30	1	-	-
HG 52	3	-	2-6	23-27	3	-	-	-	-
0.35-0.51 and 0.82-1.00 m.u.	4	-	2-5	23-27	1	-		-	-
	5	-	2-7	22-27	2	-	-	-	-
	6	-	3-4	20-27	5	-	-	-	-

of the recombinant virus R40/2/2. None of the rabbits, following infection with virus, showed any signs of distress, but exhibited development of characteristic herpetic ulcers of their left corneas. The results of virus infection in rabbtis are shown in Table 8. No spontaneously shed virus was detected in the tear films on screening for 7 days starting on day 28 post infection. Epinephrine induced reactivated virus from the rabbit eyes negative for any spontaneous shedding, was detected in all the animals between 1 and 4 days post iontophoresis. On in vitro explantation of the left trigeminal ganglia 9 weeks post infection, virus release was first detected between 16 and 26 days post explantation. In one of the animals infectious virus could be detected up to 33 days post explantation. The number of ganglion segments releasing virus varied between 1 and 5. No infectious virus was recovered from any of the right trigeminal ganglion or corneal segments during a 7 week period of in vitro culture.

One of the six segments of the left cornea from one rabbit yielded infectious virus between 35 and 49 days post explantation. The BamHI restriction profiles of the DNA of the reactivated virus from ganglia and that of corneal isolates were identical to that of the infecting virus.

Recovery of virus from the left eyes of all the rabbits upon epinephrine induction indicates a HFRc phenotype for R40/2/2. As this recombinant has an HG52 insert between 0.35 and 0.576 m.u., it appears that the sequences between 0.51 and 0.576 too do not determine the reactivation differential between McKrae and HG52.

(vi) Recombinant R43/2/2

Each of the three rabbits inoculated with $3x10^6$ pfu of the recombinant virus R43/2/2 into their left eyes could tolerate the

TABLE 8

Time (days) of release of infectious virus

Virus dose	Rabbit No.	Spontaneous shedding	Post iontophoresis	Left T.G.	No. of segments positive	Right T.G.	No. of segments positive	Left cornea	No. of segments positive
R40/2/2	1	-	1-4	26-33	3	-	-	-	-
2×10^7 pfu	2	-	1-4	16-26	1	-	-	-	-
HG 52	3	-	1-4	16-26	5	-	-	-	-
0.35-0.576	4	-	1-4	26-31	5	-		35-49	1

infecting dose without exhibiting any distress including encephalitis. Development of keratitis and corneal ulcers within 7 to 10 days post infection as revealed by visual examination of infected eye indicated replication of virus in the corneas. The results of the infections, as shown in Table 9, indicate that monitoring of tear films from the infected eyes between 28 and 35 days post infection showed no signs of any spontaneous virus shedding. Following induction with epinephrine, reactivated virus could be detected as early as 24 hr post iontophoresis and continued for a further 48 hr in two rabbits and between 3 and 5 days in the third rabbit. Evidence of latent infection of the LTG was supplied by the release of infectious virus from all the ganglia between 15 and 25 days in culture. One segment out of 6 of the LTG from rabbit number 2 while 3 and 2 segments out of 6 in rabbits number 1 and 3 respectively were positive for virus release. The ganglia were explanted into culture 10 weeks after the primary infection. None of the RTG or right corneal explants released virus during the period of in vitro culture. However, one of the 6 segments of the left cornea of one rabbit released virus between 35 and 49 days post explantation. The genomic restriction endonuclease profiles of the various isolates from eyes or ganglia remained unchanged from the infecting virus.

The recombinant R43/2/2 is, therefore, designated as HFRc irrespective of the presence of genetic information between 0.82 and 1.00 m.u. from the LFRc parent, thus indicating that the virus genes encoded therein do not control reactivation differential between McKrae and HG52.

3.8. VIRULENCE OF THE McKRAE X HG52 RECOMBINANT VIRUSES FOR RABBITS

Analysis of the data presented in Tables 2 to 9 indicates that the rabbits inoculated in the left eyes only with $5x10^5$ pfu of HSV-1

TABLE 9

Time (days) of release of infectious virus

Virus dose	Rabbit No.	Spontaneous shedding	Post ionto-phoresis	Left T.G.	No. of segments positive	Right T.G.	No. of segments positive	Left cornea	No. of segments positive
R43/2/2	1	-	1-3	15-25	3	-	-	35-49	1
3×10^6 pfu	2	-	1-3	22-25	1	-	-	-	-
HG 52	3	-	3-5	19-25	2	-	-	-	-
0.82-1.00									

strain McKrae could survive while those inoculated with $5x10^6$ and $5x10^7$ pfu died within a few days of infection or had to put down because of severe distress. On the other hand rabbits could tolerate up to $5x10^7$ pfu of HSV-2 strain HG52 without any noticeable adverse effects (Tables 2 and 3).

Much higher doses of the infecting recombinant viruses compared to the McKrae parent were tolerated by the rabbits. Up to 3, 4, 6 and 40 fold excess doses of the recombinants R10/3/4/6, R10/3/1, R43/2/2 and R40/2/2 respectively were inoculated without any signs of distress. These preliminary results from a relatively small number of samples may suggest moderation in the virulence of HSV-1 strain McKrae on insertion of HSV-2 strain HG52 genetic information.

Further this reduction in virulence of the recombinant viruses was observed irrespective of the location of the HG52 insert i.e. either in the long unique region alone (R10/3/1 and R40/2/2) or in the short region of the genome (R43/2/2) or at both locations (R10/3/4/6). The reduction in virulence of the recombinant viruses compared to their McKrae parent and irrespective of location of the HG52 insert indicates that virulence is not confined to any single region of the genome and may be multigenic.

3.9. LIMITS OF THE HG52 DNA INSERTS IN THE McKRAE X HG52 RECOMBINANTS

Of the recombinants derived from cotransfections of intact McKrae genomes with the total products of HpaI cleaved HG52 genomes, all but one (R43/2/2) had an HG52 insert in U_L which terminated at approximately 0.35 m.u. This was determined as precisely as possible by BamHI digests of recombinant virus DNAs. The left hand limit of the HG52 inserts co-maps with the left hand boundary of the HG52 HpaI d fragment (0.35 to 0.576 m.u.).

The right hand boundary of the HG52 insert ended at 0.43 m.u. in

recombinants R10/4/1 and R30/3; at 0.47 m.u. in R40/2/4; at 0.51 m.u. in R10/3/1 and R10/3/4/6; at 0.52 m.u. in R20/4/1 and at 0.576 m.u. in R40/2/2. The largest HG52 insert in U_L was observed in the recombinant R40/2/2 (0.35 to 0.576 m.u.) whose right hand end comaps with the right hand boundary of the HSV-2 HpaI d fragment. This indicated that in R40/2/2 the whole of HSV-2 HpaI d has recombined into the McKrae genome.

In R10/3/4/6 and R30/3, in addition to the HG52 insert in U_L an insert of HG52 sequences was also present in the short region of the genome. The recombinant R43/2/2 had an insert of HG52 sequences only in the short region of the genome. In the three recombinants the whole of the short region (U_S + R_S) of the McKrae genome (0.82 to 1.00 m.u.) has been replaced by HG52 DNA. There are no HpaI sites in the short region of HG52, therefore R10/3/4/6, R30/3 and R43/2/2 must have been generated by recombination between intact McKrae genomes and HG52 HpaI [S] fragments.

Recombinants R5/26/1, R47/27/1 and R7/3/3 were obtained from independent cotransfections of intact McKrae DNA and the total products of XbaI cleaved HG52 DNA. They each contained HG52 DNA inserts in U_L between 0.35 and 0.43 m.u. and must have been generated by recombination of intact McKrae genomes and sequences derived from the right hand end of HG52 XbaI c (0.0 to 0.45 m.u.).

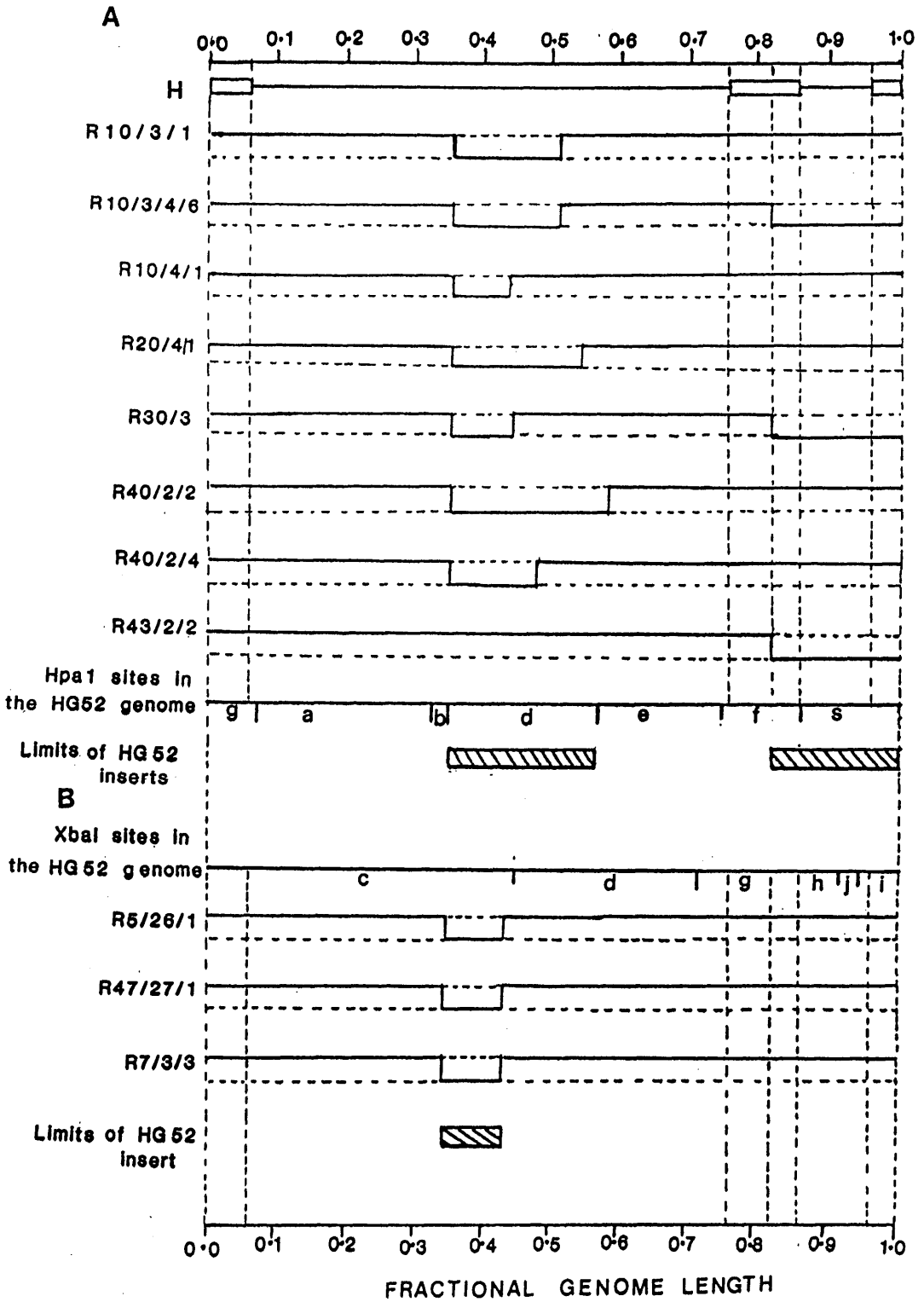
The limits of the HG52 inserts within the recombinants are shown diagrammatically in Figure 48.

3.10. LIMITS OF RECOMBINATION WITH RESPECT TO ORIGINS OF VIRUS REPLICATION (ORI_L AND ORI_S)

In recombinants derived from cotransfection experiments using HpaI cleaved HG52 genomes, the HG52 inserts were derived from HpaI d or [S] or both. In recombinants derived from cotransfections using

FIGURE 48

Diagrammatic representation of the structure of recombinants obtained from cotransfections of intact McKrae genomes with HpaI cleaved (A) and XbaI cleaved (B) HG52 genomes. 'H' indicates the prototype arrangement of the HSV genome. The horizontal dotted line represents HSV-1 (upper) and HSV-2 (lower) DNA sequences. The solid horizontal line represents the structure of the recombinant viruses. Letters between HpaI and XbaI sites of the HG52 genome refer to specific DNA fragments. ▨ indicates to the limits of the HG52 inserts for each group of recombinants.


A
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
H
R10/3/1
R10/3/4/6
R10/4/1
R20/4/1
R30/3
R40/2/2
R40/2/4
R43/2/2
Hpa1 sites in the HG52 genome
g
a
b
d
e
f
s
Limits of HG52 inserts
B
Xba1 sites in the HG52 genome
c
d
g
h
j
i
R5/26/1
R47/27/1
R7/3/3
Limits of HG52 insert
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
FRACTIONAL GENOME LENGTH

XbaI cleaved HG52 DNA, the HG52 inserts were derived from the right hand end of XbaI c. The origins of replication in HSV-1 have been located between 0.398 and 0.413 m.u. (Ori_L) (Weller et al., 1985) and at 0.87 and 0.96 m.u. (Ori_S) (Stow, 1982; Stow and McMonagle, 1983). In HSV-2 Ori_L and Ori_S are located in equivalent positions (Lockshon and Galloway, 1986) and (Whitton and Clements, 1984a). The map coordinates of HpaI d are 0.35 to 0.576 m.u.; of HpaI [S], 0.82 to 1.00 m.u. and of XbaI c, 0.0-0.45 m.u. Each of these fragments contains an origin of replication (Ori_L or Ori_S).

As evident from the structure of the recombinants (Figure 48), R10/3/1, R10/4/1, R40/2/2, R40/2/4, R5/26/1, R47/27/1 and R7/3/3 all contained Ori_L of HG52 and Ori_S of McKrae. In recombinants R10/3/4/6 and R30/3 besides Ori_L both copies of Ori_S were also derived from HG52 while in recombinant R43/2/2 both copies of Ori_S were derived from HG52 and Ori_L from McKrae. The position of Ori_S and Ori_L in relation to the limits of the HG52 inserts in the recombinants is shown diagrammatically in Figure 49.

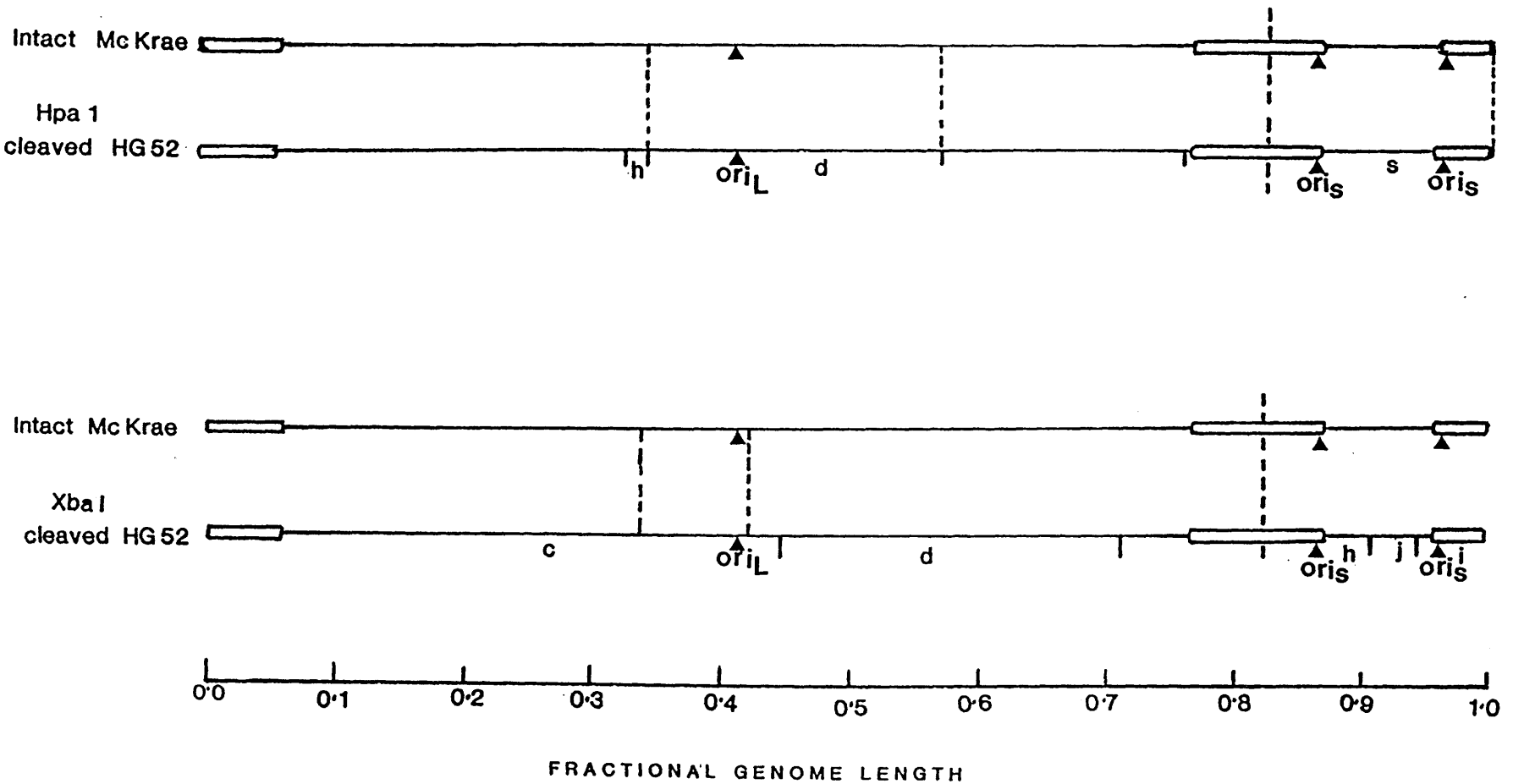
It is concluded that when McKrae X HG52 recombinants were constructed by cotransfection of intact McKrae genomes and restriction endonuclease cleaved HG52 genomes, the HG52 inserts were only derived from the restriction endonuclease fragments of HG52 containing an origin of replication. The isolation of recombinants containing both heterologous Ori_S and Ori_L indicates that both origins of replication are functional in vitro.

3.11. CONSTRUCTION OF HG52 X McRAE RECOMBINANTS

To determine whether our hypothesis that sequences from restriction endonuclease fragments containing an origin of replication were preferentially recombined, was correct, HG52 X McKrae recombinants were constructed in a reciprocal manner i.e. by using

FIGURE 49

Diagrammatic representation of the limits of the HG52 inserts in recombinants derived from cotransfections of intact McKrae genomes with either XbaI or HpaI cleaved HG52 DNA. The inserts are represented by the DNA sequences between the vertical dotted lines. The positions of HSV origins of replication Ori_L and Ori_S are represented (▲). Letters below the solid line represent HG52 XbaI and HpaI fragments.


Intact Mc Krae
Hpa 1
cleaved HG 52
h
ori L
d
ori S
s
ori S
Intact Mc Krae
Xba I
cleaved HG 52
c
ori L
d
ori S
h
j
ori S
i
0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
FRACTIONAL GENOME LENGTH

intact HG52 genomes and restriction endonuclease cleaved McKrae DNA.

Intact HG52 genomes (0.2 ug) were cotransfected onto BHK21/C13 monolayers with 2 ug per 50 mm dish of either the total products of XbaI or HpaI cleaved McKrae genomes. Progeny plaques were picked and viral DNA labelled *in vivo* with [^{32}P] orthophosphate and their restriction endonuclease profiles determined.

Of the DNA of 231 progeny plaques screened from co-transfection experiments using HpaI cleaved McKrae DNA, two recombinants. R17/1 and R368/38 were isolated, giving a recombination frequency of 0.86%. Analysis of the DNA of 174 progeny plaques from transfections using XbaI cleaved McKrae genomes did not show any recombinant genomes.

3.11.a. Clonal relationship and nomenclature of the recombinant viruses

Since both recombinants (R17/1 and R368/38) were isolated from independent transfections, they were clonally unrelated and were designated R17/1 and R368/38 denoting the plaque number picked from the transfection experiments

3.11.b. Analysis of genome structure of R17/1 and R368/38 with respect to loctions of Ori_L and Ori_S

(i) R17/1

A BamHI digest of R17/1 shown in Figure 50 is similar to that of HG52 except for the absence of BamHI g', z, e', f', l, c', d', b', m' and k' fragments (◁). These fragments constitute a major portion of the short region of HG52. The type I fragments identifiable are n, j, z and x (▶). The HSV-2 fragment n appears to be comigrating with HSV-2 BamHI m indicating an approximately 150 bp insert in the n fragment. The HSV-2 t fragment appears to be deleted by 150 bp and is now migrating slightly above HSV-2 u. However, a deletion in HSV-2 s would also result in a band of similar size. The identity of this band was

FIGURE 50

Autoradiographs of BamHI restriction digests of viral DNA [^{32}P]-labelled in vivo from recombinants R368/38. R17/1, McKrae (extreme left lane) and HG52 (extreme right lane). Letters refer to specific HSV-1 or HSV-2 DNA fragments, the physical map locations of which are shown in Figure 28. ◁ refers to missing HSV-2 fragments; ◀ represents HSV-1 fragments present; ■ represents novel fragments present in the recombinant genome.

McK
R368/38
R17/1
HG52
Bam HI

not investigated further by Southern blotting. The genome structure of R17/1 is type II in the U_L and R_L. It crosses over within BamHI g' in typeII to y in type I and back again from y in TRs of type I to g' in TRs of type II to generate a novel fragment of approximately $1.4x10^6$ mw from the type II u/g' site to the type I y/n site. This fragment is comigrating with a'of type I (■). Recombination must have taken place between the intact HG52 genome and HpaI c and HpaI g or HpaI (c + g) of McKrae. Both HpaI c and g of HSV-1 contain one copy each of Ori_S (Figure 51). The genome of R17/1, therefore, contains Ori_L of HG52 and both copies of Ori_S of McKrae. The structure of R17/1 in relation to Ori_L and Ori_S is shown diagrammatically in Figure 51.

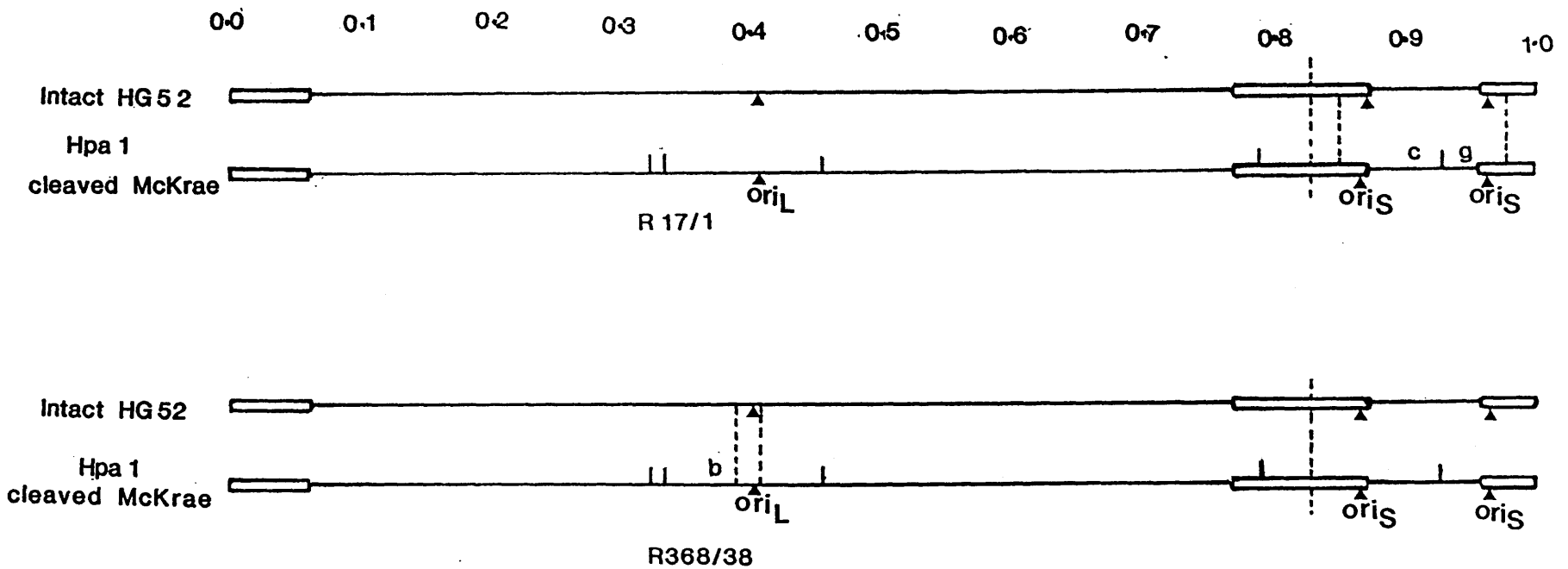
(ii) R368/38

A BamHI digest of recombinant R368/38 DNA is shown in Figure 50. The genome is essentially HG52 except for fragment b. This fragment is smaller in size (labelled as b' and is migrating slightly below a. The only type 1 band identifiable is BamHI v. The structure of the recombinant is interpreted as having an insert of BamHI v of the McKrae genome that is $1.5x10^6$ mw. The new b' thus generated runs from the l'/b site of HG52 to the g/v site of McKrae. The other small fragment generated by the insert of type I v runs from the type I v/r site to the type II b/h' site. The fragment thus generated is not apparent on the gel but its size is smaller than type I v and it may be comigrating with a'.

Weller et al (1985) located Ori_L within BamHI v (0.398 to 0.413 m.u.) of HSV-1. R368/38, therefore, contains Ori_L of McKrae and both copies of Ori_S of HG52. The structure of R368/38 is shown diagrammatically in Figure 51.

FIGURE 51

Diagrammatic representation of the limits of McKrae DNA inserts in recombinants R368/38 and R17/1 derived from cotransfections of intact HG52 genomes with HpaI cleaved McKrae DNA. The inserts are represented by DNA sequences between the vertical dotted lines. The positions of HSV origins of replication Ori_L and Ori_S are marked (▲). The short vertical lines above the line represent the relevant HpaI restriction sites on the HSV-1 genome. Letters refer to specific HSV-1 HpaI fragments.


0·0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Intact HG 5 2
Hpa 1
cleaved McKrae
c
g
oriL
oriS
oriS
R 17/1
Intact HG 52
Hpa 1
cleaved McKrae
b
oriL
oriS
oriS
R368/38

3.12. CLONING OF HSV-2 (HG52) DNA FRAGMENTS INTO A PLASMID CONTAINING AN HSV-1 ORIGIN OF REPLICATION (ORI_S)

Isolation of recombinants having intertypic inserts containing heterologous origins (S) of replication led to the conclusion that either (i) fragments containing an origin of replication are amplified in cotransfection experiments and thereby their chance of recombination with intact genomes is increased or (ii) replication and recombination in HSV are inter-linked.

To determine whether either of these possibilities was tenable the HSV-2 HindIII h fragment (0.29 to 4.00 m.u.) was recloned into the plasmid pS1 (Stow and McMonagle, 1983) at the Hind III site as described in methods (Section 2.9). Of the 96 transformed bacterial colonies screened by the miniprep alkaline lysis method (Birnboim and Doly, 1979), one was found to contain the desired plasmid. The resultant new plasmid containing HSV-2 Hind III h and HSV-1 Ori_S cloned in pAT 153 was named pS1h.

3.12.a. Genome analysis of plasmid pS1h

The presence of the HSV-2 HindIII h fragment in the plasmid pS1h was deduced by analysis of BamHI and HindIII digests of plasmid pS1h DNA. Plasmids pS1 and pZ15 were used as controls. Lambda (λ) DNA digested with AvaI was included in each digest to serve as a molecular weight marker control. As seen in Figure 52A, digestion of plasmid pS1 DNA with HindIII linearizes the plasmid to generate a single band of about 4kb in size. Digestion of plasmid pZ15 with HindIII cleaves out the HSV-2 HindIII h fragment of approximately 16kb (h), running at the top of the gel, and the linearized plasmid vector sequences of 3.6kb (v). Digestion of plasmid pS1h with HindIII generates two fragments of approximately 16kb and 4kb representing HSV-2 HindIII h and vector sequences (S) of the plasmid pS1h respectively. The second band (h) in

FIGURE 52

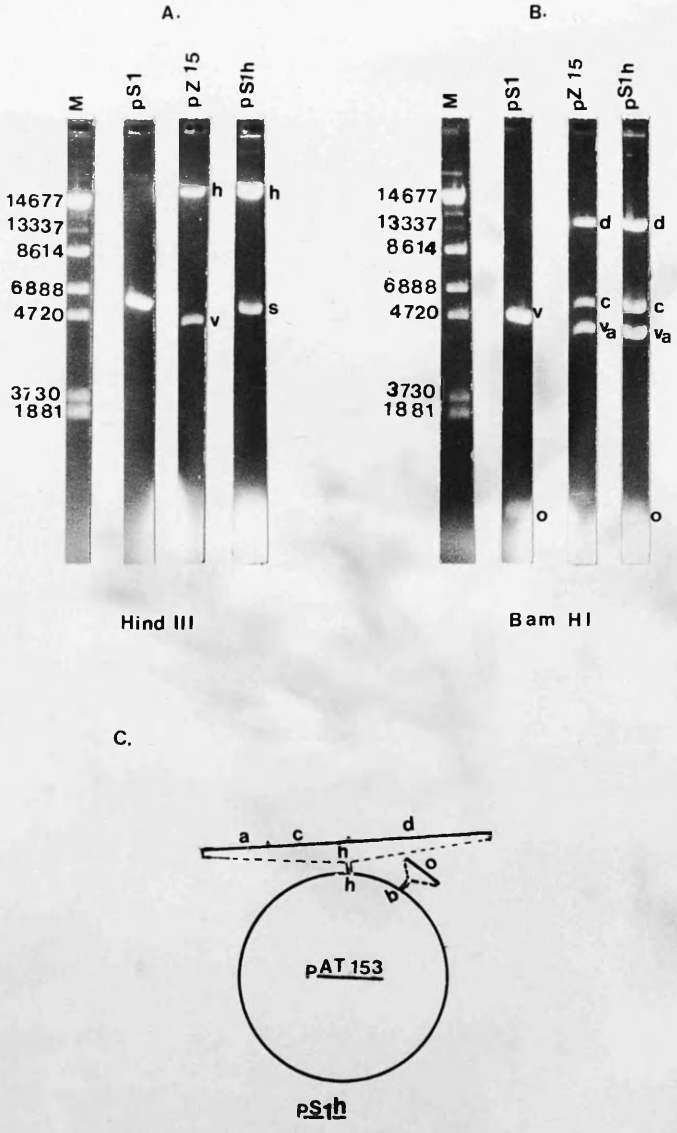
Analysis of recombinant plasmid pSlh.

Photographs showing HindIII (A) and BamHI (B) restriction digests of plasmids pSlh, pZ15 and pS1 DNA. Phage lambda DNA (1 ug) digested with AvaI was included as a molecular size marker control (M). Plasmid DNA (1 ug) was digested with the enzyme under standard conditions, electrophoresed on a 0.8% agarose gel containing ethidium bromide, visualised under U.V. illumination and photographed. The tracts in each gel are, from the left, as follows:

M = lambda 1. plasmid pS1; 2. plasmid pZ15; 3. plasmid pSlh.

Figures on left of the tract marked M indicate the size in bp of the lambda DNA fragments.

(c) Diagrammatic representation of the plasmid pSlh containing the HSV-2 HindIII h fragment (h) and HSV-1 origin (Ori_S) containing fragment (O) cloned in the HindIII and BamHI sites of plasmid pAT153 respectively. a, c and d represent the three subfragments of HSV-2 HindIII h generated upon restriction with BamHI.


A.
B.
M
pS1
pZ 15
pS1h
14677
13337
8614
6888
4720
3730
1881
h
v
s
d
c
v_a
o
Hind III
Bam HI
C.
a
c
d
h
o
p
pAT 153
pS1h

HindIII digestion of pS1h could be due to the partial digestion of a very small population of plasmid (pS1h). The slower migration of S in digests of plasmid pS1h as compared to the vector sequences v in the plasmid pZ15 digest indicates that the increase in size and hence slower mobility of S is due to the presence of an approximately 535 bp fragment of HSV-1 containing the origin of replication (Ori_S).

Confirmation of the presence of Ori_S in the plasmid pS1h came from BamHI digests of plasmids pS1, pS1h and pZ15 (Figure 52B). Digestion of the plasmid pS1 with BamHI cleaves out the origin containing HSV-1 fragment of approximately 535 bp (O) from the 3.6 kb vector sequences (v). BamHI digestion of plasmid pZ15 yields 4 bands of approximate sizes 3.2, 4.5, 10 and 3.5 kb designated as a, c, d and v respectively. Digestion of plasmid pS1h with BamHI as expected generated all the bands seen in the BamHI digest of pZ15 plus an extra band of 535 bp (O) representing the HSV-1 origin containing insert.

The HSV-2 HindIII h fragment has, therefore, been recloned into the plasmid pS1h without affecting the HSV-1 insert (O) containing an origin of replication (Ori_S).

3.13. MARKER RESCUE OF ts MUTATIONS WITH VIRUS ORIGIN OF DNA REPLICATION CONTAINING PLASMID

To determine whether the introduction of an origin of replication into a plasmid containing a HSV fragment normally devoid of an origin, enhanced the chances of the HSV sequences recombining, the plasmid pS1h described in section 2.9 was constructed. As stated in section 3.12.a it contained HSV-2 fragment HindIII h (0.29 to 0.40 m.u.) and HSV-1 Ori_S cloned in HindIII and BamHI sites respectively in plasmid pAT. The shortest distance between the sites is 346 bp.

The plasmid pS1h was used in cotransfection experiments to rescue the ts lesion in the HSV-1 mutant ts 1201 (Preston et al., 1983) which

maps at 0.33 to 0.34 m.u. As controls cotransfections of ts 1201 with HSV-2 HindIII h alone, HSV-1 EcoRI f (0.31 to 0.42 m.u.) and HSV-1 KpnI n (0.35 to 0.37 m.u.) were set up. A 1-, 5-, 10- and 15-fold molar excess of the HSV fragments to the intact ts 1201 genome was used. Transfections were carried out using the calcium phosphate/ DMSO boost method. One set of the transfections were incubated at 37°C and the second at 31°C. Plates were harvested when plaques were evident (3 to 4 days) and the progeny titrated at 31°C and 38.5°C. The results of transfection experiments are shown in Tables 10 and 11.

As shown in Tables 10 and 11 it is evident that when incubation of the transfection was at 37°C, the efficiency of rescue of the ts mutation in ts 1201 was extremely high for all the fragments. Even taking into account that there was some leak through of the mutant as evidenced by the growth of plaques at 38.5°C when ts 1201 DNA was transfected alone; the figures were still high. The average percentage marker rescue for HindIII h alone was 36.45; for HindIII h plus Ori_S was 34.4; for EcoRI f was 83 and for KpnI n was 22.7. It is also evident that there was variation between replica transfections, most notably for the KpnI n fragment. All the fragments would have been expected to rescue ts 1201. KpnI n being the smallest (3kb), it would be expected to be the least efficient as it infact appeared to be. EcoRI f and HindIII h being of virtually equal size (about 16kb) might have been expected to rescue equally efficiently but EcoRI f proved to be 2 x as efficient. EcoRI f contains Ori_L and HindIII h does not. However, as the HindIII h fragment was of HSV-2 and EcoRI f of HSV-1, the difference in rescue efficiency is not surprising. The plasmid containing HindIII h coupled to Ori_S did not rescue any more efficiently than HindIII h alone.

The high efficiency of rescue could have been due to the initial

TABLE 10

Marker rescue of ts mutations with HSV DNA fragments containing or devoid of DNA origins of replication.

The transfections were carried out as outlined in the text. Initial incubation was carried out at 37°C before titrating the progeny yield at 31°C and 38.5°C. The table shows the per cent marker rescue, obtained from cotransfections of ts 1201 DNA with variable molar excesses of HSV-1 and HSV-2 specific DNA fragments.

TABLE 10

Transfection Plate	Intact Genome	HSV DNA fragment		Titer in pfu/ml at 31°C	Titer in pfu/ml at 38.5°C	% Marker rescue
1.	ts 1201	-		1.2×10^9	$<10^4$	0.00083
2.	ts 1201	-		1.3×10^9	3×10^4	0.0023
3.	ts 1201	-		1.4×10^9	1×10^6	0.07
4.	ts 1201	-		1.3×10^9	1×10^4	0.0007
5.	ts 1201	HSV-2 HindIII h	10x *	1.5×10^9	4×10^8	26.7
6.	ts 1201	-do- -do-	10x	1.3×10^9	3.3×10^8	25.3
7.	ts 1201	-do- -do-	15x	1.3×10^9	8×10^8	61.5
8.	ts 1201	-do- -do-	15x	9.3×10^8	3×10^8	32.3
9.	ts 1201	HSV-2 HindIII h + Ori_S	10x	10^9	6.7×10^7	6.7
10.	ts 1201	-do- -do-	10x	2×10^9	1.2×10^9	60
11.	ts 1201	-do- -do-	15x	9.4×10^8	6.6×10^8	70.2
12.	ts 1201	-do- -do-	15x	5.7×10^8	2.7×10^7	4.7
13.	ts 1201	HSV-1 EcoRI f	10x	5×10^8	5.6×10^8	100
14.	ts 1201	-do- -do-	10x	9.4×10^8	1.6×10^9	100
15.	ts 1201	-do- -do-	15x	6.7×10^7	4.5×10^7	67
16.	ts 1201	-do- -do-	15x	N.D.	N.D.	N.D.
17.	ts 1201	HSV-1 Kpn1 n	10x	7.8×10^8	$<10^3$	0.013
18.	ts 1201	-do- -do-	10x	3×10^8	2×10^4	0.007
19.	ts 1201	-do- -do-	15x	1×10^9	1×10^9	90.9
20.	ts 1201	-do- -do-	15x	4×10^8	2×10^5	0.05

* 10x and 15x denote molar excess of fragment

N.D., not done.

TABLE 11

Marker rescue of ts mutations with HSV DNA fragments containing or devoid of DNA origins of replication.

The transfections were carried out as outlined in the text. Initial incubation was carried out at 31°C before titrating the progeny yield at 31°C and 38.5°C. The table depicts the per cent marker rescue of the ts mutation, from cotransfections of ts 1201 DNA with different molar excess amounts of HSV-1 and HSV-2 specific DNA fragments.

TABLE 11

Transfection Plate	Intact Genome	HSV DNA fragment			Titer in pfu/ml at 31°C	Titer in pfu/ml at 38.5°C	% Marker rescue
1.	ts 1201	-			10^7	3×10^4	0.003
2.	ts 1201	-			9.5×10^8	$<10^2$	0.0
3.	ts 1201	-			7.2×10^8	4.5×10^5	0.062
4.	ts 1201	-			1.9×10^9	$<10^2$	0.0
5.	ts 1201	HSV-2 HindIII h		1x *	1.5×10^9	1.5×10^5	0.001
6.	ts 1201	-do-	-do-	1x	10^9	7×10^4	0.007
7.	ts 1201	-do-	-do-	5x	10^9	5×10^4	0.005
8.	ts 1201	-do-	-do-	5x	2.3×10^9	7×10^5	0.03
9.	ts 1201	HSV-2 HindIII h + Ori_S		1x	1.1×10^9	1.6×10^6	0.14
10.	ts 1201	-do-	-do-	10x	10^9	1.4×10^5	0.014
11.	ts 1201	-do-	-do-	5x	N.D.	N.D.	N.D.
12.	ts 1201	-do-	-do-	5x	1.1×10^8	1.2×10^4	0.01
13.	ts 1201	HSV-1 EcoRI f		1x	1.6×10^9	4.5×10^7	2.8
14.	ts 1201	-do-	-do-	1x	1×10^{10}	10^6	0.01
15.	ts 1201	-do-	-do-	5x	5.4×10^8	6×10^5	0.11
16.	ts 1201	-do-	-do-	5x	3.8×10^8	5.5×10^6	1.4
17.	ts 1201	HSV-1 KpnI n		1x	1.2×10^9	8×10^4	0.006
18.	ts 1201	-do-	-do-	1x	1.2×10^9	$<10^2$	0.0
19.	ts 1201	-do-	-do-	5x	1.2×10^9	$<10^2$	0.0
20.	ts 1201	-do-	-do-	5x	2.8×10^8	4×10^3	0.001

* 1x and 5x denote molar excess of fragment DNA over the intact genomes

N.D., not done.

incubation temperature of the transfection or the high molar excess of the HSV fragments. For this reason in the second experiment, the incubation temperature was changed to 31°C and the fragments were cotransfected at a 1x and 5x molar excess to the ts 1201 genomes. The results indicate that there was still leak through of the ts 1201 at 38.5°C although again variation was apparent. The average marker rescue frequencies were 0.01% for HindIII h; 0.05% for HindIII h + Ori_S; 1.08% for EcoRI f and 0.002% for KpnI n. As ts 1201 leaked through at 0.02%, the only fragment which rescue efficiently was EcoRI f. The figure of 0.05% for HindIII h + Ori_S is only 2.5-fold above background and its significance is, therefore, unclear.

CHAPTER 4

DISCUSSION

4.1. RECURRENCE OF HERPES SIMPLEX VIRUS IN THE RABBIT EYE MODEL OF LATENCY

Herpes simplex virus ocular infections manifest themselves in a variety of clinical conditions ranging from self-limiting benign epithelial disease to necrotizing stromal keratitis and uveitis. Patients with HSV infections of the face and eyes typically have repeated episodes of the disease that often occur in the same location. Following infection of the eye, HSV establishes a latent infection of the trigeminal ganglia (TG) as evidenced by the presence of latent virus (recoverable by cocultivation or in vitro explantation) in the TG of a significant proportion of human cadavers (Baringer and Swoveland, 1973). The reactivated virus travels down to the neurodermatome and may cause recurrent disease. In fact recurrent infections have been shown to be due to reactivation of the endogenous virus. Studies by Gerdes et al (1981) and Asbell et al (1984) demonstrated that the DNA banding patterns of spontaneous isolates from recurrent eyelid or mouth infection with HSV-1 were identical. The identical DNA restriction patterns of spontaneous virus isolates from explanted trigeminal, superior cervical and vagus ganglia from individual cadavers (Lonsdale et al., 1979) gave rise to the hypothesis that each individual carries only one strain of HSV-1 latent in their ganglia, and that this strain is carried throughout an individual's life time.

Experimentally ocular herpes virus infection can be studied in the rabbit eye model of latency. Keratitis following inoculation of HSV onto the rabbit cornea simulates the natural history of HSV keratitis in humans (Williams et al., 1965). Latently infected rabbits like humans, do not continuously shed detectable virus but may experience periodic recurrent infections during which reactivated

virus can be found in the eye (Laibson and Kibrick, 1969; Nesburn et al., 1972). Previous studies in the rabbit eye model have shown that identifiable and reproducible ocular lesions of distinctly different size, location, and shape may result from corneal infection with different strains of HSV-1 irrespective of the dose of the virus (Wander et al., 1980) and several of the characteristics of the disease such as morphology of dendritic ulcers, severity of epithelial disease, and evidence of stromal disease are determined by the viral genome (Centifanto-Fitzgerald et al., 1982). A more serious clinical problem is the recurrence of ocular lesions in natural hosts. Factors which control recurrence are not understood; various stimuli have been implicated and the host's immune response no doubt plays a critical role (Easty, 1985).

Different strains of HSV have been shown to vary in their recurrence potential. Gerdes and Smith (1983) using the rabbit eye model of keratitis demonstrated a close relationship between strains of HSV with regard to ocular viral shedding and T.G. latency. HSV-1 strains 17 and McKrae were designated high frequency recurrence (HFRc) phenotypes as spontaneous ocular shedding of the virus was detected in 80 to 85% of the infected rabbits. Latent virus was recovered by cocultivation from 72% of the ipsilateral and 21% of the contralateral trigeminal ganglia. The five HSV-2 strains i.e. strain 186, HG52, CJ359, 2461 and 2544 studied in rabbits were all designated low frequency recurrence phenotype (LFRc) as these recurred in 0 to 25% of animals with a maximum of 45% of the eye swab cultures positive for infectious virus. HSV-2 strain HG52 recurred in 10% of animals and 0.15% of eye swabs were positive for infectious virus. Virus was isolated from 20% of the ipsilateral ganglia by the cocultivation method. The recurrence phenotype of the virus as defined in singly

infected animals remained unchanged following bilateral infection of the same animal with strains of the opposite phenotype, indicating that the differences seen in the recurrence phenotypes are maintained under identical conditions of immunity i.e. in the same animal. The restriction enzyme patterns of the recurrent virus isolates were identical to the infecting virus indicating the genetic stability of the virus during recurrence. However, in animals simultaneously infected with two strains of opposite recurrence phenotype, some recombinants were also found. These observations demonstrated that recurrence of latent virus in the rabbit keratitis model is HSV strain-dependent and the viral genes involved in establishment and maintenance of latency do not necessarily control recurrence. The findings of Gerdes and Smith (1983) described above formed the basis of the work presented in this thesis. In order to identify viral gene(s) controlling reactivation of the latent virus in the rabbit eye model, it was decided to make recombinants between HSV-1 strain 17 and HSV-2 strain HG52 and compare their capacity to establish a latent infection and reactivation potential with the HFRc, McKrae and LFRc, HG52 parental virus.

Our observations on the spontaneous recurrence of McKrae and HG52 confirmed the findings of Gerdes and Smith (1983). Spontaneous shedding of McKrae found in the tear films of 100% of latently infected rabbits is in line with the findings of Berman and Hill (1985) who observed that 96.7% of McKrae inoculated rabbits shed virus spontaneously in their tear films at least once during the period of observation (20 to 39 days post infection). Spontaneous shedding of HSV-2 strain HG52 could not be detected in the tear films of any of the rabbits inoculated with $5x10^5$ or $5x10^6$ pfu of virus (Table 2). The absence of spontaneous shedding of HG52 could be due to either (i) the presence of some genetic defect in the HG52 genome or (2) the absence

of latent infections in the rabbits inoculated with HG52. The recovery of latent virus from the TG explants of all the HG52 inoculated animals (Table 3) excludes the second possibility.

Induced reactivation of latent virus was achieved by iontophoresis of epinephrine. Epinephrine iontophoresis to the rabbit cornea has been shown to induce virus shedding into the preocular tear film reliably and with a high frequency during the latent phase of virus infection and the virus can be isolated from the tear film (Kwon et al., 1981, 1982; Hill et al., 1983). Virus shedding following iontophoresis has been shown to occur on average for 4 days with highest virus titers in the tear film on the third day post iontophoresis. Epinephrine being an adrenergic drug is believed to act on the sympathetic nerve fibres of post root ganglia resulting in reactivation of virus both in the TG and SCG (superior cervical ganglia). The virus upon reactivation travels down the axon and is shed in the tear film. However, the precise role of epinephrine in reactivation of HSV from latency is unknown.

In the present study 100% of rabbits (11/11) infected with $5x10^5$ pfu of McKrae shed virus on induction with iontophoresis of epinephrine. The average duration of shedding of virus was 4 days (range being 1 to 8 days post iontophoresis; Table 2). These findings confirm the findings of virus shedding for 3 to 4 days in the eyes of latently infected rabbits upon induction with epinephrine as reported by Kwon et al (1982). None of the six rabbits latently infected with $5x10^5$ or $5x10^6$ pfu of HG52 shed virus on induction with epinephrine iontophoresis. However, two of the rabbits inoculated with $5x10^7$ pfu of HG52 shed virus post iontophoresis.

These findings on virus shedding post epinephrine iontophoresis confirm the recurrence differential demonstrated by Gerdes and Smith

(1983) on spontaneous shedding of HSV-1 strain McKrae and HSV-2 strain HG52. The HFRc phenotype of McKrae and LFRc phenotype of HG52 appear to be unchanged and only infrequently and after high inoculating doses can HG52 be induced to reactivate from latency. As there were no differences in the groups of animals infected with McKrae and HG52, the findings on spontaneous and induced shedding point to the viral genotype determining the recurrence differential between the two strains.

Latent infection of the rabbits was confirmed by isolation of virus from explanted trigeminal ganglia. The ipsilateral TG explants of all the rabbits inoculated with McKrae or HG52 shed virus between 7 to 30 days post explantation. Our findings of isolation of virus from 100% (Table 2) of rabbits latently infected with HG52 is in contrast to the findings of Gerdes and Smith (1983) who could recover virus from only 20% of HG52 infected rabbits by cocultivation of their TGs. One possible explanation for the high incidence of recovery of HG52 in the present studies is the efficiency of the screening method i.e. straight explantation and growth, versus cocultivation with helper cells. As seen in Table 2 in some cases release of virus from TG explants was detected as late as 22 days after explantation. Such TGs are more likely to be taken as latency negative if screened by the cocultivation methods in which the ganglia are incubated before cocultivation only for 7 to 10 days. Not all the 6 segments of a trigeminal ganglion explant released virus; most cases varied between 1 and 3 segments. Tullo et al (1982b) reported that following ocular infection of rabbits with HSV, latent infection of the ophthalmic division was more frequent than the mandibular or maxillary divisions of the trigeminal ganglion. Our observation of release of virus from some but not all segments of explanted TGs support these findings in that it is possible that the explanted TG fragments releasing virus

may only have originated from the ophthalmic division of the ganglia. However, the exact anatomical origin of the segments with regard to the divisions of a TG was not recorded in the present study.

Following ocular infection of one eye with HSV, virus may spread to the other eye by the neuronal pathways (Pettit et al., 1965) and cause disease (Goodpasture and Teague, 1923; Kimura, 1962; Kaufman, 1982). Gerdes and Smith (1983) observed some strain differences in the capability of virus to spread to the contralateral TG on inoculation of rabbit corneas with different strains of HSV-1 and HSV-2. The frequency of latent infection in the contralateral TG was much less than that in ipsilateral ganglia. These findings of Gerdes and Smith (1983) are in line with the recovery of both HG52 and McKrae virus from the explants of contralateral trigeminal ganglia of the rabbits (Table 3).

Taken together the observations of spontaneous and induced shedding patterns and the capability of establishing latent infections of the trigeminal ganglia confirm the findings of Gerdes and Smith (1983) on the recurrence phenotypes of HSV-1 strain McKrae and HSV-2 strain HG52.

Some of the corneal explants from the ipsilateral eyes of rabbits latently infected with HSV-1 strain McKrae also shed virus between days 15 and 35 in organ culture. This finding is discussed in a later section.

4.2. RESTRICTION ENDONUCLEASE ANALYSIS OF THE HSV-1 STRAIN McKRAE GENOME

Since analysis of recombinant virus genome structures is based on restriction site polymorphism, it was necessary to know the restriction enzyme cleavage patterns of both parental viruses (HSV-1 strain McKrae and HSV-2 strain HG52) being used to generate

recombinants. The restriction maps for HG52 have already been published (Cortini and Wilkie, 1978). The only previous mapping data for McKrae are that of Gerdes and Smith (1983) who reported that the HSV-1 strain 17 defined HindIII m/n site, the Kpnl c/x site; a Kpnl m site and a HpaI k site were all missing in the McKrae genome. On comparison of the BglII and HpaI digests of McKrae with that of strain 17 (Figure 30) no differences in the sizes or number of fragments were observed. The Eco RI digests disclosed no site loss but both the k and l fragments were smaller than those in strain 17 (Figure 29B). Our results on HindIII digests confirmed the absence in the McKrae genome of the HindIII m/n site. The hexanucleotide for the HindIII m/n site in strain 17 begins at residues 862 (McGeoch et al., 1985) within the coding region of gene US1 encoding polypeptide Vmw 68. The BamHI a fragment was deleted by approximately $1x10^6$ mw and w was larger by 0.3 x 10^6 mw in the McKrae genome. The size alterations were not investigated further. In addition to the HindIII m/n site the KpnI c/x site and d/z site were also found to be missing in the McKrae genome. The Kpnl c/x site and d/z site in HSV-1 strain 17 lie in the coding regions of genes UL 31 and UL 46 respectively. The functions of the UL 31 and UL 46 gene products are unknown. Comparison of the Kpnl profiles of McKrae and strain 17 also revealed the presence of an extra band of approximately 2 x 10^6 mw (Figure 32). Analysis of hybridization data indicated the presence of an extra Kpnl site in the repeat sequences flanking U_L. The sequence analysis of the IE-1 gene (Perry et al., 1986) encoding Vmw 110 in strain 17 has revealed the presence of the Kpnl r/g site at residue 3260 and the 3' terminus of 1E-1 mRNA is located at residue 5301. In order to generate a fragment of approximately 3Kb, the new Kpnl site must lie outside the coding region of the IE-1 gene. Sequence analysis of TR_L/IR_L has not revealed

any other open reading frames in this region. We could not confirm the KpnI m site and HpaI k site differences claimed by Gerdes and Smith (1983). However, a different strain of HSV-1 (i.e. McIntyre) was used in their comparison studies.

The loss of HindIII m/n, KpnI c/x and KpnI d/z sites in McKrae could be due to a single base change in the hexanucleotides recognised by these enzymes. The additional KpnI site in TR_L/IR_L of McKrae, as stated earlier, seems to lie outside the coding sequences for IE-1 mRNA. This analysis is consistent with induction of similar polypeptide profiles by McKrae and strain 17 (Figure 47 tracks 2 and 3).

4.3. CONSTRUCTION OF HSV-1 STRAIN McKRAE X HSV-2 STRAIN HG52 RECOMBINANTS

Our initial approach in constructing McKrae x HG52 recombinants was to do cotransfection experiments with intact HSV-1 McKrae DNA and either plasmid cloned fragments or restriction endonuclease cleaved individual fragments of HG52 DNA isolated from agarose gels. This approach would have allowed us to cover all areas of the genome in a systematic way. Despite restriction endonuclease analysis of nearly 1000 plaques resulting from such cotransfection experiments, no recombinants were obtained. Attempts to make recombinants by double infection of cells with McKrae and HG52 virions were also unsuccessful, as restriction endonuclease analysis of 490 progeny plaques from separate mixed infections did not yield any recombinant virus. The lack of isolation of recombinants using these techniques is not fully understood. Alterations in various parameters especially the molar ratios of the intact and fragmented DNA and different multiplicities of infection were used. The lack of selection pressure in the isolation procedure no doubt plays a prominent role.

HSV-1 X HSV-2 intertypic recombinants have previously been isolated by plaque purification of progeny virus from mixed infections of cells with HSV-1 and HSV-2 virions in which the parents contained selectable markers e.g. a temperature sensitive (ts) mutation (Timbury and Subak-Sharpe, 1973; Preston et al., 1978), a ts mutation in addition to a mutation conferring resistance to a drug like phosphono acetic acid (Morse et al., 1977; Marsden et al., 1978) or a ts mutation and a plaque morphology (syn/syn$^+$) mutation (Halliburton et al., 1977). Intertypic recombinants of HSV-1 and HSV-2 have also been generated by intertypic marker rescue of ts mutations in HSV-1 with restriction endonuclease cleaved fragments, either individual (Knipe et al., 1978) or unseparated (Stow et al., 1978) of HSV-2 wild-type virus DNA. In each case the isolation of recombinants has been facilitated by a selection system i.e. sensitivity to temperature, or to a particular drug or by plaque morphology. We are unaware of any report describing isolation of intertypic recombinants of HSV without a selection system. Alternative explanations for lack of success in obtaining unselected HSV intertypic recombinants could be (i) an inherent failure in the system e.g. the BHK21/C13 cells used being inefficient for transfection or recombination; poor DNA preparation; poor technique etc. (ii) inability to detect recombinants by the method of restriction enzyme profile analysis, (iii) incompatability of McKrae and HG52 resulting in an inability to recombine freely. The results of the marker rescue experiments presented in Tables 4, 10 and 11 indicate both high efficiency of intratypic as well as intertypic marker rescue of ts mutations and may argue against the first possibility. Deduction of genome structure of HSV intertypic recombinants by restriction enzyme analysis in the past (Preston et al., 1978; Stow et al., 1978; Preston, 1981 and also this study) invalidates the second possibility. However, the possibility exists that a recombinant having

a very small insert of heterologous DNA not involving any restriction enzyme site may escape detection by this technique.

As the McKrae genome has minor differences in terms of restriction enzyme site deletions/additions and sizes of fragments compared to strain 17 it seems unlikely that its genome is unable to recombine with HG52 because of gross non-homology. As strain 17 and HG52 recombine albeit inefficiently, explanation (iii) is untenable.

It seems likely that the inability to isolate recombinants by the cotransfection of intact genomes and restriction enzyme cleaved or individual cloned fragments was due to a lack of a selection system restricted or and a low level of recombination between HSV-1 and HSV-2 genomes.

Eleven McKrae X HG52 recombinants (out of 641 progeny plaques screened) were isolated successfully in the absence of selection pressure following cotransfection of intact McKrae DNA with HpaI or XbaI cleaved unseparated products of HG52 DNA. Restriction enzyme cleaved unseparated fragments of HSV-2 DNA have successfully been used in marker rescue of intertypic ts mutations (Stow et al., 1978). It has also been reported that transfection of cells with restriction endonuclease cleaved unseparated fragments of HSV-2 did not yield any progeny virus (Stow et al., 1978) possibly due to inability of the fragments to religate and generate infectious virus. The frequency of intertypic recombination in the present study was found to be 1.8% which probably in part explains the failure to isolate recombinants when using individual or cloned fragments of HG52 DNA. All the putative recombinants were plaque purified three times before virus stocks were grown and their DNA analysed.

4.4. STRUCTURE OF THE RECOMBINANTS

The structure of the intertypic recombinants was deduced from the analysis of their DNA restriction enzyme patterns. The restriction

endonuclease maps of HSV-1 and HSV-2 differ markedly from each other in terms of the positions of sites and sizes of fragments and therefore can be used to determine the crossover points in intertypic recombinants (Halliburton, 1980). The accuracy with which the crossover points can be determined is largely related to the frequency of cleavage with a particular enzyme. The restriction enzymes XbaI, HindIII, BglII, EcoRI, HpaI and KpnI have been used in the past to analyse the DNA structure of the majority of HSV-1 X HSV-2 recombinants (Timbury and Subak-Sharpe, 1973; Halliburton et al., 1977; Preston et al., 1978; Marsden et al., 1978; Stow and Wilkie, 1978). Analysis of recombinants with these enzymes involves 56 cleavage sites in HSV-1 DNA and 40 in HSV-2 DNA (Halliburton, 1980). In the present study, BamHI was used in addition to some of the above enzymes. There are 41 BamHI cleavage sites in HSV-1 and 42 in HSV-2 DNA (Figure 28). Analysis of the BamHI restriction endonuclease patterns of the recombinant genomes determined the location of HG52 DNA inserts with considerable presion without fine mapping. Of the 11 recombinants isolated, R43/2/2 had a single crossover in the short region of the genome (crossover frequency of 9.091%). The recombinants having 2 and 3 crossovers were observed at frequencies of 72% (8/11) and 18.1% (2/11) respectively. These crossover frequencies are in contrast to the observations of Halliburton (1980) who from restriction sites polymorphism of 99 recombinants determined crossover frequencies of 19.2%, 42.4% and 6.1% for 1, 2 and 3 crossover containing recombinant genomes respectively. The small number (11) of recombinants isolated in this study has probably contributed to the observed differences between crossover frequencies.

However, within the limits of the small number of recombinants, it is obvious that the number of recombinants 10/11 containing inserts

of heterologous sequences in U_L (0.35 to 0.576 m.u.) is more than those (3/11) containing inserts in the [S] region (0.82 to 1.00) of the genome. Except for R43/2/2, all the recombinants contained inserts of HG52 sequences in U_L. One interpretation of the data could be the presence of 'hot spots' or preferential sites for recombination in particular regions of the genome. Preferential crossover sites between map units 0.40 to 0.45 and 0.60 to 0.70 reported by Morse et al (1977) from the anlyses of 28 selected intertypic recombinants concords with our findings of increased recombination frequency between 0.35 and 0.576 m.u. Halliburton (1980) from analysis of the genome structure of 79 selected intertypic recombinants observed that the most frequent crossover events lie between 0.025 and 0.250, 0.40 and 0.45, 0.825 and 0.85, and 0.650 and 0.675 m.u. but there were no obvious hot spots for recombination.

Each of the 11 recombinants isolated after restriction endonuclease analysis of 641 plaques derived from cotransfection of intact McKrae DNA with the total restriction endonuclease cleaved products of HG52 DNA contained HG52 sequences between 0.35 and 0.576 m.u. and/or 0.82 and 1.00 m.u. (Figure 48). As far as could be determined by BamHI analysis no other HG52 DNA fragments were present in any of the recombinants. The left hand end of the type II inserts in the long region of the genome always terminated at approximately 0.35 m.u. i.e., the crossover was around the BamHI e'/g site of HSV-1 such that whole of BamHI b of type II was present in all the recombinants except R43/2/2. BamHI analysis does not unequivocally determine the origin of the DNA present in the region delimited by the HSV-1 BamHI e'/g site and the HSV-2 BamHI l/b site. For the recombinants derived from HpaI cleaved HG52 fragments (R10/3/1, R10/3/4/6, R10/4/1, R20/4/1, R30/3, R40/2/2 and R40/2/4) the 0.35 map position is apparently coincidental with the HpaI h/d site of HSV-2 (Figure 48).

It could, therefore, be postulated that the left hand end of the insert was determined by the left hand end of the HpaI d fragment. However, the left hand end of the type II inserts in recombinants R5/26/1, R47/27/1 and R7/3/3 derived from cotransfection involving XbaI cleaved HG52 genomes also terminated approximately at 0.35 m.u. Recent sequencing analysis of HSV-1 strain 17 (D.J. McGeoch, personal communication) has shown that the 3' ends of the transcripts of genes UL 26 and UL 27 terminate in a tail to tail fashion (Figure 5) at this position. UL 26 codes for the packaging polypeptide p40 (Preston et al., 1983) and UL 27 codes for gB (Bzik et al., 1984). The 3' end of the mRNA of UL 26 stops at nucleotide position 52796 and the 3' end of UL 27 stops at nucleotide position 53028. The nucleotide sequence of HG52 in this region is not known but it could be envisaged that there would be a high degree of homology between gB of HSV-1 and HSV-2 thereby facilitating recombination and that the 200 bp intervening sequence (368 bp non-coding sequence) may not be highly homologous such that the crossover is likely to have occurred within gB. This model would require the viruses to tolerate a recombinant glycoprotein.

The HG52 U_L inserts in recombinants derived from HpaI cleaved HG52 DNA were of variable length. The right hand end of the largest insert in U_L seen in R40/2/2 extended up to the HG52 HpaI d/e site such that whole of the HpaI d fragment had recombined into the McKrae genome. The right hand boundary of the HG52 U_L inserts in recombinants derived from XbaI cleaved HG52 genomes terminated at 0.43 m.u. comapping with the HG52 BamHI j'/o site. Sequence analysis of HSV-1 strain 17 (D.J. McGeoch, personal communication) has revealed that the genes located between 0.35 and 0.576 m.u. include UL 26 to UL 38. UL 26, UL 27, UL 29 and UL 30 code for the packaging polypeptide p40, gB,

DBP and DNA polymerase respectively. The functions of the gene products encoded by UL 31 to UL 38 are as yet unknown.

Inserts of HG52 DNA sequences in the short region of the genome as determined by the restriction endonuclease analysis were located between 0.82 and 1.00 m.u. i.e. the entire short region. The DNA sequence comparison of the short region of HSV-1 strain 17 and HSV-2, HG52 has indicated colinearity of the genes except gG (Whitton and Clements, 1984a,b; Whitton et al., 1983; McGeoch et al., 1985, 1987).

4.5. POLYPEPTIDE ANALYSIS

The electrophoretic mobility of the infected cell polypeptides induced with HSV-1 strain McKrae was similar to that of strain 17 except for a change in mobility of Vmw 21K induced by McKrae. In an analysis of polypeptides induced by HSV isolates from human trigeminal ganglia an interstrain variability in the mobility of the 21K polypeptide was reported by Lonsdale et al (1979). Dargan and Subak-Sharpe (1984) reported a similar shift in the mobility of Vmw 21K in a study of polypeptides induced with ts^+ revertants of HSV-1. DNA sequencing of the US 11 gene encoding Vmw 21K has revealed the presence of a short 18 bp sequence which is present as 3 tandem repeats in HSV-1 strain 17 syn^+ (Rixon and McGeoch, 1984), but only as two tandem repeats in HSV-1 strain Patton (Watson and Vande Woude, 1982). The corresponding sequence in the McKrae genome is not known. It has been suggested (Rixon and McGeoch, 1984) that variation in the number of repeats of this 18 bp sequence may account for the interstrain variability in the molecular weight and hence the mobility shift of Vmw 21 reported by Lonsdale et al (1979) and hence may account for the difference in the 21K polypeptides of strain 17 and McKrae.

Close correlation has been reported between the genome structure

and the polypeptides induced by intertypic recombinants and this formed the basis of mapping of genes specifying particular polypeptides (Marsden et al., 1978; Ruyechan et al., 1979). Analysis of the polypeptides induced by the McKrae x HG52 recombinants and their genome structures has allowed the assignment of the gene coding for Vmw 29.5 of HG52 to 0.43 and 0.51 m.u. of the genome. This polypeptide had previously been mapped to 0.43 - 0.48 m.u. (Marsden et al., 1978).

4.6. REACTIVATION POTENTIAL OF THE McKRAE X HG52 RECOMBINANTS IN THE RABBIT EYE MODEL

As all the recombinants were not isolated at the same time, it was necessary to check, as they became available, their latency recurrence phenotypes in rabbits. Obviously it would have been advantageous to be able to screen the recombinants with the longest HSV-2 inserts first but this was not possible. However, as the recombinants had similar structures in terms of HSV-2 sequence inserts it was possible to limit the number of recombinants for test to 4 (R10/3/1, R10/3/4/6, R40/2/2 and R43/2/2). In this way there was as little duplication as possible. The four recombinants were fully able to produce a latent infection as evidenced by recovery of virus from explanted trigeminal ganglia between days 15 and 33 post explantation (Tables 5 to 8). The trigeminal ganglia from the four recombinant infected animals when compared to those from McKrae infected animals shed virus for the same number of days (10-15).

However, there was an initial delay in shedding of virus from the trigeminal ganglia of rabbits latently infected with the recombinants (15 to 26 days) compared to McKrae infected animals (5 to 7 days). The possible reasons for the delay in releasing virus from the left trigeminal ganglia of rabbits infected with the recombinants could be

that (a) there were fewer ganglionic cells infected with the virus and/or (b) there was less virus being released from the trigeminal ganglia explants. (c) inserts of HG52 DNA alters timing of reactivation In either case a longer time will be needed for virus amplification before detection. Since quantitation of virus in the TG was not done during these studies, it is not possible to exclude either of the above possibilities. Recovery of virus from one of the six segments of the explanted right trigeminal ganglia from one of the rabbits (Table 5) infected with R10/3/4/6 is similar to the isolation of virus from the right trigeminal ganglion of rabbits infected with the McKrae parent and concords with the findings of Gerdes and Smith (1983) in which virus was isolated from cocultivated contralateral ganglia. Recovery of virus from the contralateral ganglia on explantation indicates active spread in contrast to passive transport of HSV within the nervous system. (ii) cross contamination during explantation However, no attempts were made to quantitate virus released in the tear films or from the explanted ganglia, to determine the role of replication of virus.

Spontaneous shedding of HSV in the tear films of latently infected rabbits has been reported to be intermittent (Berman and Hill, 1985). Gerdes and Smith (1983) observed that in rabbits latently infected with McKrae virus the frequency of spontaneous shedding of virus in the preocular tear film was 85% and that 90% of animals were latently infected. However only 5.4% of eye swabs were positive for virus at a given time point. These observations of Gerdes and Smith (1983) and Berman and Hill (1985) explain our inability to detect any infectious virus when the rabbit eyes were screened for spontaneous shedding of virus. Since the eyes were screened only once (except immediately prior to iontophoresis for spontaneous shedding) albeit for 7 days, it seems likely that virus was not being shed at that particular time point. It is possible that virus could have been detected in the tear films had the eyes been screened throughout the

infection.

On induction with epinephrine iontophoresis all the rabbits inoculated with the recombinants were positive for virus shedding in their tear films and the duration of shedding was similar to that with McKrae infected rabbits (Tables 4 to 8). In other words, each of the 4 recombinant viruses exhibited a high frequency recurrence phenotype (HFRc) like their McKrae parent and not a low frequency recurrence phenptype (LFRc) like the HG52 parent. It follows that the genes encoded in the [S] region of HSV do not determine the recurrence differential and similarly the genes between 0.35 and 0.576 m.u. either alone or in concert with those in [S] are not involved in the reactivation differential from latency between McKrae and HG52 at least in the rabbit eye model except possibly a modification in timings of reactivation due to HG52 insert(s). The genes between 0.35 and 0.43 m.u. in HSV-1 strain 17 have been sequenced (Quinn and McGeoch, 1985). The genes for DNA polymerase (UL 30) and the major DNA binding protein (UL 29) are arranged in a head to head manner with an origin of DNA replication located between them. Recent sequencing has assigned two open reading frames to the left of UL 29 i.e., UL 27 which codes for gB and UL 28 whose polypeptide product has not yet been defined. The genes to the right of UL 30 have also now been defined (D.J. McGeoch, personal communication; Figures 5 and 6). The sequence of the short region of the HSV-1 genome has been determined (Murchie and McGeoch, 1982; McGeoch et al., 1985). The genes encoded in the short unique region of HSV-1 have been shown to be dispensable in vitro with the possible exception of the US 6 gene coding gD (Longnecker and Roizman, 1987; Weber et al., 1987). DNA sequence comparison of the short region of HSV-1 strain 17 and a major portion of U_S of HSV-2 HG52 has indicated a colinearity of sequences with the exception of US 4 which in HG52 is larger by 1460 bp (McGeoch et al., 1987). In both viruses

US 4 encodes glycoprotein gG. Viral mutants lacking HSV-2 (HG52) US 10, 11 and 12 and one copy of the IE-3 gene and Ori_S have been shown to grow in cell culture indicating that these genes are dispensable for lytic growth of virus (Brown and Harland, 1987). It would appear from our work that these genes are not involved in determining recurrence phenotype.

4.7. HERPES SIMPLEX VIRUS LATENCY IN RABBIT CORNEAS

Isolation of HSV from corneal explants in addition to trigeminal ganglion explants is one of the interesting findings of this study. Release of virus from the corneal explants of 3 rabbits infected with McKrae, one infected with R40/2/2 and one with R43/2/2 was observed between days 15 and 31 post explantation for McKrae and 35 and 49 for the recombinants. In general, the corneas were a longer time in culture than ganglia before the first detection of released virus and the virus was detected in only one of the six corneal segments cultured from each animal. In each case the restriction endonuclease patterns of the viral DNA released from the corneas were identical to those of the infecting virus (data not shown).

The isolation of HSV from latently infected rabbit corneas can be attributed to one of 3 possibilities; (i) virus was latent in the cells of the cornea (ii) virus was present as a chronic low grade infection undetectable by eye swab (iii) virus had recently been transported to the corneas and needed amplification by growth in cultured cells before detection.

The virus was first detected from the five positive corneal explants on days 18, 28 and 15 from 3 rabbits latently infected with McKrae virus and on 35 days post explantation from rabbits latently infected with R40/2/2 and R43/2/2. In each case, therefore, there was a considerable delay before virus isolation and the delay was similar

to that found in isolation of virus from latently infected ganglia (12, 8, 12, 26 and 15 days post explantation). The long delay in release of virus from corneas, counter argues the possibility of a chronic low grade infection or of the recent transport of a small amount of virus from the TG to the cornea which is only detectable after amplification. In either case amplification and therefore, detection of infectious virus would probably have occurred within 2 to 3 days of explantation. The homogenization of corneal tissues immediately after explantation could have demonstrated the presence of a chronic low grade persistence but paucity of material, the low frequency of isolation and the small number of positive segments precluded homogenization immediately after explantation. The isolation of virus from only one of the six segments in each case is in line with the results obtained in the human study where HSV was only released from clinically diseased segments of corneas. It could be that only certain parts of rabbit corneas were initially infected.

The corneal explants from rabbits latently infected with recombinant viruses were a longer time in culture before releasing virus compared to those latently infected with McKrae. It is possible that during the primary infection of corneas, McKrae had multiplied faster and infected more corneal cells compared to the recombinant viruses.

The frequency of HSV isolation from rabbit corneas (19.2%, 5 out of 26) compared to ipsilateral ganglia (100%, 26 out of 26) suggests that dorsal root ganglia (TG) are the preferred site for establishment of a latent infection following eye infection, but that some corneal cells also support and maintain HSV in a latent state. In vitro studies have shown that cultured rabbit corneal cells are capable of supporting a latent infection (Cook and Brown, 1986, 1987) and support the finding of latent virus in corneal explants. The isolation of

virus from rabbit corneas after a long period of time in organ culture taken together with isolation from only a portion of the corneas strongly suggests that HSV can become latent in some corneal cells.

4.8. HERPES SIMPLEX VIRUS VIRULENCE

Heterogeneity among strains of HSV in relation to virulence has been known for some time. The correlation of data on virulence of intertypic recombinants with their genome structure has been used to identify the genes involved in type specific virulence (Thompson et al., 1983, 1985; Rosen and Darai, 1985; Rosen et al., 1985, 1986).

Doses of 5×10^6 pfu and 5×10^7 pfu of McKrae inoculated onto rabbit corneas caused death or could not be tolerated (Section 3.2). On the other hand doses of at least 5×10^7 pfu of HG52 per animal could be used with no adverse effects. Similarly rabbits were able to tolerate at least 2×10^7 pfu of one recombinant virus suggesting that the inserts of HG52 information had reduced considerably the virulence of McKrae.

It is possible that the particle:pfu ratio of the virus stocks could have modified the virulence of the recombinants. The particle:pfu ratios of the parental virus and the recombinants ranged from 10^1 to 10^3 and the virus with the highest particle:pfu ratio (R43/2/2) did not cause death at 3×10^6 pfu/inoculation. It is also possible that reduction in virulence of the recombinants could have been due to inability of the virus to replicate in the corneas or the inability to spread in the nervous system. Although exact virus levels were not titrated in either the eye or ganglia, there are several observations which suggest that reduced replication by the recombinant viruses did not occur. First the length of the acute phase of the disease was identical in most cases for eyes infected with the parental McKrae or HG52 strains or the recombinant viruses. If

replication in the eye had been restricted, the duration of the acute phase as evidenced by the presence of characteristic herpetic corneal ulcers on visual examination by fluorescein staining would have been decreased. The fact that 100% of the recombinant infected rabbits were shown to be latently infected as well as the length of time required to recover virus from the explanted TG in conjunction with shedding of virus from the contralateral TG in one of the rabbits inoculated with R10/3/4/6 (Table 7), strongly suggest that replication in the cornea or spread of virus in the TG was not restricted.

Moderation in virulence of the McKrae genomes was observed irrespective of the location of HG52 sequences in [S] or U_L or both regions of the genome, indicating the involvement of genes in both regions in the determination of virulence.

Studies on virulence of HSV have implicated different regions of the genome. Centifanto-Fitzgerald et al (1982) have shown that the 0.710 to 0.830 m.u. region of HSV-1 contains genes that control the pattern of corneal involvement in the case of herpetic eye disease in rabbits. Thompson et al (1983, 1985) using mice and Rosen et al (1985) using tree shrews have reported that HSV DNA from 0.710 to 0.830 m.u. is associated with the functions responsible for neurovirulence. On the other hand Halliburton et al (1987) on intraperitoneal inoculation of mice with 31 HSV intertypic recombinants having crossovers along the length of the genomes observed an attenuation in virulence independent of the parental viruses and concluded that factors controlling virulence of HSV are multigenic. Similarly Sedarati and Stevens (1987) from studies on the virulence phenotype of three strains of HSV-1 in mice concluded that the avirulent phenotype was related to different loci in the 3 viruses.

Our studies do not implicate the region of the HSV-1 genome

(0.710 to 0.830) mapped by Centifanto-Fitzgerald (1982) or Thomson et al (1983, 1985) but support the concept that virulence of HSV is controlled multigenically, an observation already reached for HSV1 x HSV2 intertypic recombinants and for intertypic strains of HSV-1 in mice (Halliburton et al., 1987; Sedarati and Stevens, 1987).

4.9. STRUCTURE OF McKRAE X HG52 RECOMBINANTS IN RELATION TO ORIGINS OF VIRUS DNA REPLICATION

On cotransfection of intact McKrae genomes and the total products from restriction endonuclease cleaved HG52 genomes, 11 identifiable recombinants were isolated. The restriction endonuclease profile analysis of the recombinant genomes indicated that when XbaI cleaved HG52 DNA fragments were used, only sequences from the right hand end of XbaI c (0.0 to 0.45 m.u.) were inserted into the McKrae genome. When HpaI cleaved HG52 fragments were used, only information from HpaI d (0.35 to 0.576 m.u.) or [S] or HpaI d plus [S] were inserted. The common feature between XbaI c, HpaI d and the whole of the [S] region is that each contains an origin of replication, either Ori_L or Ori_S. There are no other shared characteristics in terms of gene coding between the sequences in [S] and those between 0.35 and 0.576 m.u. Isolation of recombinants containing both Ori_L and Ori_S of HSV-2 suggests that both origins i.e. Ori_L and Ori_S are functional in vitro.

We propose that the preferential insertion of HG52 sequences from the above listed restriction fragments into the McKrae genomes is due to the presence of an origin of replication in each of these fragments. Preferential recombination in these regions of the genome could be due to (a) amplified replication of fragments containing an origin such that these fragments are increased in number and are therefore more readily available for recombination and/or (b) an interrelationship between replication and recombination exists such

that in the process of replication, recombination is more efficient due to dispersed DNA strands being freely available (c) heterogeneity along the genome in terms of potential for recombination. The absence of an origin of replication in the other restriction endonuclease cleaved fragments would preclude possibilities (a) and (b).

If preferential recombination was due to amplification of the origin containing fragments i.e. increased copy number making them more available, it would not be necessary for the inserted fragment always to contain an origin of replication. If abundance was the only criterion for recombination, then it would be expected that any region of the origin containing fragment would be equally available for recombination. When XbaI fragments were used for cotransfection, only Ori_L containing sequences from the extreme right (0.35 to 0.45) of the XbaI c fragment (0.0 to 0.45 m.u.) were inserted. This would suggest that preferential recombination between origin containing fragments and intact genomes may be related to replication and the frequency could be inversely proportional to the distance from the origin: the highest degree of recombination around Ori_L being concentrated between 0.35 and 0.43 m.u.

It could also be postulated that the preferential recombination demonstrated is due to a high degree of homology in these regions of the genome. The fact that proteins involved in replication, i.e. the major DNA binding protein and HSV DNA polymerase are located between 0.38 to 0.43 m.u. and are presumably highly conserved in HSV-2 may give credence to this possibility. The genes in the short region of HSV-1 and HSV-2 are also highly conserved. However, there are other regions of the genome which are highly conserved between HSV-1 and HSV-2 and yet no recombination has been demonstrated between them.

Experiments to generate HG52 x McKrae recombinants by cotransfection of intact HSV-2 DNA with HpaI digested McKrae DNA has

so far led to the isolation of two recombinants. One has a McKrae insert between 0.85 and 0.98 m.u. and contains two copies of Ori_S of McKrae. HpaI cleaves McKrae at 0.93 m.u. in [S] into c and g fragments. This recombinant has therefore been generated by recombination between c plus g and the intact HG52 genome (presumably due to inefficient cleavage by HpaI such that the c/g site has not been cut). The second recombinant contains approximately 2.3 Kb of type 1 sequences. The 2.3 Kb contains the long origin of replication of type 1 (located within the BamHI v fragment). The reason for the type I insert being so small is not immediately apparent. The insert presumably contains most if not all the UL 29 and UL 30 genes at either side of Ori_L. Recombination must therefore have taken place within these presumably highly conserved genes or in adjacent homologous sequences.

The fact that there were only 2 recombinants identified from 405 progeny plaques screened, and that both contained a heterologous origin of replication is consistent with our hypothesis that the presence of a replication origin in a fragment appreciably enhances its chances of recombination after cotransfection.

The frequencies of intertypic marker rescue of ts mutations were found to be independent of the presence of an origin of virus DNA replication in the rescuing fragment of heterologous DNA (Tables 10 and 11) This may indicate that DNA origin of virus DNA replication, Ori_S present in the plasmid pS1h was functionally inactive thereby not increasing the copy number of the HSV DNA fragment. The parent plasmid pS1 (pAT + Ori^S) was found to be active in transfection experiments (Stow and McMonagle, 1983) and therefore invalidates the possibility of Ori_S being inactive. However, the possibility of Ori_S being inactivated during the recloning process cannot be ruled out.

Alternatively, as proposed, if recombination is linked with replication of HSV, the inability to obtain enhanced recombination with the plasmid pSlh may be due to the presence of 346 bp plasmid pAT DNA sequences intervening Ori_S and the HSV-2 HindIII h fragment.

Recombinants from transfection involving XbaI cleaved fragments had HG52 inserts only extending as far as 0.35 m.u. on the left hand side and 0.43 m.u. on the right hand side, when the fragment from which the insert was derived i.e. c extended from 0.0 to 0.45 m.u. The limitation on the extent of the insert being concentrated about Ori_L i.e., 0.41 m.u., gives credence to the proposal that replication and recombination are interlinked and fits in with the marker rescue results obtained with the plasmid pSlh.

Recombination has been shown to be linked with replication in adenoviruses and T4 phage. Using ts mutants in the adenovirus DNA polymerase and DNA binding protein, it has been observed that no recombination occurs until 24 hr post infection at which time a limited amount of DNA replication was detected (Stillman et al., 1982). Similarly Young et al (1984) using two ts mutants of adenovirus having different restriction site alleles demonstrated that the recombinant products could be detected just after the onset of DNA replication. If virus replication was blocked with viral DNA inhibitors such as cytosine arabinoside no recombinants were detected, whereas reversal of the block allowed recombination to occur. The precise relationship between adenovirus DNA replication and recombination is unknown. Two possibilities have been put forward: (1) some polypeptides such as DNA polymerase and the DNA binding protein may be needed in both pathways or (2) the single stranded DNA produced by replication might serve as a substrate for recombination (Flint et al., 1976; Meselson and Radding, 1975; also see Figure 53) which would be analogous to the situation in bacteriophage T4, where DNA

replication and recombination are tightly linked (Mosig, 1983; Stahl, 1979; Figure 54).

It can be argued that by analogy to adenovirus, recombination in HSV may also occur contemporaneously during the course of the replicative cycle. In this context it is worth mentioning the role of the major DNA binding protein in HSV replication. It has been observed that the HSV major DBP keeps single stranded DNA in an extended configuration during HSV DNA replication (Ruyechan, 1983) and it can be argued that this extended single strand of DNA might act as a substrate for homologous recombination with the input DNA of the heterologous strain of HSV.

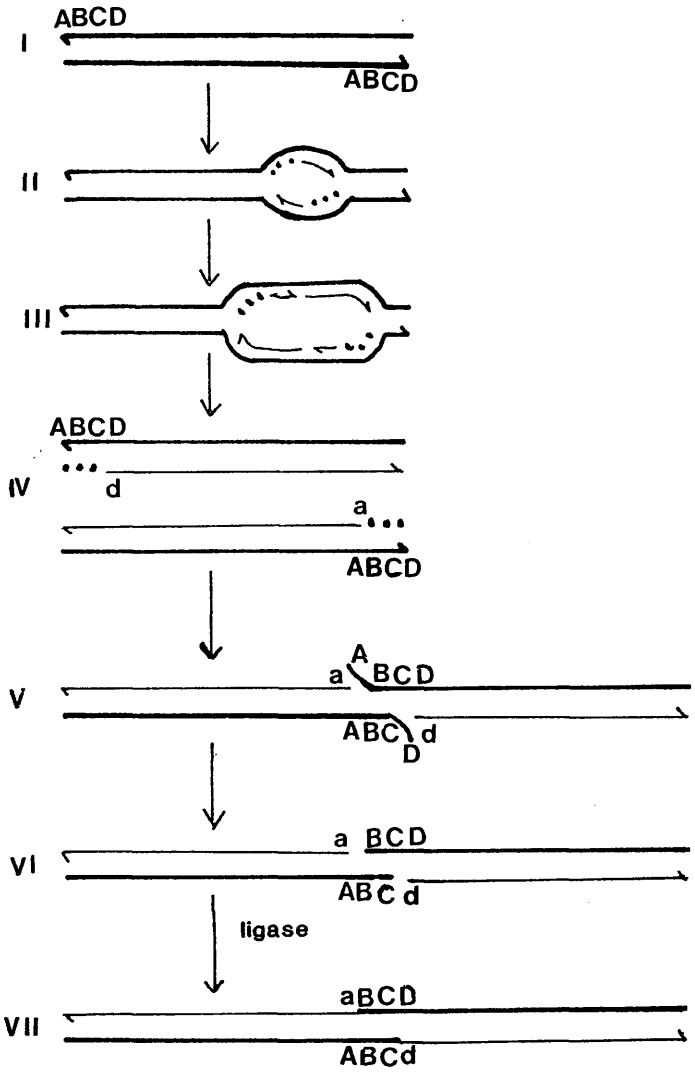
4.10. CONCLUSIONS

The present studies were carried out to investigate the viral gene(s) controlling reactivation of herpes simplex virus from latency. Using the rabbit eye model of latency HSV-1 strain McKrae and HSV-2 strain HG52, on induction with iontophoresis of epinephrine, were found to recur with a frequency of 100% and 10% (2 out of 21) respectively. Both strains produced latent infections in trigeminal ganglia with equal frequency (100%). Intertypic recombinants between McKrae and HG52 strains were constructed and screened in the rabbit eye model for their reactivation potential in order to map the gene(s) controlling reactivation differential from latency. Genome structure analysis of the recombinant viruses revealed some interesting observations with respect to recombination per se in HSV. The important conclusions of the present studies are as follows:

1. It has been demonstrated that the presence of HSV-2 HG52 DNA inserts between 0.35 and 0.576 m.u. and 0.82 and 1.00 m.u. in the HSV-1 McKrae genome do not preclude the reactivation

FIGURE 54

Role of recombination in T4 DNA replication (Broker, 1973). i, Linear duplex DNA; ii, formation of bidirectional replicating loop (a single origin is shown for simplicity); ——, parental DNA; ——, newly replicated DNA; ⟶ 3' terminus; ●, gene 32 protein; iii, further replicated DNA showing discontinuous synthesis; iv, two replicated DNA molecules; v, recombination at incompletely replicated ends; vi, presumed trimming of single-stranded ends by gene 46 and 47 products; vii, covalently sealed recombinant molecule.


I
ABCD
ABCD
II
III
IV
ABCD
d
a
ABCD
V
A
a
BCD
ABC
D
d
VI
a
BCD
ABC
d
ligase
VII
aBCD
ABCd

potential of the McKrae genome. In other words genes located between 0.35 and 0.576 and 0.82 and 1.00 m.u. either alone or in concert are not essential for in reactivation from latency.

2. Isolation of infectious virus from corneal explants (of latently infected rabbits) after a long period of *in vitro* culture indicates that HSV may be capable of establishing a latent infection in corneal cells. These findings substantiate the previous work on the isolation of HSV from diseased human corneas.

3. The preliminary observations from a limited number of rabbits inoculated with wild-type McKrae, HG52 or McKrae x HG52 recombinants suggest that the presence of HG52 DNA inserts in McKrae x HG52 recombinants results in moderation in virulence of the McKrae genome. The reduction in virulence of the McKrae genome was independent of the location of the insert indicating that the gene(s) controlling virulence are not confined to a particular part of the genome i.e. virulence is controlled multigenically.

4. Isolation of McKrae x HG52 intertypic recombinants containing both HG52 origins of DNA replication (Ori_S and Ori_L) in a predominantly McKrae genome indicates that both origins are functional *in vitro*.

5. Restriction enzyme analysis of plaque purified McKrae x HG52 or HG52 x McKrae intertypic recombinants has revealed that the inserts of heterologous DNA were derived from limited areas of the genome and the inserts always contained a heterologous origin of replication Ori_L or Ori_S or both. These results indicate that either the (1) presence of an origin of replication in a

restriction fragment amplifies the copy number of the fragment thereby increasing the chance of at least part of that fragment recombining with intact genomes in cotransfection experiments or (2) recombination and replication may be interlinked. The results of preliminary experiments carried out to investigate the role of the origin of DNA replication in fragment amplification by ligating an origin of replication to a heterologous DNA fragment normally devoid of an origin counter argues the involvement of fragment amplification in the recombination process and gives credence to the proposal that in HSV replication and recombination may occur simultaneously.

4.11. FUTURE PROSPECTS

The work described in this thesis has shown that the HSV genes encoded between 0.35 and 0.576 and 0.82 and 1.00 map units do not control reactivation from latency in the rabbit eye model. Future work will require to address itself to the role of the genes in the long region outwith 0.35 to 0.576 map units. However as considerable effort has been put into constructing recombinants covering these regions of the genome without any success, it would seem sensible to introduce a selectable marker into the system before constructing recombinants. The introduction of the Lac-Z gene into specific endonuclease cloned fragments will allow the selection of blue coloured recombinant plaques from the progeny from transfections. In this way a systematic screening of the genome can be carried out and selected recombinants tested in vivo for their reactivation potential.

A similar approach can be taken in determining genes controlling virulence. The findings on a small number of recombinants tested indicates that virulence may be multigenic but of course different animal model systems and different routes may give variable results.

Unexpectedly the construction of intertypic recombinants to study latency reactivation has shown that in cotransfection experiments involving intact genomes of one parent and the total endonuclease cleavage products of a second parent, the only recombinants isolated were those derived from restriction endonuclease fragments containing an origin of replication. This suggested either amplification of a fragment increasing its chances of recombining or that recombination and replication were interlinked. Preliminary results with a plasmid containing an origin of repliction, linked to a HSV fragment not normally containing an origin, point to amplification not being the controlling factor in the observed preferential recombination and possibly to a close relationship between replication and recombination. It would obviously be pertinent to expand the experiments using a range of fragments linked either directly to an origin of replication or with intervening plasmid sequences. Comparison of the recombination potential of such fragments compared to fragments naturally containing an origin will hopefully throw some light on the replication/recombination relationship. These experiments can be carried out with selectable markers such as temperature sensitivity or drug resistance. This should facilitate the analysis which otherwise without a selectable marker, would involve the restriction endonuclease analysis of thousands of plaques to determine recombinant structures.

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SUMMARY

The effect of the deposition of carbonaceous residues on the performance of two different Pt/γ-Al_2O_3 bifunctional reforming catalysts has been studied during continuous flow experiments performed at 500°C and 9.5 atm. using n-heptane as a model hydrocarbon feedstock. With both catalysts, the deposition of carbonaceous residues resulted in an overall decline in catalytic activity. This decline occurred most rapidly during the early stages of the reaction but progressively slowed with increasing time-on-stream until eventually a stable catalytic activity was attained. Significant variations in catalyst selectivity were observed to occur as a consequence of the deposition of carbonaceous residues. Increases in isomerisation selectivity of 11% and 16% occurred for the two catalysts. Corresponding decreases occurred in the aromatisation selectivities (4% and 10%) and hydrocracking selectivities (5% and 6%) of the catalysts, whilst the selectivities with respect of hydrogenolysis remained essentially constant. These selectivity changes are interpreted in terms of a coking process in which the metallic function of the bifunctional reforming catalysts is more selectively poisoned than the acidic function. It is therefore deduced that carbonaceous residues preferentially reside on the metallic function of bifunctional reforming catalysts. Differences in the nature of the individual product yield and selectivity changes observed for the two different catalysts with the deposition of carbonaceous residues, are interpreted in terms of the different metal loadings of the catalysts.

In addition, pulse-flow experiments have been performed with n-heptane at 500°C in the absence of hydrogen gas. These experiments have revealed that, under these conditions, the catalytic activity is enhanced by the presence of surface carbonaceous residues. The nature of the yield and selectivity