ISOLATION OF HERPES SIMPLEX VIRUS TYPE 1 VARIANTS DEVOID OF *HIND III* RE SITES AND THEIR USE IN INTRASTRAIN RECOMBINATION STUDIES

By

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A thesis presented for the Degree of
Doctor of Philosophy

in

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May 1992

I dedicate this manuscript to my:

	PARENTS	**Islahuddin Shaikh**
		Afrida K Shaikh
	WIFE	**Farzeen**
and	DAUGHTER	**Hiba**

ACKNOWLEDGEMENTS

I am grateful to Professor John H Subak-Sharpe for the provision of facilities in the Institute of Virology, for his interest and concern throughout and for his overall supervision.

I would particularly like to thank my supervisor, Dr S Moira Brown, for her guidance, friendship and enthusiasm throughout the course of this work and critical reading of this manuscript. In addition, I am extremely grateful to Moira for being a constant source of support and encouragement in every possible way during the period of my illness and subsequent surgery, when I needed it most. I am also grateful to her husband, Mr Alasdair Brown, whose cheerfulness and welcoming smiles have always provided me both encouragement and happiness throughout my stay in Glasgow.

Thanks are due to the past and present members of labs 209 and 210 for providing a friendly and pleasant environment to work in and making my stay enjoyable. In particular, I would like to thank Dr Alasdair R MacLean and Dr June E Harland for their invaluable friendship, helpful suggestions and technical guidance.

I would like to acknowledge all the members of the Institute of Virology for useful discussions, help and advice. I am especially grateful to Drs Derrick J Dargan, Roger D Everett, Duncan J McGeoch, John McLauchlan, Christine A MacLean, Valerie G Preston, Frazer J Rixon, Nigel D Stow and Philip Taylor.

I wish to thank Dr Christine MacLean for carrying out the immunoprecipitation experiments, Miss Elizabeth McKay for running few of the polyacrylamide gels towards the end of the project, Dr June Harland for making figures 64 to 69 and Drs S M Brown, A R MacLean, J E Harland, Amina Fu, D J McGeoch, A J Davison, V G Preston, B C Barnett, L Robertson, E A R Telford, H Weir and Miss L Coulter for allowing me to quote their unpublished work in this manuscript.

I also very much appreciate the friendship and help of Dr Derrick Dargan, Ms Fiona Jamieson, Miss Elizabeth McKay, Dr Satish K Batra, Dr Fazil Junejo, Dr Mubashir Zia, Dr Waqar Mufti, Dr Nanda Kumar, Dr Asad Elfigi and Dr Muhammad Shibu through the years of my stay in Glasgow as well as in the United Kingdom.

Thanks are also due to all the members of washroom, media, cytology and administrative departments who have always been friendly and helpful during the entire course of this project.

I am of course deeply indebted to my parents, Mr Islahuddin Shaikh and Mrs Afrida K Shaikh for their love and both moral and financial support, without which this work would have ended before it had begun. I also thank all the other members of my family for their support and sincere prayers to succeed in an otherwise contrary situation.

Finally, I would like to take this opportunity to express my heart-felt thanks for the love, sweet smiles and support given by my wife, Farzeen and daughter, Hiba.

The author was the recipient of a Quid-e-Azam Scholarship granted by the Government of Pakistan, Ministry of Education, Islamabad. During the academic year 1989-1990, the author was financially supported by a Charles Wallace Trust Award administered by the British Council. Unless otherwise stated, all the results described herein were carried out by the author's own efforts.

Moin Ul Fareed (May 1992).

SUMMARY

The work presented in this thesis describes the construction of *Hind III* restriction endonuclease (RE) cleavage site-deletion variants of HSV-1 strain 17+ and their use as unselected markers in intrastrain recombination studies. In addition, the isolation of a *Bgl II* RE site-deletion variant and a HSV-1 genome containing an additional *Hind III* site along with the use of these extra markers in recombination experiments are also described. Furthermore, several other deletion/insertion variants were isolated and their preliminary characterization was carried out.

The HSV-1 strain 17+ *Xba I* site negative variant 1702 was used as the parental virus to delete the *Hind III* sites. There are 10 *Hind III* sites on the *wt* HSV-1 genome. Site-directed mutagenesis was used to delete the following 7 *Hind III* sites :

the 0.08 m.c. *Hind III* site, located within the UL5 ORF; the 0.1 m.c. *Hind III* site, which lies within the UL6 gene; the two *Hind III* sites at 0.18 m.c., located within the gene UL13; the 0.26 m.c. *Hind III* site, located within the UL19 ORF; the 0.64 m.c. *Hind III* site, which lies within the UL46 coding sequences; and, the 0.91 m.c. *Hind III* site, which lies within the promoter region of gD (US6). The variant (1733) devoid of the 4 *Xba I* sites plus the 7 *Hind III* sites showed normal growth properties *in vitro*. However, like the parental 1702 virus, 1733 is also *tk*- and produces truncated gC.

During the process of *Hind III* site-deletion variant (the variants 1721 to 1733) isolation, two other variants (1734 and 1739) were spontaneously isolated. The variant 1734 was devoid of the 0.272 m.c. *Bgl II* site and showed no other alteration in its RE DNA profiles, growth properties or polypeptide profile compared to those of the parental viruses. This variant

was recombined with 1733 to generate a variant (1738) lacking 1 *Bgl II*, 7 *Hind III* and 4 *Xba I* sites. The variant 1738 had very similar growth properties and identical polypeptide profile to those of the parental 1702, 1721 and 1733 viruses.

An HSV-1 (strain 17^+) variant 1708 containing an additional *Xba I* site at 0.74 m.c. has previously been described. The variant 1739 isolated herein contained an additional *Hind III* site at 0.374 m.c. However, like the parental 1702 and 1721 viruses, 1739 was devoid of the 4 *Xba I* sites. The restriction endonuclease analysis revealed no other deletion/insertion within the genome of 1739. In addition, 1739 showed normal growth characteristics *in vitro*. To obtain extra unselected markers in recombination studies, recombination experiments between the variants 1708 and 1739 were carried out and a variant (1743) containing an additional *Xba I* site & an additional *Hind III* site was generated. The variant 1743 also showed normal *in vitro* growth properties, was tk^- and produced truncated gC.

Intrastrain recombination experiments used the variants 1738 and 1743 as the parental viruses, differing in 14 unselected markers (1 *Bgl II*, 8 *Hind III* and 5 *Xba I* sites). The 1008 progeny were analysed for the presence or absence of these restriction endonuclease sites. The data obtained from these experiments yielded the following conclusions : (1) HSV is highly recombinogenic; (2) the high recombination frequencies (RFs) obtained herein between tightly linked markers demonstrate the necessity to have more frequent markers within the genomic region(s) of interest to measure correct RF values; (3) possibly all the four genomic isomers (P, I_L, I_S, I_{SL}) take part in the process of HSV recombination; (4) no 'hot spots' of recombination were discovered.

In addition to the isolation of HSV-1 (strain 17^+) RE site-deletion/insertion variants, a number of other variants showing DNA deletions or insertions were also isolated. Most of the deletion variants involved the

repeat elements of the HSV-1 genome, suggesting that possibly these elements are more prone to deletion among the non-essential viral regions in tissue culture. These variants include : the variant 1714 with a 759 bp deletion from both repeats of the L component (TR_L, IR_L), thus removing most of the RL1 ORF and making it non-neurovirulent in mice; the variant 1721X193 with a deletion of approximately 9.9 Kbp between 0.74 and 0.83 m.c. involving the U_L/IR_L sequences; and, the variant 1727X31 with a deletion of approximately 6.6 Kbp between 0.94 and 1.00 m.c., which involved the U_S/TR_S region. The variants 1721X193 and 1727X31 were not analysed any further because of lack of time.

The insertion variants showed a high range of variation among the inserts i.e. from 356 bp to 30 Kbp. The variant 1740*In* containing an insert of 356 bp was analysed and sequence of the insert determined. Since purification and characterization of other insertion variants were not carried out, it is not known whether the large inserts are stable or unstable within their genomes. Moreover, the location and/or nature of these inserts was not determined, again due to constraint imposed by time.

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ABBREVIATIONS

The abbreviations concerning media and solutions are given elsewhere (see Section 2A : Materials). The abbreviations for commonly used chemical elements or compounds are not given on the assumption that the reader would be familiar with them.

A	adenine
'a'	small reiterated sequence
ACV	acyclovir
Ad	adenovirus
AIDS	acquired immune deficiency syndrome
APS	ammonium persulphate
Ara-A	adenosine arabinoside
ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphate nuclease
BCdR	5-bromo-2'-deoxycytidine
BCV	buciclovir
BHK21/C13	baby hamster kidney 21, clone 13 cells
bp	base pairs
BMV	bovine mammilitis virus
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
BUdR	5-bromo-2'-deoxyuridine
C	cytosine
°C	centigrade, degree (thermometer of Celsius)
CAT	chloramphenicol acetyltransferase
CAV	cell associated virus
CCV	channel catfish virus
CD4+	the predominant haematopoietic cell types that are targets for HIV replication (T-lymphocytes, cells of monocyte macrophage lineage and dendritic cells)
Ci	curie(s)
CIP	calf intestinal phosphatase
cm	centimetre
CMV	cytomegalovirus

cpe	cytopathic effect
CRV	cell released virus
CT	calf thymus
CV-1	a heteroploid cell line derived from the kidney of an adult male African green monkey for use in transformation studies on Rous Sarcoma virus
dATP	2'-deoxyadenosine 5'-triphosphate
DBP	DNA-binding protein
DC	donor chromatid
dCTP	2'-deoxycytidine 5'-triphosphate
DEAE	diethylaminoethanol
dGTP	2'-deoxyguanosine 5'-triphosphate
DHPG	2'-nor-2'-deoxyguanosine (BW759U)
D_L	duplicated sequences near the left end of UL (EBV genome)
dl	deletion (for example, a deletion/mutant virus)
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxyribonucleoside 5'-triphosphate
ds	double-stranded (DNA)
D_R	duplicated sequences near the right end of UL (EBV genome)
DR_1	direct repeat (terminal; 17-21bp) of the *'a'* sequence
DR_2	direct repeat 2 (12bp) of the *'a'* sequence
DR_4	direct repeat 4 (37bp) of the *'a'* sequence
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
dUMP	2'-deoxyuridine 5'-monophosphate
dUTPase or DUTPase	deoxyuridine triphosphate nucleotidohydrolase (2'-deoxyuridine 5'-triphosphate nuclease)
E	early (gene)
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
ECMV	equine cytomegalovirus
EDTA	ethylenediamine tetra-acetic acid, sodium
e.g.	for example
EHV-1	equid (equine) herpesvirus 1

EM	electron microscopy
FFWI	fusion from within
FFWO	fusion from without
FGF	fibroblast growth factor
G	guanine
g	gram(s)
gB	glycoprotein B
gC	glycoprotein C
gD	glycoprotein D
gE	glycoprotein E
gG	glycoprotein G
gG2	glycoprotein G of HSV-2
gH	glycoprotein H
gI	glycoprotein I
gK	glycoprotein K
gL	glycoprotein L
GL	genome length
GMEM	Glasgow modified Eagle's medium
GTPase	guanosine-5'-triphosphate nuclease
h	hour(s)
HA	hydroxylamine
HCMV	human cytomegalovirus
HEBS	hepes buffered saline
Hep 2	a heteroploid cell line derived from tumours produced in irradiated and cortisone-treated weanling rats after injection with epidermoid carcinoma tissue from larynx of a 56-year-old human male
HFL	human foetal lung cells
HHV-1	human herpesvirus 1 (HSV-1)
HHV-2	human herpesvirus 2 (HSV -2)
HHV-3	human herpesvirus 3 (VZV)
HHV-4	human herpesvirus 4 (EBV)
HHV-5	human herpesvirus 5 (HCMV)
HHV-6	human herpesvirus 6
HHV-7	human herpesvirus 7

HIV	human immunodeficiency virus
HPV	human papilloma virus
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HVA	herpesvirus ateles
HVS	herpesvirus siamiri
ICP	infected cell polypeptide
icr	immune cytolysis-resistant (mutant virus)
IE	immediate-early (gene)
i.e.	Id est (Latin=that is); in essence
IEC	immediate-early complex
I_L	inversion of L component (VIRAL ISOMERS)
in	insertion (for example, an insertion/mutant virus)
In	innominate RE fragment
Int	a basic, phage-encoded protein (M_r 40,330) with topoisomerase activity (Integrase)
IR_L	internal repeat long
IR_S	internal repeat short
I_S	inversion of S component (VIRAL ISOMERS)
I_{SL}	inversion of both L and S components (VIRAL ISOMERS)
Kb	kilobases (ssDNA or ssRNA i.e. 1000 bases)
Kbp	kilobase pairs (dsDNA or dsRNA i.e. 1000 base pairs)
KD	kilodaltons
L	late (gene); long (genome component)
l	litre
Lac Z	an *E coli* reporter gene encoding ß-galactosidase
LATs	latency-associated transcripts
LMTK	mouse L cells
M	molar
mA	milliamperes
MAbs	monoclonal antibodies
mar	monoclonal antibody-resistant (mutant virus)
m.c	map coordinates
MCMV	murine cytomegalovirus

MCP	major capsid protein
MCS	multiple cloning sites
MDBP	major DNA-binding protein
mg	milligrams
MHC	major histocompatibility complex
min	minute
MIR	major internal repeat
mix	mixture
ml	millilitre
mM	millimolar
mm	millimetre
mmol	millimoles
moi	multiplicity of infection
M_r	molecular weight
mRNAs	messenger ribonucleic acids
MTR	morphological transforming region
n.	nucleotide number(s)
NA	nitrous acid
ng	nanogram(s)
nm	nanometres
NP40	Nonidet P40
NPT	non-permissive temperature
NT	nick-translation
NTG	5-methyl-N'-nitro-N-nitrosoguanidine
NTP	ribonucleoside 5'-triphosphate
OBP	origin-binding protein
OD	optical density
ORF	open reading frame
ORFs	open reading frames
Ori	origin of replication
Ori_L	long region origin of replication
Ori-Lyt	lytic (HCMV) origin of replication
Ori_S	short region origin of replication
oz	ounce
P	prototype (VIRAL ISOMERS)

p	protein
^{32}-P	phosphorus-32 (radioisotope)
PAA	phosphonoacetic acid
PAA^{R}	phosphonoacetic acid resistant (mutant virus)
pac 1	conserved sequence within U_b element of the *'a'* sequence
pac 2	conserved sequence within U_c element of the *'a'* sequence
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFA	phosphonoformic acid
pfu	plaque forming units
pH	potential of Hydrogen*
pi	post infection
pmol	picomole(s)
PMSF	phenylmethylsulphonyl fluoride
pol	polymerase
Pr	protease
PRV	pseudorabies virus
PT	permissive temperature
R	purine moiety
RC	recipient chromatid
RE	restriction endonuclease
RF	recombination frequency
RFs	recombination frequencies
RL or R_L	repeat long (gene or genome region)
RNA	ribonucleic acid
RPC-1	RNA-protein complex 1
rpm	rounds (revolutions) per minute
RR	ribonucleotide reductase
RR_1	large subunit of RR
RR_2	small subunit of RR
R_S	repeat short (genome region)
r.t	room temperature
S	short (genome component)
sd	sterile and distilled (water)
Sp 1	GC-rich DNA sequence (binding site)
ss	single-stranded (DNA)

SDS	sodium dodecyl sulphate
syn	syncytial plaque morphology locus (syn^+ = non-syncytial; syn or syn^- = syncytial)
T	thymine
TEMED	N,N,N',N'-tetramethylene diamine
TIF	trans-inducing factor
TK or *tk*	thymidine kinase
TR	terminal repeat(s)
TRF	transcription factor, cellular (Oct-1, OTF-1, OBP100, NFIII, etc.)
TR_L	terminal repeat long
TR_S	terminal repeat short
Tris	tris(hydroxymethyl)aminomethane
ts or *ts*	temperature-sensitive
TTP	thymidine 5'-triphosphate
U_b	unique region between DR_1 and DR_2 of the 'a' sequence
U_c	unique region between DR_4 and DR_1 of the 'a' sequence
U1102	HHV-6 strain isolated from a Ugandan AIDS patient
UL or U_L	unique long (gene or genome component)
US or U_S	unique short (gene or genome component)
UV	ultra-violet
V	volts
Vero	African green monkey kidney cells
vhs	virion host shutoff
Vmw	apparent molecular weight of virus-induced protein
vol	volume
VP	virion protein
v/v	volume/volume
VZV	varicella-zoster virus
wt	wild type
w/v	weight/volume
X	(5'-GCTGGTGG-3') specific sequence that stimulates recombination in phage λ

X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside, a chromogenic substrate for ß-galactosidase
Xis	a small (M_r 8,630), basic, phage-encoded protein (excisionase)
Y	pyrimidine moiety
Z-29	HHV-6 strain isolated from Zairian AIDS patients
µCi	microcurie
µg	microgram
µl	microlitre
µM	micromoles
λ	lambda

AMINO ACID SYMBOLS

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

CHAPTER 1:

INTRODUCTION

INTRODUCTION

The aim of the research described in this thesis was to isolate herpes simplex virus type 1 (HSV-1) Glasgow strain 17+ genomes lacking *Hind III* restriction endonuclease (RE) sites and to use deleted RE sites as unselected markers to study intrastrain recombination in HSV-1. With this objective, seven *Hind III* sites have been deleted from an HSV-1 strain 17+ genome already devoid of the four normally occuring Xba I sites. Unselected recombination experiments have been carried out between one parent in which 7 *Hind III* sites plus 4 *Xba I* sites plus 1 *Bgl II* site have been deleted and a second parent with the full complement of *Hind III* sites (10), *Xba I* sites (4) and *Bgl II* sites (12) plus one additional *Hind III* site and one additional *Xba I* site and the resulting progeny analysed for the presence or absence of specific sites. Several other HSV-1 deletion/insertion variants have also been isolated and characterized. The aim of this introduction therefore is to give a summary of HSV biology with detailed accounts of subjects particularly relevant to this project, namely, HSV-genetics and HSV-recombination.

SECTION 1A : THE HERPESVIRUS GROUP

1. THE FAMILY HERPESVIRIDAE - DESCRIPTION AND CLASSIFICATION

The family herpesviridae, consists of over 80 distinct viruses that have a similar morphology (Fenner, 1976), but with a host range varying from vertebrates to molluscs and even fungi (Kazama and Schornstein, 1972; Nahmias, 1972; Roizman *et al.*, 1981). Since most species are infected by a single member of the herpesviridae and several different herpesviruses have been isolated from the better-studied vertebrates, such as man, it seems likely that many more members of this family are still awaiting to be discovered. The morphological features essential for membership of herpesviridae are:

(i) **The core**, which is an electron-dense fibrillar spindle (Epstein, 1962) surrounded by the double-stranded linear DNA of the viral genome (Chai, 1971; Furlong *et al.*, 1972; Nazerian, 1974).

(ii) **The capsid**, which is icosahedral in shape and consists of 162 hexagonal and pentagonal capsomeres (150 hexons and 12 pentons) surrounding the core (Wildy *et al.*, 1960).

(iii) **The tegument** is an ill-defined layer of proteinaceous material arranged between the capsid and the envelope (Schwartz and Roizman, 1969; Nayak, 1971; Roizman and Furlong, 1974). The tegument comprises approximately 65% of the virion by volume (Schrag *et al.*, 1989).

(iv) **The envelope** is a bilayered lipid membrane containing numerous virus-specified glycoprotein spikes. The envelope surrounds the entire structure of the virion and is derived from budding through the inner nuclear membrane (Morgan *et al.*, 1959; Spring *et al.*, 1968 ; Asher *et al.*, 1969 ; Spear and Roizman, 1972).

Because of the presence of a diverse array of viruses within the family herpesviridae, classification is difficult. In addition to being morphologically identical, herpesviruses share the ability to establish and maintain a latent infection in their hosts. Therefore, a hierarchical system has been devised on the basis of their biological characteristics. Three sub-families, alpha, beta and gammaherpesvirinae have been established using the biological properties such as host range, duration of the lytic cycle, cytopathology and characteristics of their latent infection (Roizman *et al.*, 1978; Matthews, 1982; Roizman, 1982). The herpesviruses have also been classified on the basis of the structural arrangements of their genomes (Honess and Watson, 1977; Roizman *et al.*, 1981; Roizman and Batterson, 1985). By this criterion, herpesviruses have been divided into six groups, representatives of which, are shown in Figure 1.

(i) **Alphaherpesvirinae**

Members of this group usually cause acute, self-limiting disease in their natural hosts. They have a short reproductive cycle which results in destruction of infected cells *in vitro*. After the primary infection, the viruses establish latent infections in the central nervous system of their hosts and often reactivate to produce recurrent disease. Human herpesviruses 1,2 and 3 [herpes simplex virus type 1 and 2 (HSV-1 & 2) and varicella-zoster virus (VZV)], equid herpesvirus 1 (EHV-1; Randall *et al.*, 1953), bovine mammilitis virus (BMV; Martin *et al.*, 1966) and pseudorabies virus (PRV; Gustafsohn, 1970) are the prominent members of this sub-family.

(ii) **Betaherpesvirinae**

The viruses of this group have a narrow host range *in vivo* and usually cause subclinical infections. However, they may cause severe disease in immunocompromised patients and neonates. They have a relatively longer reproductive cycle compared to alphaherpesviruses. Latent infections are established in secretory glands, lymphoreticular cells, kidneys

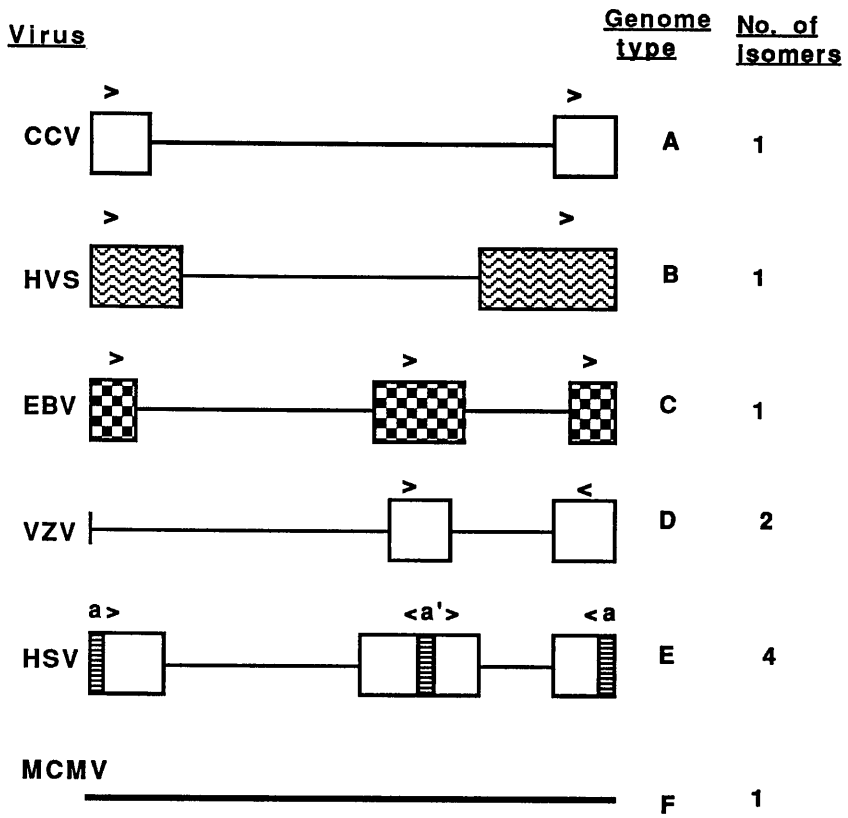

Figure 1. **Structural Arrangements Of Herpesvirus Genomes**

Examples of genome types (groups) A to F are channel catfish virus (CCV), herpesvirus saimiri (HVS), Epstein-Barr virus (EBV), varicella zoster virus (VZV), herpes simplex virus (HSV) and murine cytomegalovirus (MCMV) respectively. Lines represent unique sequences. Open boxes represent larger repeats (greater than 1 Kbp) whereas shaded boxes are repeats of small reiterated sequences. The direction of repeats (direct or inverted) is shown by arrowheads. The terminal direct and internal inverted repeats of HSV, the "a" sequence, are also shown.

and other tissues. This group includes human cytomegalovirus (HCMV; human herpesvirus 5), human herpesvirus 6 (HHV-6; Salahuddin *et al.*, 1986; Lawrence *et al.*, 1990), murine cytomegalovirus (MCMV) and possibly human herpesvirus 7 (HHV-7; Frenkel *et al.*, 1990).

(iii) **Gammaherpesvirinae**

Gammaherpesviruses replicate in B or T lymphocytes *in vitro* and have a narrow host range *in vivo* which is often restricted to the same family or genus to which the host belongs. They establish latency in lymphoid tissues. Human herpesvirus 4 (Epstein-Barr virus; EBV), herpesvirus siamiri (HVS) and herpesvirus ateles (HVA) belong to this sub-family.

2. THE HUMAN HERPESVIRUSES

It has been known for many years that humans are infected naturally by five herpesviruses. However, this number has recently risen to seven : HSV-1, HSV-2, VZV, EBV, HCMV, HHV-6 (Salahuddin *et al.*, 1986) and HHV-7 (Frenkel *et al.*, 1990). Most of these viruses cause asymptomatic or mild infections. However, in immunocompromised individuals they may become very serious. Generally, they produce skin lesions in the superficial areas and replicate in cells of the epidermis and dermis.

HSV-1

Herpes simplex virus type 1 infections are widespread in man and generally give rise to oral or facial lesions (Fiddian *et al.*, 1983). The virus may cause gingivostomatitis, herpetic Whitlow, conjunctivitis and keratitis and acute necrotising encephalitis. Mostly, man acquires a primary infection during childhood, which may be asymptomatic or lead to the development of a range of clinical symptoms such as fever, sore throat, ulcerative and vesicular lesions, oedema, localized lymphadenopathy and malaise. The primary infection is often followed by a latent infection where the virus resides in the neurons of sensory ganglia and possibly rarely in brain tissue (Baringer and Swoveland, 1973; Fraser *et al.*, 1981). The virus may reactivate to produce recurrent disease (cold sores) which are triggered by factors such as stress, fatigue and exposure to sunlight. It is now well established by RE profiles of viral DNAs cleaved with various restriction endonucleases that each individual has a single distinct HSV-1 strain (Lonsdale *et al.*, 1979) and both primary and recurrent infections are caused by the same strains of HSV producing evidence for reactivation of the latent virus (Whitley, 1985; Corey and Spear, 1986).

HSV-2

HSV-2 is commonly associated with genital infections, however, HSV-1 has also been isolated from genital lesions (Dowdle *et al.*, 1967; Kalinyak *et al.*, 1977; Peutherer, 1978; Chaney *et al.*, 1983). HSV-2 is also a causative agent of neonatal herpes and has been implicated in the aetiology of cervical carcinoma (Klein, 1976; Spear and Roizman, 1980; Park *et al.*, 1983). HSV-1and HSV-2 share similar biological properties and homology in about 50% of their DNA sequences (Kieff *et al.*, 1972; Wilkie *et al.*, 1978; Davison and Wilkie, 1983). HSV-2 often establishes latency in the sacral ganglia with periodic recurrence of lytic infection (Baringer, 1974; Reeves *et al.*, 1976; Walz *et al.*, 1977).

VZV

Varicella-zoster virus causes two common, well-defined diseases: Chickenpox (varicella) upon primary infection, and shingles (herpes zoster) after reactivation of latent virus from dorsal root ganglia (Gilden *et al.*, 1978). Chickenpox is commonly a mild disease of childhood with clinical symptoms such as fever and rash of skin lesions leading to pustules. It is transmitted by the respiratory route and direct contact. Shingles is a local vesicular condition caused by reactivation of the latent VZV within a dermatome, mainly affecting adults and often extremely painful. VZV may rarely cause a range of neurological complications of poorly understood pathogenesis (Kennedy, 1987). The virus is difficult to propagate in cell culture (Weller *et al.*, 1958; Grose *et al.*, 1979) and a method to obtain high titre cell-free virus has not been found as yet (Becker *et al.*, 1965; Cook and Stevens, 1968; Gershon *et al.*, 1973).

EBV

Epstein-Barr virus was first discovered in the lymphoblastoid cells of the tumour known as Burkitt's lymphoma (Epstein *et al.*, 1964) and was subsequently found to be the causative agent of infectious mononucleosis or glandular fever (Evans *et al.*, 1968; Evans and Niederman, 1976). Beside EBV's involvement in Burkitt's lymphoma, it is also strongly associated with nasopharyngeal carcinoma (zur Hausen *et al.*, 1970; Epstein and Achong, 1986). The virus can produce a latent infection in B lymphocytes and epithelial cells.

HCMV

Human cytomegalovirus infections are mainly asymptomatic. However, more serious complications occur in patients with acquired immune deficiency syndrome (AIDS) and in immunosuppressed individuals. The virus

can be transmitted by close personal contact and by blood transfusion (Adler, 1983) or organ transplantation (Pass *et al.*, 1983). HCMV is frequently transmitted from mother to child (Dworsky *et al.*, 1983). Prenatal transmission of HCMV is often associated with severe generalised infection and the surviving infants usually suffer from deafness and mental retardation (Pass *et al.*, 1980; Stagno and Whitley, 1985). The isolation of HCMV from biopsies of cervical carcinoma (Melnick *et al.*, 1978, 1979) and adenocarcinoma of the colon (Hashiro *et al.*, 1979) has led to the speculation of its association with these cancers. In addition, it has also been linked to various human malignant tumours, such as neuroblastoma, Wilm's tumour (Wertheim and Voute, 1976), prostate cancer (Sanford *et al.*, 1978) and testicular cancer (Mueller *et al.*, 1988). Usually the virus reactivates from the latent state during pregnancy, multiple blood transfusions or immunosuppression because of a variety of situations such as malignant disease and chemotherapy (Weller, 1971; Plummer, 1973; Gold and Nankervis, 1976; Ho, 1982).

HHV-6

Human herpesvirus 6 was first isolated from human lymphocytes (Salahuddin *et al.*, 1986). Initially, the virus was termed human B-lymphotropic virus (HBLV) because it was reported to replicate only in B lymphocytes (Josephs *et al.*, 1986; Salahuddin *et al.*, 1986). However, recent reports have shown that it mainly infects and replicates in T-cells *in vitro* (Tedder *et al.*, 1987; Agut *et al.*, 1988; Lusso *et al.*, 1988). Antibodies against HHV-6 have been found in most children under the age of 2 (Briggs *et al.*, 1988; Knowles and Gardner, 1988; Okuno *et al.*, 1989) and a recent report has also shown the prevalence of these antibodies in monkeys (Higashi *et al.*, 1989). In humans, the virus causes exanthem subitum (roseola infantum; Takahashi *et al.*, 1988; Yaminishi *et al.*, 1988), a transient childhood illness characterized by high fever and skin rash. Various strains of HHV-6 have since been isolated from the peripheral blood of human immunodeficiency virus (HIV) infected individuals and healthy adults (Downing *et al.*, 1987; Tedder *et al.*, 1987; Lopez *et al.*, 1988; Pietroboni *et al.*, 1988; Harnett *et al.*, 1990; Levy *et al.*, 1990). For instance, HHV-6 strain Z-29 was obtained from Zairian AIDS patients (Lopez *et al.*, 1988) whereas strain U1102 was isolated from the peripheral blood of a Ugandan with AIDS (Downing *et al.*, 1987). The virus has also been isolated from the peripheral blood of individuals immunocompromised following organ transplantation (Ward *et al.*, 1989). The majority of the adult human population have antibodies to HHV-6 (Saxinger *et al.*, 1988; Okuno *et al.*, 1989) usually developed within the first year of life

(Briggs *et al.*, 1988). Antigenic cross-reactivity among the HCMV and HHV-6 strains (Larcher *et al.*, 1988; Yamamoto *et al.*, 1990), DNA hybridization (Efstathiou *et al.*, 1988) and selective sequencing studies of HHV-6 (Lawrence *et al.*, 1990; Josephs *et al.*, 1991; Neipel *et al.*, 1991) have indicated a closer resemblance of this virus to the betaherpesvirus HCMV than to any other characterized herpesvirus. The viral DNA has also been reported to bear a superficial resemblance to the DNA from channel catfish virus (CCV) and equine cytomegalovirus (Martin *et al.*, 1991).

HHV-7

Human herpesvirus 7 has recently been isolated from human lymphocytes (Frenkel *et al.*, 1990). This virus was spontaneously isolated from a culture of uninfected $CD4^+$ cells obtained from a healthy individual during the course of a HIV-1 study. Frequent isolation of HHV-7 from the saliva of healthy adults has also been reported (Wyatt and Frenkel, 1992). Preliminary characterization of HHV-7 DNA has suggested that the virus is related to HHV-6 and HCMV, although distinct enough from them to classify as a different herpesvirus. In addition, using monoclonal antibodies and Western blot analysis, it has recently been shown that HHV-6 and HHV-7 are antigenically distinct viruses (Wyatt *et al.*, 1991). Moreover, HHV-7 is also a prevalent virus that infects children at a later age as compared to HHV-6. However, HHV-7 has not, as yet, been reported to be the causative agent of any specific human disease.

3. LATENCY

A unique and important feature of herpesviruses is the ability to establish and maintain a latent infection within their natural hosts in a non-infectious state. The virus may remain in a latent state for years and then reactivate to produce the disease accompanied with viral shedding to infect fresh individuals or hosts. Following primary infection, the virus establishes latency either in the neurodermatome (for example, HSV-1) or it may become lymphotropic, an example of which is EBV.

Latency in HSV was first proposed by Goodpasture (1929) and then both clinical and experimental evidence in animals and man has established that neuronal cells within sensory ganglia provide a home for the latent virus (Stevens and Cook, 1971; Baringer and Swoveland, 1973; Cook *et al.*, 1974; Baringer, 1975; Stevens, 1975, 1978; Galloway *et al.*, 1979; McLennan and Darby, 1980; Hill, 1985). The virus travels intra-axonally down nerves to ganglionic neurons after infection at peripheral skin sensory nerve cell sites (Cook and Stevens, 1973). Clinically latent HSV has been

recovered from sensory and autonomic ganglia (Baringer and Swoveland, 1973; Warren *et al.*, 1977, 1978) and also from cultured trigeminal nerve roots of human cadavers (Warren *et al.*, 1982). Isolation of latent HSV from peripheral non-neural sites, such as, ear skin (Hill *et al.*, 1980), footpad (Al-Saadi *et al.*, 1983; Clements and Subak-Sharpe, 1983, 1988; Subak-Sharpe *et al.*, 1984a,b; Al-Saadi *et al.*, 1988), explanted corneal tissues (Openshaw, 1983; Cook *et al.*, 1987) and body secretions (Kaufman *et al.*, 1967; Douglas and Couch, 1970) in humans and animal models has also been reported. Reactivation of latent HSV can be triggered by fever, UV irradiation, hormonal changes, stress and other stimuli (Wildy *et al.*, 1982; Hill, 1985). Temperature-sensitive *(ts)* mutants of both HSV-1 and HSV-2 have been used extensively to understand latency and reactivation (Al-Saadi *et al.*, 1983; Kennedy *et al.*, 1983). Using *ts* mutants, McLennan and Darby (1980) have identified latent virus in the neuronal cell bodies of mice that reactivated at the permissive temperature (PT). However, viral antigen could only be detected at the non-permissive temperature (NPT) by immunofluorescence techniques. Superinfection with *ts* mutants at the NPT has also resulted in reactivation of latent HSV (Brown *et al.*, 1979; Lewis *et al.*, 1984).

Latent HSV DNA was first detected by Puga *et al.* (1978) in sensory ganglia from latently infected mice. The latent viral genomes are maintained in a non-linear configuration, probably in circular or concatemeric forms (Rock and Fraser, 1983, 1985; Efstathiou *et al.*, 1986). However, the latent DNA does appear to exist in an episomal state rather than being integrated into the host cell DNA (Mellerick and Fraser, 1987; Deshmane and Fraser, 1989).

Expression of HSV genes during a productive infection occur in a coordinated and temporally regulated fashion (see Sections1C and 1D). HSV-1 encodes more than 70 genes, which are transcribed from both strands of the DNA (Watson *et al.*, 1979; Roizman and Batterson, 1985; McGeoch *et al.*, 1988b). However, viral gene expression and transcription is limited and very different during the latent phase compared to that of productive infection. To understand the molecular events during the establishment, maintenance and reactivation stages of latency, both *in vitro* and *in vivo* models have been developed (O'Neill, 1977; Colberg-Poley *et al.*, 1979, 1981; Wigdahl *et al.*, 1981, 1982, 1983, 1984; McDermott *et al.*, 1984; Nilheden *et al.*, 1985; Russell and Preston, 1986; Tenser and Edris, 1987; Wilcox and Johnson, 1987, 1988; Leist *et al.*, 1989; Wilcox *et al.*, 1990; Speck and Simmons, 1991). In an *in vitro* latency system, Russell *et al.* (1987) have demonstrated that little, or no,

viral gene expression is required for the establishment of latency. However, the HSV-1 immediate early gene 1 (IE-1) product, Vmw110 has been implicated in the reactivation event since an HSV-1 mutant, *dl*1403 (containing a deletion in IE-1; Stow and Stow, 1986) fails to reactivate HSV-2 in vitro on superinfection (Russell *et al.*, 1987). Furthermore, using adenovirus vectors expressing Vmw110, reactivation of HSV was observed (Harris *et al.*, 1989; Zhu *et al.*, 1990). In addition, the viral thymidine kinase (TK; Coen *et al.*, 1989; Efstathiou *et al.*, 1989; Tenser *et al.*, 1989) and ribonucleotide reductase (RR; Jacobson *et al.*, 1989; Katz *et al.*, 1990) may also have a role in reactivation of latent HSV. Studies with an insertion mutant, *in*1814 (Ace *et al.*, 1989) have also demonstrated that the establishment of latency does not require viral gene expression (Steiner *et al.*, 1990; Valyi-Nagyi *et al.*, 1991). Similar studies have also provided evidence for divergent molecular pathways leading to productive and latent infections (Leib *et al.*, 1989; Katz *et al.*, 1990; Speck and Simmons, 1991).

The detection of latency-associated transcripts (LATs) from sensory ganglia latently infected with HSV-1 (Deatly *et al.*, 1987; Rock and Nesburn, 1987; Rock *et al.*, 1987; Spivack and Fraser, 1987; Stevens *et al.*, 1987) or HSV-2 (Mitchell *et al.*, 1990; Burke *et al.*, 1991; Tenser *et al.*, 1991) has provided strong evidence that the HSV genome does not remain completely silent during the latent phase of infection. These transcripts originate from within an 8.3Kb transcription unit located in the long repeats of the viral genome (Spivack and Fraser, 1987; Wagner *et al.*, 1988). Three LATs of 2.0, 1.5 and 1.4Kb have been detected, of which the smaller transcripts are spliced from the larger (Wagner *et al.*, 1988a, b; Wechsler *et al.*, 1988). The large transcript (2.0Kb) is stable, nonpolyadenylated and localized within the ganglionic cell nuclei (Spivack and Fraser, 1987; Wagner *et al.*, 1988a, b). The last 750 bases of the stable LAT are complementary (antisense) to the HSV-1 IE-1 gene (ICP0; Wagner *et al.*, 1988; Wechsler *et al.*, 1988) and overlap the 3'-end of IE gene 1 (Spivack and Fraser, 1987; Stevens *et al.*, 1987). Studies with deletion variants of HSV-1 have suggested that LATs are dispensable for establishment or maintenance of the latent infection (Javier *et al.*, 1988; Sedarati *et al.*, 1989; Steiner *et al.*, 1989), but may play a role in reactivation of the virus from the latent state (Leib *et al.*, 1989; Steiner *et al.*, 1989; Hill *et al.*, 1990).

4. TRANSFORMATION AND ONCOGENESIS

Most of the herpesviruses are capable of inducing tumours and cell transformation. The human herpesviruses, HSV-1 and HSV-2 (Duff and Rapp, 1971, 1973), HCMV (Albrecht and Rapp, 1973) and VZV (Gelb *et al.*, 1980) have been observed to change the properties of rodent cells in tissue culture to a malignant phenotype.

Herpes simplex virus type 2 has long been associated with cervical carcinoma (Naib *et al.*, 1966; Rawls *et al.*, 1969; Nahmias *et al.*, 1970; Adam *et al.*, 1973), although a direct link is still unclear (reviewed by Rawls, 1985). Beside seroepidemiological research (Rawls *et al.*, 1969; Nahmias *et al.*, 1970; Adam *et al.*, 1972), *in vitro* cell transformation systems have been developed to investigate the oncogenic potential of HSV (Duff and Rapp, 1971; Munyon *et al.*, 1971). Three distinct morphological transforming regions (MTR1, MTR2 and MTR3) have been identified in HSV-1 and HSV-2 DNA sequences (Camacho and Spear, 1978; Reyes *et al.*, 1979; Galloway and McDougall, 1981; Jariwalla *et al.*, 1980, 1983; Galloway *et al.*, 1984). The MTR1 region has been mapped in the *Xba I* f [0.29-0.45 m.c (map coordinates)] RE fragment of HSV-1 DNA (Reyes *et al.*, 1979; Galloway and McDougall, 1983). The MTR2 and MTR3 regions are contained in the *Bgl II* n (0.58-0.63 m.c) and *Bgl II* c (0.41-0.58 m.c) RE fragments of the HSV-2 genome (Reyes *et al.*, 1979; Macnab and McDougall, 1980; Galloway *et al.*, 1984; Cameron *et al.*, 1985; Jones *et al.*, 1986). Both MTR2 and MTR3 are required for oncogenesis (Jariwalla *et al.*, 1983, 1986). However, no specific gene has been identified in these regions and no evidence exists to support that an HSV gene is involved in the transformation process (Macnab, 1987). In addition, HSV does not seem to carry a viral oncogene analogous to those found in retroviruses or DNA tumour viruses, such as papova or adenoviruses. On the other hand, HSV-specific antigens (Macnab *et al.*, 1980; Suh *et al.*, 1980) and DNA sequences (Galloway *et al.*, 1980) have been found in transformed cell lines. However, no viral protein has consistently been observed. Furthermore, HSV-transformed cells *in vitro* failed to retain HSV-2 DNA sequences (Galloway and McDougall, 1983), which is in contrast to the detection of such sequences from cervical cancer biopsies (Park *et al.*, 1983). The difference in these observations may however be because of the respective epithelial (cervical cancer) and fibroblastic (*in vitro*) cellular origins (Macnab, 1987).

The exact role of HSV-2 in cervical carcinoma remains obscure. However, cellular transformation with MTRs or other HSV-specific sequences occurs at a low frequency, particularly not by a single-step mechanism

(Macnab, 1987). In addition, HSV-DNA sequences are detected in only a small percentage of cervical carcinomas (Park *et al.*, 1983; Galloway and McDougall, 1983; Macnab *et al.*, 1985) compared to the detection of human papilloma virus (HPV) DNA sequences in up to 80% of cervical carcinomas (Macnab *et al.*, 1986). Therefore, if there is any role played by HSV in cervical carcinoma, it is more likely that it acts in conjunction with various other factors including HPV (Durst *et al.*, 1983; Boshart *et al.*, 1984).

SECTION 1B : GENOME STRUCTURE OF HERPESVIRUSES

1. OVERVIEW

Members of the family *Herpesviridae* have large and complex DNA genomes. The DNAs are linear duplex molecules (Ben-Porat and Kaplan, 1962; Russell, 1962; Becker *et al.*, 1968) ranging in molecular weight from 80 to 150 X 10^6 (120 to 230 Kbp) depending on species (Kieff *et al.*, 1971; Roizman and Furlong, 1974). Genetic studies and sequence analysis of several herpesviruses have shown homologies as well as distinctive features questioning the classifications of the family currently in use (see Section 1A and Figure 1).

The complete DNA sequences of the four human herpesviruses, EBV, VZV, HSV-1 and HCMV have now been published (Baer *et al.*, 1984; Davison and Scott, 1986a; McGeoch *et al.*, 1988b; Chee *et al.*, 1990). The DNA sequences for selective regions of HSV-2 have also been published (see for example, Davison and Wilkie, 1981; McGeoch, 1989; McGeoch *et al.*, 1991). The gross structures of the remaining human herpesviruses, HHV-6 and HHV-7 (the literature so far on HHV-7 consist of only three papers by Frenkel *et al.*, 1990, Wyatt *et al.*, 1991 and Wyatt & Frenkel, 1992) are only now emerging. The genome structures of the six human herpesviruses are shown in Figure 2. The complete sequences for two other herpesviruses, channel catfish virus (CCV) and equine herpesvirus type 1 (EHV-1) have also been determined and their interpretation has led to interesting observations regarding classification and evolution of herpesviruses (see below).

The DNA sequence of the B95-8 EBV genome was the first among the sequences for herpesviruses determined so far (Baer *et al.*, 1984). The genome contains 172,282 residues, however, because of the presence of a 13.6 Kbp deletion in B95-8, a complete EBV genome must be around 186 Kbp (Raab-Traub *et al.*, 1980; Laux *et al.*, 1985). The EBV DNA has a base

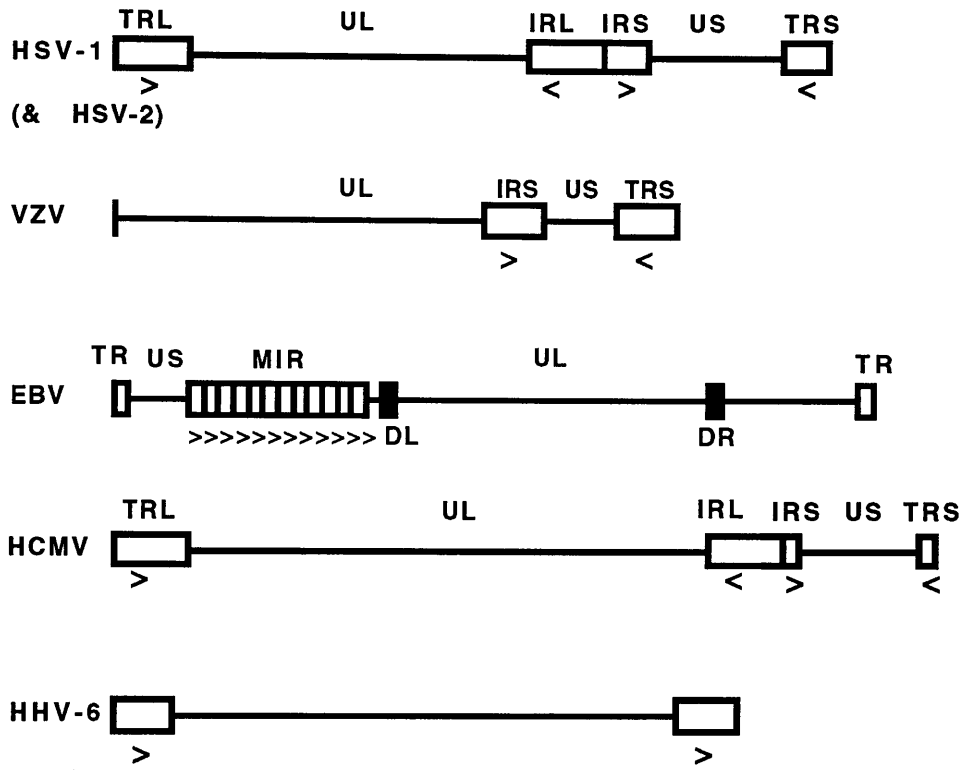

Figure 2. **Genome Structures Of The Human Herpesviruses**

The gross genomic structures of the six human herpesviruses are shown. Solid lines represent the unique DNA sequences. The repeat elements are shown as boxes with relative orientations indicated by arrowheads. Refer to the text for relevant references.

composition of 59.94% G+C (Baer *et al.*, 1984) and its gross genome structure differs markedly from those of other characterised human herpesviruses (see Figure 2). The terminal repeats (TRs) of the genome are made up of several directly repeated copies of a 540 bp sequence. Another set of large directly repeated elements, each of 3072 bp, is located internally near one end of the genome and termed as the major internal repeat (MIR). The TR and MIR families vary in copy number. The MIR family divides the genome into the two unique regions, U_S and U_L. Towards the extremities of U_L are located two identical regions (D_L and D_R), each of 1 Kb, lying in the same orientation. A family of tandemly reiterated sequences (whose sequences are related but not identical) is found adjacent to both D_L and D_R (Raab-Traub *et al.*, 1980; Laux *et al.*, 1985).

The complete VZV DNA sequence has been published in 1986 as 124,884 residues (Davison and Scott, 1986a). The VZV genome is one of the smallest among herpesviruses (Dumas *et al.*, 1980) with a base composition of 46.02% G+C (Ludwig *et al.*, 1972; Davison and Scott, 1986a). The gross genome structure is similar to those of HSV-1 and HSV-2 and is composed of two covalently linked unique regions, U_L (104,836 bp) flanked by inverted terminal repeats, TR_L and IR_L (88.5 bp each) and U_S (5,232 bp) flanked by an inverted repeat sequence, TR_S/IR_S (7,319.5 bp each) (Ecker and Hyman, 1982; Straus *et al.*, 1982; Gilden *et al.*, 1982; Davison, 1984; Davison and Scott, 1985). Like HSV, VZV virions contain four genome isomers, however, the two isomers (termed major) with both orientations of U_S but 'frozen' U_L (prototype) are found in 95-98% of the total VZV DNA molecules. The minor population with an inverted orientation of U_L is found only in 2-5% of the molecules (Davison, 1984; Kinchington *et al.*, 1985; Hayakawa and Hyman, 1987). A similar occurrence regarding genome isomers has also been reported for a related alphaherpesvirus, pseudorabies virus (PRV; DeMarchi *et al.*, 1990). VZV and HSV-1 are closely related viruses in terms of genome structures and isomerization (Davison and McGeoch, 1986). However, an important difference between the two genomes is the absence of terminal redundancy in VZV (Davison, 1984).

The structure of the HSV-1 genome is described elsewhere [see Section 1B (2)].

HCMV is the largest known viral genome (approximate M_r 1.5 X 10^8) that replicates in the cell nucleus (Kilpatrick and Huang, 1977; Geelen *et al.*, 1978). The HCMV (strain AD169) DNA molecule is 229,354 bp in size with a base composition of 57.2% G+C (Weston and Barrell, 1986; Kouzarides *et*

al., 1987; Chee *et al.*, 1990). The structure of the HCMV genome is similar to that of HSV in its two linked unique segments, termed L and S. The L (U_L, 166.9 Kbp) and S (U_S, 35.4 Kbp) are bounded by inverted repeat sequences (TR_L and IR_L, each 11.2 Kbp; TR_S and IR_S, each 2.5 Kbp) and the genome exists as four isomers differing in the relative orientation of L and S sequences (Weststrate *et al.*, 1983; Honess, 1984; Chee *et al.*, 1990). The virus also contains an equivalent of the HSV 'a' sequence (Spaete and Mocarski, 1985). The HCMV genome appears to be largely filled with functional open reading frames (ORFs) which encode around 180 proteins. Hamzeh *et al.* (1990) have identified a region in the HCMV genome active as an origin of replication during lytic infection. These sequences, termed Ori-Lyt, do not resemble any of the known herpesvirus origin of replication sequences and, therefore, need to be explored further.

The genome of herpes simplex virus type 2 has not been completely sequenced, as yet. However, more than 50 Kbp of HSV-2 DNA sequence has already been determined (Davison and Wilkie, 1981; McLauchlan and Clements, 1983; Swain and Galloway, 1983, 1986; Whitton *et al.*, 1983; Swain *et al.*, 1985; Whitton and Clements, 1984a, b; Lockshon and Galloway, 1986; Draper *et al.*, 1986; McGeoch *et al.*, 1987, 1991; Stuve *et al.*, 1987; Tsurumi *et al.*, 1987; Worrad and Caradonna, 1988) and further sequence analysis is being continued (Dr D J McGeoch, personal communication). DNA hybridization studies (Davison and Wilkie, 1983), and sequence analysis (McGeoch *et al.*, 1987) have shown that HSV-1 and HSV-2 are closely related viruses and their genome sequences are closely colinear. Coding sequences of corresponding genes exhibit extensive similarities (70-80%), whereas noncoding sequences, such as, repeat regions show greater differences (McGeoch *et al.*, 1986a, 1987, 1991).

Sequence determination for over 60 Kbp of HHV-6 DNA (Lawrence *et al.*, 1990; Josephs *et al.*, 1991; Neipel *et al.*, 1991) has revealed that the organization of genes and the gene layout is more closely related to HCMV than to any other human herpesvirus (Efstathiou *et al.*, 1988; Neipel *et al.*, 1991). The genome of HHV-6 is approximately 165 Kbp (163 Kbp for HHV-6 strain U1102 and 170 Kbp for strain Z-29) with a base composition of 43% G+C. The genome is composed of a single unique sequence (ranging from 142-155 Kbp, depending upon the viral strain) flanked by a set of large direct repeats (10.5 Kbp long in strain U1102 whereas 12-13 Kbp long in strain Z-29) (McGeoch, 1989; Lawrence *et al.*, 1990; Martin *et al.*, 1991a, b; Neipel *et al.*, 1991). In addition to the variation in length of the unique and

terminal repeat structures (Pellett *et al.*, 1990; Lindquester and Pellett, 1991), immunological and molecular biological analysis has also revealed differences among the various HHV-6 strains, suggesting the probable existence of two or more types (groups) of HHV-6, like HSV types 1 and 2 (Ablashi *et al.*, 1991 and the references therein). HHV-7 appears to be most related to HHV-6 (Frenkel *et al.*, 1990; Wyatt *et al.*, 1991).

CCV DNA sequence has recently been determined completely by Dr A J Davison (MRC Virology Unit, Glasgow). The genome is 134,226 bp in size and composed of a unique region (97,114 bp) flanked by a set of direct repeats (18,556 bp each). CCV has been classified as an alphaherpesvirus (Roizman, 1982). However, the sequence data has revealed that the CCV proteins do not show significant similarities to proteins of any alpha, beta or gammaherpesviruses suggesting a different evolutionary origin than those of other herpesviruses. The data also suggests that CCV should be placed in a fourth subfamily of *Herpesviridae* (Davison, 1991b, 1992; Dr A J Davison, personal communication).

The complete DNA sequence of EHV-1 has also been determined recently (Drs A J Davison and E Telford, personal communication). The DNA genome is 150 Kbp in length and consists of a unique long (U_L) and a unique short (U_S) component (Whalley *et al.*, 1981; Ruyechan *et al.*, 1982). The S component is flanked by an inverted repeat (TR_S/IR_S) (Whalley *et al.*, 1981) and the virion DNA contains equimolar proportions of the two isomers (orientation of U_S). Sequencing data has shown close genetic relationship with VZV and HSV-1. However, five of the EHV-1 genes are found to be unique in that no homologues could be detected in VZV or HSV-1 (Telford *et al.*, 1992 : submitted for publication; Drs A J Davison and E Telford, personal communication).

2. THE STRUCTURE OF THE HSV-1 GENOME

The herpes simplex virus type 1 genome comprises 152,260 residues with an overall 68.3% composition of guanine and cytosine (Davison and Wilkie, 1981; Preston and McGeoch, 1981; Murchie and McGeoch, 1982; Quinn and McGeoch, 1985; Dalrymple *et al.*, 1985; McGeoch *et al.*, 1985, 1986a, b, 1988b; Davison and Scott, 1986b; McGeoch and Davison, 1986; Nikas *et al.*, 1986; Perry *et al.*, 1986; Perry and McGeoch, 1988) and has a molecular weight of 100 X 10^6 (Becker *et al.*, 1968; Frenkel and Roizman, 1971; Kieff *et al.*, 1971; Wilkie, 1973). Sheldrick and Berthelot (1974) have observed self-annealing HSV-1 DNA molecules by electron microscopy and

suggested that the molecules were composed of two covalently linked components, designated L (long) and S (short). Each component contained unique sequences (U) bracketed by inverted repeat sequences (TR_L, IR_L, IR_S and TR_S respectively). At the termini of the HSV genome, a short direct repeat termed the 'a' sequence, was also observed. At least one additional copy of the 'a' sequence is located at the junction of L and S, in the opposite orientation to the terminal copies (Wadsworth *et al.*, 1976; Wagner and Summers, 1978; Davison and Wilkie, 1981). The size of the 'a' sequence varies from strain to strain (250-550 bp) (Wadsworth *et al.*, 1976; Locker and Frenkel, 1979; Mocarski and Roizman, 1981). The ends of the terminal 'a' sequences possess one overhanging residue with a free 3' hydroxyl group (Mocarski and Roizman, 1982).

Sheldrick and Berthelot (1974) further suggested that the recombination events between the terminal and inverted repeats of the molecule may give rise to four possible isomeric forms of the HSV DNA, resulting as a consequence of inversion of the L or S component. Restriction endonuclease analysis (Clements *et al.*, 1976; Wilkie and Cortini, 1976) and partial denaturation mapping (Hayward *et al.*, 1975; Delius and Clements, 1976) established that these four isomeric forms do exist in equal proportions. Isomerization of the genome occurs by intramolecular recombination at the 'a' sequences, possibly during viral DNA replication (Weber *et al.*, 1988). However, HSV mutants with genomes 'frozen' have been isolated in all four isomeric forms and shown to be able to replicate independently, indicating that inversion is not essential for viral DNA replication (Jenkins and Roizman, 1986). The isomers are termed as P (prototype), I_S (inversion of S component), I_L (inversion of L component) and I_{SL} (inversion of both S and L components) (Figure 3; Hayward *et al.*, 1975; Wilkie and Cortini, 1976; Roizman, 1979).

Earlier studies of HSV-1 DNA, based on buoyant density centrifugation have shown the overall G+C value of 67% (Kieff *et al.*, 1971; Halliburton *et al.*, 1975) with variations in different regions of the genome. Sequence analysis have revealed that those values are not accurate. The HSV-1 strain 17^+ has a base composition of 68.3% G+C, which is not constant throughout the genome; U_L is 66.9% G+C; U_S is 64.3% G+C; R_L is 71.6% G+C and the 6.6 Kbp R_S is 79.5% G+C (McGeoch *et al.*, 1985, 1986a, b, 1988b; Perry and McGeoch, 1988). HSV-2 has been reported to have a slightly higher G+C content (Roizman, 1982), which is now confirmed to some extent by a recent sequence analysis of the R_L and selective U_L regions (McGeoch *et*

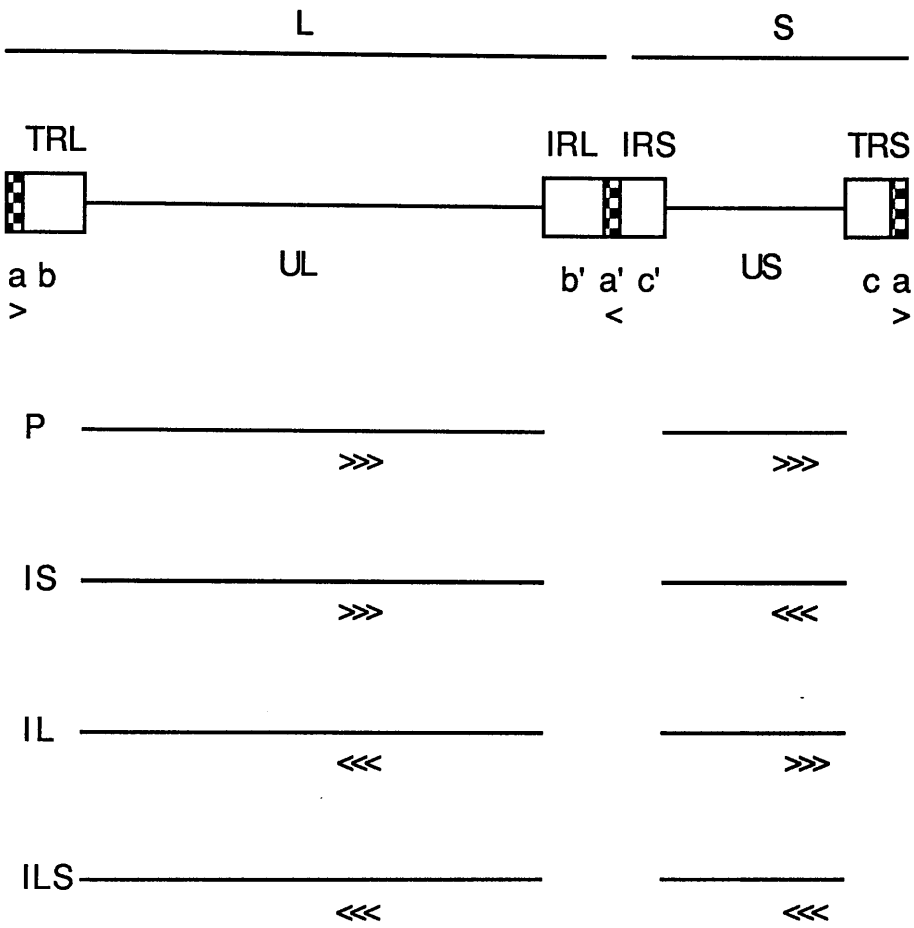

Figure 3. **The Structure Of Herpes Simplex Virus Genome**

The HSV genome is composed of two components, long (L) and short (S). L and S are flanked by inverted repeats (TRL & IRL and IRS & TRS respectively). The "a" sequence is present as a direct repeat at the genome termini and in an inverted orientation at the L-S junction (indicated by an arrowhead, < >). The four genomic isomers are represented as P (prototype), IS (inversion of the short component), IL (inversion of the long component) and ILS (inversion of both L and S components). The arrowheads (<<< >>>) indicate the direction of the genome component.

al., 1991). The occurrence of G+C residues in herpesvirus genomes has two main consequences : (a) GC-rich codons in HSV-1 proteins encode a high content of amino acids, such as, alanine, proline, glycine and arginine, and (b) the presence of a low number of nonsense codons in untranslated reading frames.

In addition to the 'a' sequences and inverted repeats, the HSV-1 genome also contain families of multiple copies of short, directly repeated sequences (Davison and Wilkie, 1981; Rixon *et al.*, 1984). These families have been observed in U_S, R_S and R_L, varying in their copy numbers and with a high content of G+C (McGeoch *et al.*, 1985, 1986a; Perry, 1986). These families have also been observed in other herpesvirus genomes analysed by large-scale sequencing (McGeoch, 1989).

3. THE NUCLEOTIDE SEQUENCE OF HSV-1

The determination of the complete nucleotide sequence of HSV-1 strain 17^+ genome (McGeoch *et al.*, 1985, 1986a, b, 1988b; Perry and McGeoch, 1988) has provided a database to understand the genomic organization in terms of gene content, layout and coding potential. The HSV-1 genome comprises 152,260 residues, although variations exist because of small reiterations and the 'a' sequence differing in copy number. The sequence analysis has revealed the presence of a large number of open reading frames (ORFs), coresponding to proposed HSV-1 genes. The genome was found to be densely packed with ORFs, capable of encoding proteins, with 89% of U_L (107,943 bp) and 97% of U_S (12,979 bp) occupied (Rixon and McGeoch, 1985; McGeoch *et al.*, 1985, 1988b). The original interpretation of the HSV-1 DNA sequence (McGeoch *et al.*, 1988b) has shown a total of 72 genes encoding 70 distinct polypeptides (two are repeated) : 56 in U_L (termed UL1-UL56), 12 in U_S (US1-US12) and one in each copy of R_L (9,214 bp) and R_S (6,677 bp). However, an additional gene (encoding a protein termed ICP34.5) has been recognized recently (Chou and Roizman, 1986, 1990) in the long repeat (R_L) region (a copy in each repeat) of the HSV-1 genome. This gene, along with a previously recognized gene (IE1), have now been termed as RL1 and RL2 respectively (McGeoch *et al.*, 1991). Therefore, the total number of genes encoded by the genome of HSV-1 has risen to 74 (71 unique polypeptides) suggesting a possibility for the presence of undiscovered genes, particularly if they are small, contain complex intron/exon structures or their coding sequences overlap extensively with the protein coding regions of neighbouring genes.

Studies on HSV-1 nucleotide sequence will allow the precise identification of viral proteins and determination of their function. For instance, Chou and Roizman (1986) reported the presence of a promoter-regulatory domain and the possible transcription initiation sites of a gene in the 'a' sequence and proposed the protein coding region of this gene in R_L, upstream of the IE110 gene, in their DNA sequence of HSV-1 strain F. Using a synthetic oligopeptide [a tripeptide; $(Ala\text{-}Thr\text{-}Pro)_{10}$] representing part of the proposed protein from sequence data, Ackermann *et al.* (1986b) raised antisera and demonstrated a protein of 358 amino acids with an apparent molecular weight of 43,500 and designated as gamma-$_1$ 34.5 (ICP34.5). However, McGeoch *et al.* (1988b) were unable to detect an intact corresponding region in the genome of HSV-1 strain 17$^+$ (Perry and McGeoch, 1988). On the other hand, deletion variants in both HSV-1 strain 17$^+$ and HSV-2 strain HG52 have provided evidence of the presence of a gene responsible for a neurovirulence factor in the same region of the genomes (Taha *et al.*, 1989a, b, 1990; MacLean, A. R. *et al.*, 1991a, b). The dispute regarding the status of ICP34.5 has finally been resolved by the publication of a correct version of sequence data of HSV-1 strain F (Chou and Roizman, 1990) showing a large number of errors in their previous sequence of the coding region containing ICP34.5. The new coding ORF for strain F ICP34.5 contained 263 codons. Furthermore, Chou *et al.* (1990) have verified the correctness of their revised sequence and reported the absence of neurovirulence factor in HSV-1 variants defective in both copies of ICP34.5 gene. Finally, Dolan *et al.* (1992) have also revised the DNA sequence of the region representing ICP34.5 and found that the old version (Perry and McGeoch, 1988) contained a 4 bp difference in comparison with the new version. They have verified the new version using different plasmid clones and found the sequence compatible with those sequenced by Chou and Roizman (1990). The strain 17$^+$ ICP34.5 protein is proposed to encode 248 amino acids with a molecular weight of 26,184 (Dolan *et al.*, 1992). Moreover, a protein representing ICP34.5 of HSV-1 strain 17$^+$ has also been detected recently using immunoblot techniques (Dr A R MacLean, personal communication). In addition, sequence analysis of the long repeat regions in the genome of HSV-2 strain HG52 has revealed an HSV-2 counterpart of ICP34.5 (McGeoch *et al.*, 1991). However, HSV-2 RL1 (ICP34.5) contains a set of repeated sequences lying within an intron. The HSV-2 RL1 gene has been proposed to encode a 261 amino acid protein and is 62.7% identical to its counterpart in HSV-1 (McGeoch *et al.*, 1991).

Similarly, potential gene functions of predicted protein sequences of an unknown gene and processes of herpesvirus genome evolution can be investigated by comparing the computer analysis of known sequences in data banks. Recently, McGeoch and Barnett (1991) have observed that the predicted coding sequence of a murine cellular protein is strongly similar to the sequences of HSV-1 and HSV-2 ICP34.5. However, the significance of this observation remains obscure. In addition, it was observed that a close relationship exists between herpesvirus thymidine kinases and a cellular deoxycytidine kinase (Harrison *et al.*, 1991) suggesting a possible evolutionary origin of herpesvirus thymidine kinase.

The HSV-1 genome also contains a 3700-bp region, downstream of the IE110 gene, whose function remains obscure. This region initiates transcription of LAT species (Stevens *et al.*, 1987; Wagner *et al.*, 1988a, b) found in latently infected neuronal cells [see Section 1A (3)]. To date, no protein product encoded by this region has been reported (McGeoch, 1989; McGeoch *et al.*, 1991). A similar, 3900-bp region has also been reported in HSV-2 strain HG52 (McGeoch *et al.*, 1991). However, the sequence of this region, outside the RL2 gene (IE118, a counterpart of HSV-1 IE110), was not found conserved.

HSV studies with deletion/insertion variants (Longnecker and Roizman, 1986, 1987; Stow and Stow,1986; Brown and Harland, 1987; MacLean and Brown, 1987b,c; Umene, 1987; Harland and Brown, 1988; Steiner *et al.*, 1988, 1989; Mullaney *et al.*, 1989; Taha *et al.*, 1989a, b; MacLean, A. R. *et al.*, 1991a, b; MacLean, C. A. *et al.*, 1991), *ts* mutants (Preston *et al.*, 1983; Addison *et al.*, 1984; Wu *et al.*, 1988; Al-Kobaisi *et al.*, 1991), virion host shutoff (*vhs*) mutants (Kwong *et al.*, 1988) and analysis of intertypic recombinants (Marsden *et al.*, 1978; Morse *et al.*, 1978) have already identified various viral proteins and their functions. Several genes have been demonstrated to be non-essential in tissue culture. However, a large number of genes are still uncharacterized. Table 1 and Figure 4 summarise the HSV-1 genes encoding proteins with known functions and map locations of open reading frames respectively. The organization of RL1, RL2 and LAT are shown in Figure 5.

SECTION 1C : THE LYTIC CYCLE OF HSV

The lytic cycle of HSV can be divided into a number of phases, although it is a continuous process : Attachment or adsorption, penetration

TABLE 1

(Adapted from McGeoch *et al.*, 1988b and McGeoch, 1989)

Gene	No. of residues	M_r [1]	Properties or functions	Status[4]	Reference[5]
RL1	263	43500[2]	ICP34.5 protein;	ne	Ackerman *et al.*, 1986; Chou and Roizman,
	248	28184[3]	Neurovirulence factor		1986, 1990; Chou *et al.*, 1990; MacLean,
					A.R. *et al.*, 1991; McGeoch *et al.*, 1991; Dolan *et al.*, 1992
RL2	(19)		IE transcriptional		
	(222)		regulatory protein	ne	
	(534)		(IE110; IE-1)		
	775 Total	78452			
UL1	224	24932	Hydrophobic N terminus;	e	Hutchinson *et al.*, 1992
			proposed virion glycoprotein L		
UL2	334	36326	DNA repair enzyme;	ne	Mullaney *et al.*, 1989
			Uracil DNA glycosylase		
UL3	235	25607	Hydrophobic N terminus	ne	Baines and Roizman, 1991
UL4	199		Unknown	ne	Baines and Roizman, 1991
UL5	882	98710	DNA replication;	e	
			possibly responsible for the DNA helicase activity		
UL6	676	74087	Virion protein;	e	
			possible role in DNA packaging		
UL7	296	33057	Unknown	-	
UL8	750	79921	DNA replication	e	
UL9	851	94246	DNA replication; OBP	e	
UL10	473	51389	Multiply hydrophobic;	ne	Baines and Roizman, 1991;
		possible	membrane-inserted protein		MacLean, C. A. *et al.*, 1991
UL11	096	10486	Myristylated virion protein	ne	MacLean, C.A. *et al.*, 1989; MacLean,
					C.A. *et al.*, 1992
UL12	626	67503	Deoxyribonuclease;	ne	Weller *et al.*, 1990
			possible role in DNA packaging or processing		
UL13	518	57193	Predicted protein kinase	ne	L Coulter, personal communication
UL14	215	23454	Unknown	-	
UL15	(343)		Possible role in packaging	-	Dolan *et al.*,1991
	(392)		nascent DNA into capsids		
	735 Total	80918			
UL16	373	40440	Unknown	ne	Baines and Roizman, 1991

TABLE 1 (continued)

Gene	No. of residues	M_r [1]	Properties or functions	Status[4]	Reference[5]
UL17	703	74577	Unknown	-	
UL18	318	34268	Virion capsid protein; VP23-		Rixon *et al.*, 1990
UL19	1374	149075	Major capsid protein	e	
UL20	222	24229	Multiply hydrophobic; possible role in viral egress	ne*	Baines *et al.*, 1991
UL21	535	57638	Unknown	-	
UL22	838	90361	Virion glycoprotein H	e	
UL23	376	40918	Thymidine kinase	ne	
UL24	269	29474	Unknown	ne	
UL25	580	62666	Virion protein; possible role in the formation of full capsids & virus entry into cells	e	Preston, V. G. 1990
UL26	635	62466	Capsid protein?; Protease; role in DNA packaging	e	Liu and Roizman,1991a, b;Preston, V.G. *et al.*, 1992
UL26.5	329		Substrate of the UL26 gene product; located entirely within the UL26 ORF	-	Liu and Roizman,1991a
UL27	904	100287	Virion glycoprotein B	e	
UL28	785	85573	Probably structural; possible role in the formation of mature capsids	e	Addison *et al.*, 1990
UL29	1196	128342	DNA replication; MDBP	e	
UL30	1235	136413	DNA polymerase	e	
UL31	306	33951	Unknown	-	
UL32	596	63946	Locus of immune cytolysis resistance mutation; structural protein	e	
UL33	130	14436	Structural protein; involved in DNA packaging	e	Al-Kobaisi *et al.*, 1991
UL34	275	29788	Virion protein; hydrophobic C terminus; Probable substrate of the viral protein kinase	-	Purves *et al.*, 1991
UL35	112	12095	Possible capsid protein	-	McNabb and Courtney, 1992
UL36	3164	335841	Virion protein; large tegument protein	e	
UL37	1123	120549	Unknown	-	

TABLE 1 (continued)

Gene	No. of residues	M_r [1]	Properties or functions	Status[4]	Reference[5]
UL38	465	50260	Virion protein; required for capsid assembly; VP19C	e	Pertuiset *et al.*, 1989; Rixon *et al.*, 1990
UL39	1137	124043	Large subunit of ribonucleotide reductase	ne*	
UL40	340	38017	Small subunit of ribonucleotide reductase	e	
UL41	489	54914	Virion host shut-off protein	ne	Fenwick and Everett, 1990
UL42	488	51156	DNA replication; DBP (65K)	e	
UL43	434	44905	Multiply hydrophobic	ne	MacLean, C. *et al.*, 1991
UL44	511	54995	Virion glycoprotein C; possible role in virus adsorption to cells	ne	Herold et al., 1991
UL45	172	18178	Hydrophobic N terminus	ne	Visalli and Brandt, 1991
UL46	718	78239	Unknown; May modulate activity of UL48 protein	ne	Barker and Roizman, 1990
UL47	693	73812	Possible tegument protein	ne	Barker and Roizman, 1990; McLean *et al.*, 1990
UL48	490	54342	Major tegument protein; activator of IE genes	-	
UL49	301	32252	Virion protein; VP22	-	Elliott and Meredith, 1992
UL49.5 or UL49A	091		membrane inserted protein	e	Barker and Roizman, 1992; Barnett *et al.*, 1992
UL50	371	39125	Deoxyuridine triphosphatase	ne	
UL51	244	25468	Unknown	ne	Barker and Roizman, 1990
UL52	1058	114416	DNA replication; possibly responsible for the DNA primase activity	e	
UL53	338	37570	Multiply hydrophobic; syn locus; proposed virion glycoprotein K	-	Hutchinson *et al.*, 1992; Ramaswamy and Holland, 1992
UL54	512	55249	IE transcriptional regulatory protein (IE63; IE-2)	e	
UL55	186	20491	Unknown	ne	
UL56	197	21182	Unknown	ne	
IE175	1298	132835	IE transcriptional regulatory protein(IE175; IE-3)	e	

TABLE 1 (continued)

Gene	No. of residues	M_r [1]	Properties or functions	Status[4]	Reference[5]
US1	420	46521	IE protein (IE68; IE-4)	ne	
US2	291	32468	Unknown	ne*	
US3	481	52831	Protein kinase	ne	
US4	238	25236	Virion glycoprotein G	ne	
US5	092	09555	Putative glycoprotein	e	
US6	394	43344	Virion glycoprotein D	e	
US7	390	41366	Virion glycoprotein I	ne	
US8	550	59090	Virion glycoprotein E	ne	
US9	090	10026	Tegument phosphoprotein	ne	
US10	312	34053	Virion protein	ne	
US11	161	17756	Unknown; Localized in nucleolus	ne	
US12	088	09792	IE protein (IE12; IE-5)	ne	

Table 1. **Properties of HSV-1 encoded proteins**

1. M_r : Molecular weight for unprocessed polypeptide chain.
2. Apparent molecular weight of ICP34.5 protein in HSV-1 strain F.
3. Molecular weight of ICP34.5 protein in HSV-1 strain 17^+.
4. e, essential; ne, nonessential; * , necessity depends on culture conditions or temperature.
5. References not mentioned are cited in the original papers of McGeoch *et al.* (1988b) and McGeoch (1989).

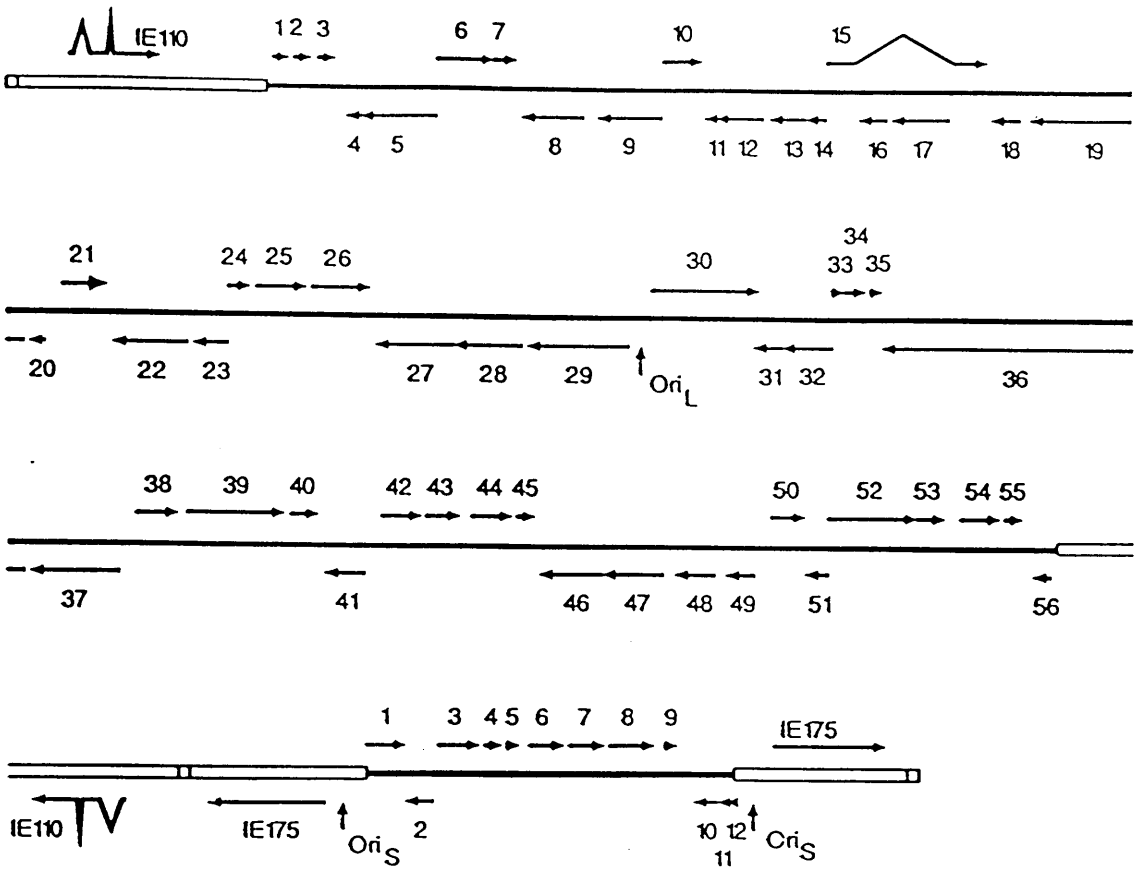

Figure 4. **Genome Organization Of The HSV-1 Genome**
(reproduced from McGeoch, 1989)

The genome of HSV-1 is shown on four successive lines (40 Kbp each) with unique regions represented by solid lines and major repeat sequences as open boxes. Locations of reading frames are shown by *arrows*, with splicing within coding regions indicated. In the topthree lines genes UL1 to UL56 are shown as 1 to 56, and in the bottom line genes US1 to US12 as 1 to 12. Locations of the three origins of DNA replication are indicated.

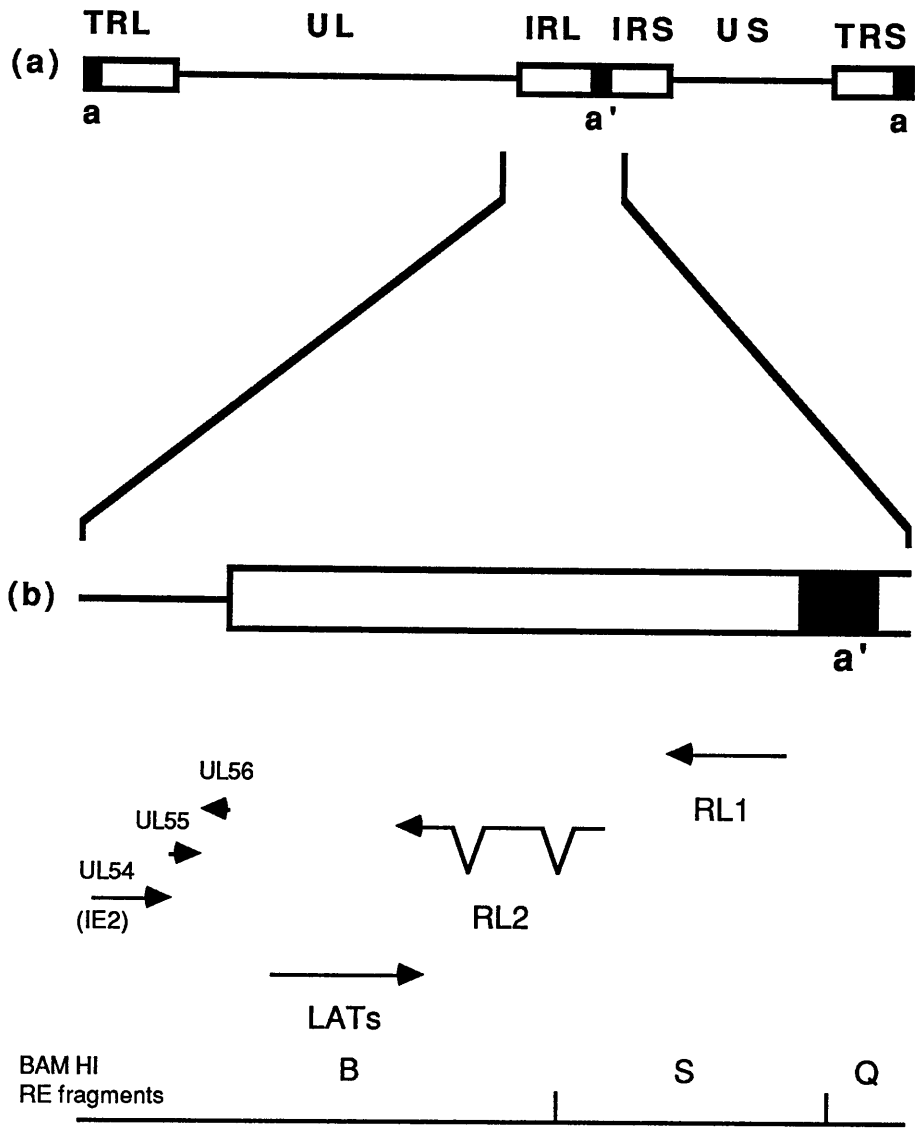

Figure 5. **Organization Of The HSV-1 Genes, RL1, RL2 And LATs**

(a) Structure of the HSV-1 genome showing U_L, U_S, TR_L, IR_L, IR_S, TR_S and the *"a"* sequences.

(b) Expanded region containing the RL1, RL2, LATs, the *"a"* sequence at the L-S junction and the terminal region of U_L. The location of HSV-1 genes UL56, UL55, UL54, RL1, RL2 and LATs are shown below with relative orientations indicated by arrowheads. The bottom line represents the *Bam HI* fragments spanning these genes.

and uncoating, transcription and translation, DNA replication, encapsidation of virus DNA, envelopment and release. The mechanistic details of the HSV lytic cycle are still poorly understood. However, the sequence of various events occuring during virus replication has been examined by time-course experiments (Nii *et al.*, 1968), inhibition of virus replication with antiviral agents (Friedman *et al.*, 1975) and by using *ts* mutants (Schaffer *et al.*, 1974; Dargan and Subak-Sharpe, 1983). During virus replication, three groups of genes are expressed in a coordinated and temporally regulated fashion : immediate-early (IE) or alpha, early (E) or beta and late (L) or gamma (Honess and Roizman, 1974; Clements *et al.*, 1977). The gene expression is followed by DNA replication, assembly of capsids and release of virus particles from the cell. The entire growth cycle from the virus adsorption onto the host cell surface to the release of new virus particles is completed within 16-20 hours (Wilkie, 1973).

The initial stages of infection, transcription and expression of genes and their products are described in this section (1C), whereas factors involved in HSV DNA replication along with assembly and release of new virus particles are discussed in the following section (1D).

1. VIRAL ADSORPTION

The adherence of virus particles to susceptible cells probably occurs through interaction between virus-envelope glycoproteins and cell-surface receptors (Vahlne *et al.*, 1978; Little *et al.*, 1981). The genome of HSV-1 encodes at least seven distinct glycoproteins, designated gB, gC, gD, gE, gG, gH and gI, found on the virus envelope and in virus-infected cell membranes (Spear *et al.*, 1970; Heine *et al.*, 1972; Dargan, 1986; Longnecker *et al.*, 1987; Roizman and Sears, 1990). Recently, two novel HSV-1 glycoproteins, termed gK and gL have also been reported and found to be involved in virus-induced membrane fusion (Hutchinson *et al.*, 1992; Ramaswamy and Holland, 1992). Three of these glycoproteins, gB, gD and gH, are essential for viral replication or infectivity since lethal effects of mutations have been observed in the genes encoding them (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Weller *et al.*, 1983a; McGeoch and Davison, 1986; Cai *et al.*, 1987, 1988; Ligas and Johnson, 1988; Desai *et al.*, 1988). Viruses with *ts* mutations in gB (Haffey and Spear, 1980; Little *et al.*, 1981) or gH (Desai *et al.*, 1988) and mutants lacking the entire gD (Ligas and Johnson, 1988) or gB (Cai *et al.*, 1988) have been shown to retain the ability to adsorb to the cell surface without initiating a productive infection. The remaining HSV-1

glycoproteins have been shown to be dispensable for viral replication in tissue culture (Heine *et al.*, 1974; Longnecker and Roizman, 1987). However, evidence is accumulating to present a better candidate for a viral glycoprotein essential for adsorption : as mutations in gC and/or gC-negative mutants have been shown to have a 10-fold reduction effect on virion adsorption (Campadelli-Fiume *et al.*, 1990; Herold *et al.*, 1991; Sommer and Courtney, 1991). A similar situation also appears to apply for pseudorabies virus (PRV), a herpesvirus of swine (Robbins *et al.*, 1986a, b; Mettenleiter *et al.*, 1990; Zsak *et al.*, 1991).

It is, however, possible that other factors, such as, cell-surface receptors, non-glycoprotein components or a combination of several glycoproteins could also play a facilitating role in the process of viral adsorption. For instance, heparan sulphate (a chemically and structurally similar compound to heparin, except that heparin is more highly sulphated; Hook, *et al.*, 1984; Lindahl and Kjellen, 1987), a ubiquitous glycosaminoglycan found on proteoglycans in cell plasma membranes, in extracellular matrices and in basement membranes has been reported to serve as a receptor for HSV adsorption (Nahmias and Kibrick, 1964; Miller, 1982; WuDunn and Spear, 1989; Herold *et al.*, 1991). Another factor, termed fibroblast growth factor (FGF), may also play a role in viral adsorption (Kaner *et al.*, 1990; Baird *et al.*, 1990), however, contrasting results have also been obtained (Herold *et al.*, 1991; Muggeridge *et al.*, 1992).

2. PENETRATION AND UNCOATING

As mentioned above, gB, gD and gH are essential for virus replication, although they are not required for virion adsorption. These glycoproteins, possibly all three, are involved in penetration. An HSV-1 *ts* mutant, *ts*B5, has shown defective penetration because of a defect in glycoprotein gB (Sarmiento and Spear, 1979; Haffey and Spear, 1980). Studies on *ts*B5 have also shown that the mutant does not form homodimers at NPT, which are normally formed by the *wt* HSV-1 gB, indicating that oligomerization is essential for infectivity (Claesson-Welsh and Spear, 1986; Chapsal and Pereira, 1988). Similarly, gB-negative mutants, gD-negative mutants and a *ts* mutant of gH, *ts* Q26 (Weller *et al.*, 1983; Desai *et al.*, 1988) have been shown to be defective in virus entry (Cai *et al.*, 1987; Ligas and Johnson, 1988). Neutralizing monoclonal antibodies (MAbs) have also been used to provide additional evidence for the participation of these proteins in penetration. Anti-gB, anti-gD and anti-gH MAbs have been shown to reduce

or block cell fusion (Noble *et al.*, 1983; Gompels and Minson, 1986; Minson *et al.*, 1986; Highlander *et al.*, 1988). In addition, virions neutralized with these MAbs could attach to cells but not penetrate (Fuller and Spear, 1987; Highlander *et al.*, 1987, 1988; Fuller *et al.*, 1989).

Following internalization, the viral capsids are transported to the nuclear pore, where, after uncoating the viral DNA is released into the nucleus (Knipe *et al.*, 1981; Batterson and Roizman, 1983). It seems likely that virion functions would be involved in these processes, as a *ts* mutant with mutation in the HSV-1 gene UL36 has been blocked in release of DNA at the NPT (Batterson *et al.*, 1983).

3. EFFECT OF HSV INFECTION ON HOST CELL MACROMOLECULAR SYNTHESIS

In cells infected with HSV-1 or HSV-2, the shutoff of host macromolecular synthesis occurs through a complex multistep process (Fenwick and Walker, 1978; Read and Frenkel, 1983; Roizman and Sears, 1990). The gene, termed virion host shutoff (*vhs*), encodes a structural protein and at early times post infection causes gross alterations in host functions and macromolecular synthesis by inducing degradation of cellular mRNAs (Sydiskis and Roizman, 1966, 1967; Nishioka and Silverstein, 1977, 1978; Fenwick and Walker, 1978; Fenwick *et al.*, 1979; Schek and Bachenheimer, 1985; Strom and Frenkel, 1987; Roizman and Sears, 1990). Studies with mutants defective in the *vhs* gene have revealed its location in HSV-1 UL41 (Read and Frenkel, 1983; Kwong *et al.*, 1988). The *vhs* gene seems to be more efficient in HSV-2 than its counterpart in HSV-1 (Powell and Courtney, 1975; Fenwick *et al.*, 1979), although HSV-2 strain HG52 has been found less efficient at host shutoff than HSV-1 strain 17^+ (Marsden *et al.*, 1978). More recently, HSV-2 strain HG52 has been found to encode a truncated *vhs* product, thus accounting for its inability to induce a strong shutoff function (Everett and Fenwick, 1990).

The *vhs* gene is dispensable *in vitro* (Fenwick and Everett, 1990). Moreover, *de novo* viral protein synthesis is not required for a functional gene (Nishioka and Silverstein, 1978; Schek and Bachenheimer, 1985; Strom and Frenkel, 1987). The activity of *vhs* is not restricted to cellular mRNAs, as the protein induces rapid turnover of viral RNAs of all kinetic classes (Oroskar and Read, 1987; Strom and Frenkel, 1987). Therefore, a defective *vhs* containing virus mutant has been shown to produce viral mRNAs with longer half-lives as compared to those produced by the *wt* virus (Kwong

and Frenkel, 1987; Strom and Frenkel, 1987). The mutation in *vhs* has also affected the ability of the virus to shutoff host protein synthesis and to degrade pre-existing cellular mRNAs.

Contrary to the inhibition of cellular DNA synthesis and mRNA degradation during HSV infection, certain cellular genes are synthesised, including stress and heat shock proteins (Notarianni and Preston, 1982; Patel *et al.*, 1986; Latchman and Kemp, 1987). A possibility of virion component involvement including the HSV-1 IE gene Vmw65 in the stimulation of certain cellular gene transcription has also been implicated (Kemp *et al.*, 1986).

4. HSV TRANSCRIPTION

Herpes simplex virus genomes are transcribed in the host cell nucleus (Wagner and Roizman, 1969) by pre-existing host RNA polymerase II (Alwine *et al.*, 1974; Ben-Zeev *et al.*, 1976; Costanzo *et al.*, 1977). The transcripts are capped at their 5'-termini, methylated at internal residues and polyadenylated at the 3'-termini (Bachenheimer and Roizman, 1972; Silverstein *et al.*, 1973, 1976; Bartoski and Roizman, 1976; Moss *et al.*, 1977). The polyadenylation signal, AATAAA and a consensus sequence, YGTGTTYY are important for correct processing and efficient termination of these transcripts, respectively (Proudfoot and Brownlee, 1976; McLauchlan and Clements, 1983b; McLauchlan *et al.*, 1985).

In contrast to the common finding of splicing events during eukaryotic mRNA processing including those of adenoviruses, papovaviruses and herpesviruses, such as, CMV and EBV, only a few HSV mRNAs are spliced (Frink *et al.*, 1981, 1983; Watson *et al.*, 1981; Costa *et al.*, 1985; Perry *et al.*, 1986; McGeoch *et al.*, 1988b). Splicing usually allows the generation of multiple individual mRNAs with common 5' and 3' termini, thereby providing economic use of template DNA. Three of the five HSV-1 IE mRNAs (1, 4 and 5) plus a U_L gene (UL15) are spliced (Watson *et al.*, 1981; Costa *et al.*, 1985; Perry *et al.*, 1986; McGeoch *et al.*, 1988b; Dolan *et al.*, 1991). HSV-1 IE-1 contains two introns within the coding sequence (Perry *et al.*, 1986) whereas IE-4 and IE-5 share a common intron within their 5' untranslated regions located within TR_S and IR_S (Watson *et al.*, 1981; Rixon and Clements, 1982). The UL15 gene contains a single intron bounded by two ORFs in separate exons (Costa *et al.*, 1985; McGeoch *et al.*, 1988b; Dolan *et al.*, 1991). The latency-associated transcripts (LATs) also appear to be spliced, although no genuine ORF has been reported to date [Wechsler *et al.*, 1988; Farrell *et al.*, 1991; McGeoch *et al.*, 1991; see Section 1A (3)]. HSV-1 gC has also been

reported to be spliced (Frink *et al.*, 1981, 1983) in infected cells but the coding region for gC (UL44) has not revealed any intron/exon within the sequence (McGeoch *et al.*, 1988b). The reason for the splicing events in HSV-1 does not seem to be related to gene compression since the introns do not lie within overlapping reading frames. As a matter of fact, US10 and US11 are the only known potential protein-coding ORFs with overlapping sequences in U_S (Rixon and McGeoch, 1984). Moreover, the arrangement of 11 of the 13 mRNAs in U_S is as four nested groups with unique 5' termini and common 3' co-termini (Hall *et al.*, 1982; McLauchlan and Clements, 1983; Rixon and McGeoch, 1985). However, these transcripts are unspliced with partial overlaps among their coding sequences. In addition, 11 proposed overlaps of coding sequences within U_L, located in two major clusters of genes, UL5 to UL14 and UL30 to UL33 have also been reported (McGeoch *et al.*, 1988b).

Transcription of HSV DNA takes place in a coordinately regulated manner (Honess and Roizman, 1974, 1975; Clements *et al.*, 1977). The expression of viral genes during lytic infection can be divided into three major temporal classes, the immediate-early (IE), early (E) and late (L) (reviewed by Wagner, 1985; Everett, 1987a). The first group of genes to be expressed are termed IE or alpha, whose protein products are involved in the efficient expression of E or beta genes. The IE and E proteins in turn induce and regulate the expression of L or gamma genes (Honess and Roizman, 1974, 1975; Clements *et al.*, 1977; Jones and Roizman, 1979; Preston, 1979a, b; Everett, 1984a, b, 1986, 1987a; McMahan and Schaffer, 1990). The L genes are further divided into early-late (beta-gamma or gamma-1) and 'true' lates (gamma-2). The true-lates are distinguished from the early-late by the fact that they are hardly detectable during the absence of viral DNA replication (Jones and Roizman, 1979; Hall *et al.*, 1982; Godowski and Knipe, 1985). The mechanisms which control the expression of these temporal shifts are still not fully understood. However, several virus-encoded proteins have been demonstrated to play important roles in the regulatory processes mediating the shifts from IE to E and E to L phases. The temporal regulation of differential viral gene expression is discussed in more detail in the following sections.

5. IMMEDIATE-EARLY GENES

As mentioned above, IE genes are transcribed by the cellular RNA polymerase II at the onset of viral infection. IE gene transcription can be differentiated from those of E and L genes by two important features. First,

their expression is stimulated by a virion polypeptide component, Vmw65 or alpha-TIF (alpha-*trans*-inducing factor; VP16; ICP25; UL48) in association with an IE-specific regulatory sequence (ATGCAAAT and TAATGARAT consensus elements) recognized by a host transcription factor, Oct-1 or alpha H1 (NFIII; OTF-1; OBP100; TRF) (Post *et al.*, 1981; Batterson and Roizman, 1983; Campbell *et al.*, 1984; Pruijin *et al.*, 1986; Kristie and Roizman, 1987; McKnight *et al.*, 1987a, b; Sturm *et al.*, 1987; Baumruker *et al.*, 1988; Grester and Roeder, 1988; O'Hare and Goding, 1988; Preston *et al.*, 1988; Kristie *et al.*, 1989). Second, IE transcription does not require *de novo* viral or host protein synthesis (Kozak and Roizman, 1974; Honess and Roizman, 1974; Clements *et al.*, 1977). There are five HSV-1 IE mRNAs, produced in small quantities during the normal lytic infection of permissive cells. However, large amounts can be produced by the addition of protein inhibitors, such as, cycloheximide or anisomycin, from the time of virus infection (Clements *et al.*, 1977, 1979; Preston, 1979a; Easton and Clements, 1980). Normally, IE gene expression peaks around 2-3 hours post adsorption but IE mRNAs can still be detected at late times (Harris-Hamilton and Bachenheimer, 1985; Godowski and Knipe, 1986). However, when protein synthesis is blocked, E and L gene transcription does not occur, as IE proteins are required for the shift from IE to E and E to L mRNA synthesis (Preston, 1979a). Under these 'immediate-early' conditions, IE mRNA transcripts were collected, separated by gel electrophoresis and ^{32}P-labelled to map to the HSV-1 genome (Clements *et al.*, 1979; Watson *et al.*, 1979; see Figures 4, 5 and 6). The 5 IE genes are termed as IE1 (alpha 0), IE2 (alpha 27), IE3 (alpha 4), IE4 (alpha 22) and IE5 (alpha 47) and their polypeptide products as Vmw110 (ICP0), Vmw 63 (ICP27), Vmw175 (ICP4), Vmw 68 (ICP22) and Vmw 12 (ICP47) respectively (see for reviews, Wagner, 1985; Everett, 1987a). The IE mRNAs 1 and 3 are contained entirely within the long and short repeat regions of HSV genome and are therefore diploid (Watson *et al.*, 1979; Anderson *et al.*, 1980; Rixon *et al.*, 1982). The 5' termini of IE4 (US1) and IE5 (US12) are located within TR_S/IR_S and thus share a common promoter and 5'untranslated leader sequence but have different coding sequences which are located in the unique component (S) of the genome (Watson *et al.*, 1981; Rixon and Clements, 1982; Figure 6). The IE2 gene (UL54) is located in U_L and is the only IE gene not associated with the repeat region of the viral genome. Of the 5 IE genes, IE1, 4 and 5 are spliced (see above, Section 1C : 4).

Various *cis*-acting elements and *trans*-activating factors seem to play major roles in IE transcription and subsequent gene regulation. A *cis*-

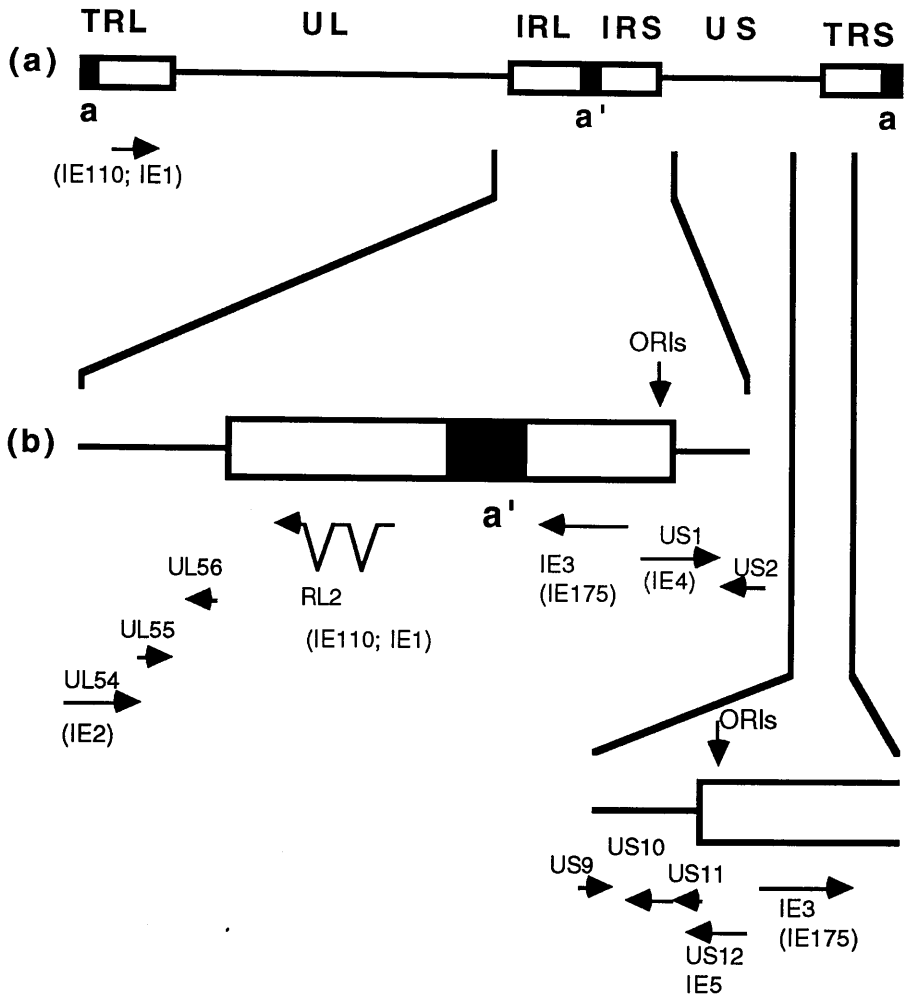

Figure 6. **Organization of the HSV-1 IE GENES**

(a) Structure of the HSV-1 genome showing U_L, U_S, TR_L, IR_L, IR_S, TR_S, IE-1 (IE110) and the "a" sequences.

(b) Expanded repeat and unique regions showing the locations of the HSV-1 genes UL54 (IE2), UL55, UL56, RL2 (IE1), IE3 (IE175), US1 (IE4), US2, US9, US10, US11 and US12 (IE5). The IE1 and IE3 are present in two copies. The arrowheads indicate the direction of the genes.

acting consensus sequence 5'-TAATGARAT-3' (where R is a purine residue) is present in one or more copies, in either orientation, in the upstream regulatory regions of all HSV-1 and HSV-2 IE genes and is essential for the *trans*-inducing activity of Vmw65 (Mackem and Roizman, 1982a; Cordingley *et al.*, 1983; Preston *et al.*, 1984; Campbell *et al.*, 1984; Bzik and Preston, 1986; O'Hare and Hayward, 1987). Deletion/insertion mutagenesis allowed the identification of two distinct domains in the IE genes : a promoter region and a regulatory domain (Mackem and Roizman, 1982a, b, c; Cordingley *et al.*, 1983; Preston *et al.*, 1984). The promoter region has been located upstream from the mRNA transcription initiation site and allows expression, but not regulation, of downstream coding sequences (Mackem and Roizman, 1982a, c; Cordingley *et al.*, 1983). A TATA box has also been located 20-30 bp upstream of the mRNA start site (Benoist *et al.*, 1980). The regulatory region is further divided into two domains : a *cis*-acting element and an enhancer-like sequence. The latter allows high levels of transcriptional activity (Post *et al.*, 1981; Mackem and Roizman, 1982a, c; Cordingley *et al.*, 1983; Lang *et al.*, 1984). The enhancer-like sequences and the *cis*-acting element play additive roles to obtain maximum transcriptional activity (Bzik and Preston, 1986).

The *trans*-inducing factor (Vmw65) is encoded by UL48 gene and is a phosphorylated protein (Campbell *et al.*, 1984; Dalrymple *et al.*, 1985; Pellet *et al.*, 1985). The polypeptide has an apparent molecular weight of 65,000 and has been shown to be a major virion structural component required for virus assembly (Marsden *et al.*, 1976; Hall *et al.*, 1982; Ace *et al.*, 1988). Mutational analysis has revealed two important regions within the DNA sequence of UL48 gene. The amino-terminal 411 amino acids are required for binding activity of Vmw65 to cellular factors (Greaves and O'Hare, 1989; McKee *et al.*, 1990) and the carboxy-terminal domain containing amino acids 411 to 490 is required for *trans*-inducing activity (Sadowski *et al.*, 1988; Triezenberg *et al.*, 1988; Cousens *et al.*, 1989).

Although Vmw65 is not a DNA binding protein (Marsden *et al.*, 1987), it interacts with cellular factor/s (Oct-1 or OTF1) to form a multiple protein-DNA complex, designated as the immediate-early complex (IEC), at or around the TAATGARAT element (Preston *et al.*, 1988; O'Hare and Goding, 1988; Gerster and Roeder, 1988). The IE complex appears to mediate IE gene expression (O'Hare and Goding, 1988; Ace *et al.*, 1988).

The regulatory region of IE genes also contain Sp1 binding sites (GC-rich sequences) and G+A-rich elements which may modulate transcriptional activity or may act as enhancer sequences in the formation of

the IE complex (Briggs *et al.*, 1986; O'Hare and Hayward, 1987;). Further studies are required to understand the exact mechanism of action and various factors associated with the formation of the IE complex.

6. IMMEDIATE-EARLY POLYPEPTIDES

Despite the availability of extensive literature on immediate-early polypeptides, their mechanisms of action and detailed genetic control systems operating during a productive infection are still not fully understood. Two of the IE polypeptides, Vmw63 and Vmw175 have been shown to be essential for virus replication, as *ts* or lethal mutations in their respective genes have resulted in a failure to transcribe E and L genes (Marsden *et al.*, 1976; Preston, 1979a; Watson and Clements, 1978, 1980; Dixon and Schaffer, 1980; Everett, 1984, 1986, 1987a; Sacks *et al.*, 1985; McCarthy *et al.*, 1989). The remaining three IE genes (1, 4 and 5) have been shown to be dispensable in tissue culture (Post and Roizman, 1981; Harland and Brown, 1985; Sears *et al.*, 1985; Umene, 1986; Longnecker and Roizman, 1986; Stow and Stow, 1986; Brown and Harland, 1987; Sacks and Schaffer, 1987), although IE1 (Vmw110) has been shown to activate gene expression in transfection assays and have a role in efficient viral growth in tissue culture (Everett, 1984, 1989; Stow and Stow, 1986; Cai and Schaffer, 1989). With the exception of the IE5 product, Vmw12, all IE proteins are phosphorylated and found within the host cell nucleus (Marsden *et al.*, 1978, 1982; Preston, 1979a; Ackermann *et al.*, 1984). The characteristics and functions of these proteins are hereby described briefly.

(a) Vmw110 (ICP0)

The RL2 gene product, Vmw110 has been shown to activate the expression of all kinetic classes of HSV-1 genes (IE, E and L) and several cellular genes in transient expression assays (Everett, 1984, 1986, 1987b; O'Hare and Hayward, 1985a, b; Gelman and Silverstein, 1985, 1986; Quinlan and Knipe, 1985; Mavromara-Nazos *et al.*, 1986; Mosca *et al.*, 1987; Sekulovich *et al.*, 1988; Cai and Schaffer, 1989). Although no *ts* mutants in the RL2 gene have so far been isolated, HSV-1 and HSV-2 variants with deletions in either one copy (Poffenberger *et al.*, 1983; Harland and Brown, 1985) or both copies (Stow and Stow, 1986; Sacks and Schaffer, 1987) of the gene have been isolated, suggesting that the gene is non-essential for viral replication *in vitro*. The deletion variant, *dl* 1403 (Stow and Stow, 1986) has shown impaired growth at low moi, with a 50-100-fold reduction in growth yield compared to *wt* virus. However, the variant has grown normally at high

moi and expressed normal quantities of viral proteins. Therefore, the defect in the deletion variant at low moi was due to a reduced ability to initiate a productive infection and was overcome by increasing the number of infectious virus particles (Stow and Stow, 1986, 1989). A possible conclusion therefore is, that Vmw110 ensures sufficient gene expression for a productive infection at low moi, which Vmw175 alone is unable to achieve and needs a synergistic activation for the expression of E and L genes (Stow and Stow, 1986, 1989; Everett, 1986, 1987a, 1989). On the other hand, a recent study using Vmw110-negative mutants indicate that a cellular function can substitute for the Vmw110 polypeptide to enhance viral gene expression and DNA replication, suggesting a facilitating role of Vmw110 in virus replication in cells not expressing the substitute function (Cai and Schaffer, 1991).

Experiments have demonstrated that Vmw110 is a potent transcriptional activator and that it can activate the transcription of some herpesvirus genes in a synergistic manner when present together with Vmw175 (Everett, 1984, 1986; O'Hare and Hayward, 1985; Gelman and Silverstein, 1985). However, the degree of synergy may vary with cell type, promoter DNA sequence and method of transfection (Everett, 1988b). *In vitro* mutagenesis of the RL2 gene has been carried out to define functional domains responsible for transactivation. Two functional domains have been defined : one contains a *'potential zinc finger'* and is located in the second exon of the gene and the other is found near the carboxy terminus (Everett, 1989). The latter is more important in synergistic activation with Vmw175. The region containing the *potential zinc finger* was found crucial for *trans*-activation function of Vmw110 during virus replication in the absence of Vmw175 (Everett, 1989) and for reactivation of latent virus in an *in vitro* system (Harris *et al.*, 1989).

The presence of two introns in the coding region of the RL2 gene is unusual as there are very few introns in the HSV-1 genome (Perry *et al.*, 1986; McGeoch *et al.*, 1988b). Because of the presence of RL1 (ICP34.5) and LATs in the same region of the genome, it was thought that RL2 coding sequences and its introns may play a role in the transcription of these other genes. However, HSV-1 variants lacking IE1 introns have shown normal gene expression during infection (Everett, 1991) and no change was found in the pathogenicity of the virus or latency potential (Natarajan *et al.*, 1991).

(b) **Vmw63 (ICP27)**

This is an essential regulatory IE polypeptide for viral replication in tissue culture as *ts* mutations in the UL54 gene have resulted in

overproduction of Vmw110 and Vmw175 at the NPT (Sacks *et al.*, 1985). Furthermore, no effect was found in the expression of E gene or DNA replication. However, the L gene expression was severely affected, indicating a regulatory role of Vmw63 in late gene expression (Rice and Knipe, 1988). Similar results were obtained with variants containing deletions in UL54 coding sequences (McCarthy *et al.*, 1989) except that a variant replicated a reduced amount of viral DNA under nonpermissive conditions. In transient expression assays, Vmw63 has been shown to both activate and repress various HSV promoters in the presence of Vmw110 and Vmw175 (Everett, 1986; Rice and Knipe, 1988, 1990; Sekulovich *et al.*, 1988). However, under certain conditions Vmw63 alone can transactivate the gB (a late gene) promoter (Rice and Knipe, 1988). More recently, it has also been reported that the expression of gB can be induced to *wt* levels in infected cells in the absence of Vmw63 (McCarthy *et al.*, 1989; Rice and Knipe, 1990). Furthermore, genetically separable regions within the UL54 gene for the stimulation of gamma-1 genes, expression of gamma-2 genes, repression of IE and E gene expression and stimulation of viral DNA replication have recently been suggested (McCarthy *et al.*, 1989; Hardwicke *et al.*, 1989; Su and Knipe, 1989; Rice and Knipe, 1990). Further studies are required to confirm and determine the exact locations of such regions.

(c) **Vmw175 (ICP4)**

Being an essential polypeptide and a critical *trans*-activator of most HSV genes, Vmw175 is the most extensively studied protein among the immediate-early's. This is a 175-KDa (as estimated by SDS-PAGE) phosphoprotein, found in at least three different molecular weight forms within infected cells (Courtney and Benyesh-Melnick, 1974; Pereira *et al.*, 1977; Wilcox *et al.*, 1980; Preston and Notarianni, 1983) which may reflect different levels of phosphorylation on serine and threonine residues (Faber and Wilcox, 1986a). However, sequence determination has revealed that the protein is 1,298 amino acid residues long with a mass of 132.835 KDa (McGeoch *et al.*, 1986, 1988b). As mentioned in section 5 (above), IE gene 3 is diploid, being located entirely in the repeat region of the HSV-1 genome (McGeoch *et al.*, 1986, 1988b), therefore, only one copy of IE3 is sufficient for gene expression and subsequent DNA replication in tissue culture (Longnecker and Roizman, 1986; Brown and Harland, 1987). Vmw175 accumulates in specific regions of the nucleus of infected cells (Pereira *et al.*, 1977; Cabral *et al.*, 1980; Knipe *et al.*, 1987). Its association with the tegument region of purified virions (Yao and Courtney, 1989) as well as the inner surface of the plasma membrane of virus

infected cells (Yao and Courtney, 1991) has also been demonstrated. The existence of the IE175 protein product as a highly elongated homodimeric complex has also been reported (Metzler and Wilcox, 1985; Faber and Wilcox, 1986b).

Both genetic analysis and biochemical assays have been carried out to investigate the role of Vmw175 in the expression and regulation of all kinetic classes of HSV genes including autoregulation and down-regulation (repression of gene expression). HSV-1 mutants containing lesions or defects within IE gene 3 have particularly been useful in these studies. For example, a temperature-sensitive mutant, *tsK*, overproduces the IE gene products and fail to activate early or late gene expression at the NPT (Preston, 1979a). Similarly, various *ts* and deletion/insertion mutants of IE gene 3 have shown the inability to express early and late genes and to autoregulate IE gene expression (Watson and Clements, 1978, 1980; Dixon and Schaffer, 1980; DeLuca *et al.*, 1984; DeLuca and Schaffer, 1988; Paterson and Everett, 1988a). The fully intact protein is therefore required for the expression of E and L genes and viral DNA replication. These analyses have further demonstrated that Vmw175 is required continuously for gene expression and regulation (Watson and Clements, 1978, 1980; Preston, 1979a; Dixon and Schaffer, 1980). Further evidence of Vmw175 functions have been obtained by using transient expression assays where a cloned IE3 gene was cotransfected with the gene for chloramphenicol acetyltransferase (CAT) linked with gD or tK promoters and the effects were monitored (Everett, 1984a, b, 1986; O'Hare and Hayward, 1985a, b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1985, 1986). Vmw175 can repress its own promoter (O'Hare and Hayward, 1985b) as well as other IE promoters under certain conditions in transfection assays (O'Hare and Hayward, 1985b; DeLuca and Schaffer, 1985; Gelman and Silverstein, 1986, 1987a, b). Further studies have recognised the binding site essential for repression activity by Vmw175 at the cap site of the IE3 promoter (Muller, 1987; DeLuca and Schaffer, 1988; Paterson and Everett, 1988a, b; Roberts *et al.*, 1988; DiDonato and Muller, 1989). However, the other IE genes except IE1 have no Vmw175 binding site in their cap site regions. The IE1 Vmw175 binding site is similar to that of the IE3 site, but on the upstream side from the TATA box (Kristie and Roizman, 1986) and has been implicated to be involved in Vmw110 repression in transfection assays (Gelman and Silverstein, 1987a, b; Resnick *et al.*, 1989). However, contrary results have recently been obtained (Everett and Orr, 1991) during a normal infection in tissue culture, questioning the reliability of results

obtained in transfection assays. Further studies are required to understand the processes involved in repression of other IE genes by Vmw175 during the course of a normal infection.

The use of *in vitro* deletion/insertion mutations of Vmw175 in DNA-binding assays has revealed important sequences specific for Vmw175 binding to DNA. Vmw175 has been shown to interact specifically and directly with DNA sequences containing the consensus sequence ATCGTC found in or near HSV gene promoters including the transcription start site of the IE3 gene (Faber and Wilcox, 1986; Beard *et al.*, 1986; Muller, 1987; Michael *et al.*, 1988; Tedder *et al.*, 1989; Kattar-Cooley and Wilcox, 1989; Imbalzano *et al.*, 1990). It appears that both proximal and distal parts of the consensus DNA binding site are crucial for specific binding by Vmw175 and that the entire sequence of the binding sites may not be essential (Pizer *et al.*, 1991; Everett *et al.*, 1991). However, the exact role of these DNA binding domains in Vmw175 functions remains unclear, although it was found that during *in vitro* Vmw175 activation for transcription, these sequences have also contributed in the process of *trans*-activation (Tedder *et al.*, 1989). Mutational analysis further revealed that a highly conserved region, termed region 2, among the corresponding proteins of related alphaherpesviruses is crucial for both *trans*-activation and autoregulation by Vmw175 (Paterson and Everett, 1988a, b, 1990; Shepard *et al.*, 1989; Paterson *et al.*, 1990; Wu and Wilcox, 1990, 1991) and that a relatively small proteolytic fragment of Vmw175 is sufficient for DNA binding (Paterson, 1989; Everett *et al.*, 1990). Moreover, region 4 (there is a conceptual division of Vmw175 polypeptide into five regions) of the related proteins of alphaherpesviruses, such as, HSV-1, VZV, EHV-1 and PRV, also share extensive homology and seems to be involved in autoregulation by Vmw175 (McGeoch *et al.*, 1986; Cheung, 1989; Grundy *et al.*, 1989; Vleek *et al.*, 1989; Paterson *et al.*, 1990). Finally, it appears that Vmw175 acts like a *trans*-acting enhancer in gene expression, as some DNA binding sites have been found dispensable (Everett *et al.*, 1991; Smiley *et al.*, 1992). Recently, it has been shown that the DNA binding domain of Vmw175 forms dimers, although parts involved in the dimerisation process are, at present, unknown (Everett *et al.*, 1991).

(d) **Vmw68 (ICP22)**

To date, no *ts* mutants have been isolated in IE gene 4. However, a deletion mutant lacking the carboxy terminal third of Vmw68 has been constructed (Post and Roizman, 1981; Sears *et al.*, 1985) and shown to grow poorly in tissue culture (dependent on the cell line used) with reduced

expression of at least one L gene and was not neurovirulent in mice (Sears *et al.*, 1985).

(e) **Vmw12 (ICP47)**

This is the only IE gene that is not phosphorylated and found predominantly in the cytoplasm of the infected cells (Preston, 1979b; Marsden *et al.*, 1982). No *ts* mutants of IE gene 5 have so far been isolated and isolation of viable mutants lacking the entire gene have indicated that Vmw12 is not essential for growth in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

7. EARLY GENES

Early mRNAs are produced prior to the onset of virus DNA replication and their production is dependent on the presence of some IE gene products (Wagner, 1972; Clements *et al.*, 1977). E genes map across the entire length of the viral genome (Clements *et al.*, 1977; Wagner, 1985) and a number of their products are involved in priming the infected cell for DNA replication (Wagner, 1985). The expression of early genes begins at about 3 hours post adsorption and peaks around 4-6 hours post infection (Honess and Roizman, 1974). E genes can be divided into two subclasses, beta-1 and beta-2, on the basis of the kinetics of their expression. For instance, the large sub-unit of ribonucleotide reductase (RR_1) can be detected very early in infection and has been classified as a beta-1 gene product, although it has also been detected in the presence of cycloheximide and in *ts* Vmw175 mutants that do not normally express E proteins at the NPT (Preston, 1979a; Roizman and Batterson, 1985; DeLuca *et al.*, 1985; Wymer *et al.*, 1989). On the other hand, E genes including gB and gD, can be detected at early times but are not fully expressed until after the onset of DNA replication (Gibson and Spear, 1983; Johnson and Everett, 1986a). These DNA replication-dependent genes have been described as beta-2 or early-late (EL), 'leaky-late' (beta-gamma) and gamma-1 (Roizman and Batterson, 1985; Wagner, 1985; Harris-Hamilton and Bachenheimer, 1985).

In order to identify sequences necessary for the *trans*-activation of E gene promoters by viral gene products, selective promoter sequences were either deleted and linked to indicator genes (McKnight *et al.*, 1981; Jones *et al.*, 1985) or replaced by synthetic linker DNAs (McKnight and Kingsbury, 1982). These mutants were then analysed under conditions where no IE products were available and promoter elements were detected by the cellular transcription machinery. As a result, functional elements such as a

TATA box, a CAAT box, upstream GA-rich region for gD, GC-rich (Sp1 binding site) region for TK were detected (McKnight and Kingsbury, 1982; Everett, 1983, 1984, a, b; Eisenberg *et al.*, 1985; El Kareh *et al.*, 1985; Coen *et al.*, 1986). However, the TATA box seems to be the most important element among these promoters, as indicated by deletion/insertion studies using gD or TK gene (Everett, 1984b). Moreover, foreign genes (for example, a rabbit ß-globin gene) inserted into the HSV genome were shown to be regulated in a similar manner as that of a viral gene (Everett, 1983, 1984b; Smiley *et al.*, 1987). This implies that E gene promoter activity is not exclusively dependent on virus-specific sequences. In addition, IE polypeptides may not entirely depend on E gene promoter sequences for their transactivation (Coen *et al.*, 1986; Everett, 1987b, 1988b) but do so by interacting with cellular transcription factors.

8. LATE GENES

Late gene transcripts have been subdivided into two classes : the early-late (leaky-late or gamma-1) and true-late (gamma-2). Gamma-1 transcripts can be detected prior to viral DNA replication whereas gamma-2 transcripts are barely detectable (< 1%) before the onset of DNA synthesis (Holland *et al.*, 1979, 1980; Roizman and Batterson, 1985; Wagner, 1985; Johnson, 1987). Both types of late gene expression are abundantly found after the onset of viral DNA replication (Clements *et al.*, 1977; Holland *et al.*, 1980; Conley *et al.*, 1981) and their expression depends on the presence of IE and E gene products (Watson and Clements, 1980). The accumulation of late gene products peak around 10-16 hours post adsorption (Munk and Sauer, 1964; Roizman, 1969; Wilkie, 1973).

UL19 (major capsid protein; MCP; Vmw155; VP5) and UL27 (gB) represent gamma-1 genes whereas US11 (21K/22K proteins), UL44 (gC) and UL47 (81K/82K proteins) are examples of true-late or gamma-2 genes (Hall *et al.*, 1982; Rixon and McGeoch, 1984; McGeoch *et al.*, 1985, 1986, 1988b; Wagner, 1985; Roizman and Batterson, 1985; Johnson *et al.*, 1986; MacLean, C. A. *et al.*, 1987; Everett, 1987b; McLean *et al.*, 1990).

In contrast to E gene promoters, which use upstream sequences from the TATA box for full expression, late gene expression requires the TATA box (Johnson and Everett, 1986b) and the sequences downstream from it (Mavromara-Nazos and Roizman, 1989). In transient transfection assays, a cloned gD promoter lacking upstream sequences from the TATA box was linked with an origin of HSV-1 DNA replication and the E promoter of gD was

observed to be regulated as a true late gene (Johnson and Everett, 1986b; Johnson *et al.*, 1986). Similar results were obtained with the true late US11 promoter (Johnson and Everett, 1986a, b; Johnson *et al.*, 1986), as well as gC promoter (Homa *et al.*, 1986) suggesting that IE, E and L gene expression and regulation requires distinct upstream promoter sequences.

The mechanisms of L gene expression and regulation are still unclear. Studies with mutant viruses containing lesions in Vmw63 and Vmw68 (Sacks *et al.*, 1985; Sears *et al.*, 1985) have indicated the importance of these polypeptides in late gene expression. However, dependence of L gene expression on viral DNA replication is confusing. If it is due to the increase of promoter copy number, then E gene expression should also depend on DNA replication. It is, therefore, possible that the replicating DNA template contains a switch for L gene expression, or the replication machinery itself is required *in situ* to initiate L gene transcription.

SECTION 1D : HSV DNA REPLICATION

HSV DNA replication requires temporal regulation of gene expression (see Section 1C). IE genes activate E gene expression, which are involved in viral DNA synthesis and are abundantly expressed in the absence of DNA replication (Swanstrom *et al.*, 1975). Following the onset of DNA replication, L genes are expressed in large quantities (Swanstrom and Wagner, 1974; Powell *et al.*, 1975), many of which are structural components of the virion (Spear and Roizman, 1972; Heine *et al.*, 1974; see Section 1D : 4).

Upon infection, linear HSV DNA molecules enter the cell nucleus and are thought to be circularised (Jean *et al.*, 1977; Jacob and Roizman, 1977), possibly because of direct ligation of the termini to form a joint fragment in which the two terminal '*a*' sequences share a DR_1 element (Jean and Ben-Porat, 1976; Davison and Wilkie, 1983; Poffenberger and Roizman, 1985). The ligation of the terminal ends is carried out by either a host cell enzyme or a virion component via the complementary 3'-single base extensions on the L and S termini of the viral genome (Mocarski and Roizman, 1982a, b; Davison and Rixon, 1985) as *de novo* protein synthesis is not required for the fusion of the termini (Ben-Porat and Veach, 1980; Poffenberger and Roizman, 1985). Moreover, despite the absence of a terminal redundancy, VZV is also assumed to carry out the process of ligation (Davison, 1984, 1991a).

The amount of the input DNA, which enters the replicative cycle varies from strain to strain as well as cell type infected, both *in vitro* and *in vivo*. The reported amount of HSV-1 parental DNA found involved in replication varies from less than 5% to greater than 50% (Jacob and Roizman, 1977; Khan *et al.*, 1978) while in PRV-infected cells, most of the input DNA takes part in replication (Ben-Porat *et al.*, 1976). Electron microscopic studies have shown that a significant proportion of 'loops and eyes' are visible in the DNA molecules at multiple but separated loci (Frenkel and Roizman, 1972; Friedman *et al.*, 1977; Ben-Porat and Rixon, 1979).

The initiation of DNA replication takes place between 1-3 hours post infection, the replication rate increases during the following 7-9 hours and the first round of replication is completed by 16 hours post infection (Munk and Sauer, 1964; Wilkie, 1973; Rixon *et al.*, 1983). Circular molecules of HSV and PRV DNA have been observed at different times post infection and immediately after the completion of the first round of replication by electron microscopy and restriction endonuclease analysis (Jacob *et al.*, 1979; Jongeneel and Bachenheimer, 1980, 1981; Rixon *et al.*, 1983). Numerous shapes of viral DNA have been identified, which may represent 'replicative intermediates' leading to head-to-tail concatemers, generated as a result of a proposed rolling-circle mechanism of DNA replication (Jean and Ben-Porat, 1976; Ben-Porat *et al.*, 1976; Hirsch *et al.*, 1976; Jacob and Roizman, 1977; Jean *et al.*, 1977; Jacob *et al.*, 1979; Ben-Porat and Rixon, 1979; Stow, 1982; Rabkin and Hanlon, 1990). Moreover, HSV DNA synthesised at late times (5-9 hours post infection) has been described as a 'large tangled mass' (Ben-Porat and Rixon, 1979), which may have generated from homologous recombination between nascent DNA circles. Homologous recombination in HSV takes place at a high rate and appears to be dependent on DNA replication (Brown *et al.*, 1992; this thesis; see also Section 1F). Therefore, the latter mechanism of viral replication cannot be ruled out (Schaffer *et al.*, 1974; Smiley *et al.*, 1980; Weber *et al.*, 1988).

Whether circularisation or template amplification occurs before DNA synthesis for packaging into progeny virus remains unclear. However, it is thought that extensive concatemers occur late in infection (Jacob *et al.*, 1979) and that inversion of the L and S components may take place by site-specific recombination via the '*a* ' sequences (Mocarski *et al.*, 1980; Mocarski and Roizman, 1981; Smiley *et al.*, 1981) leading to the formation of 4 equimolar isomers (Sheldrick and Berthelot, 1974; Wadsworth *et al.*, 1975; Preston *et al.*, 1978; Jenkins and Roizman, 1986). Insertion of '*a* ' sequence-

containing fragments into the thymidine kinase (TK) locus caused additional inversion events (Mocarski and Roizman, 1981; Smiley *et al.*, 1981). Recombination studies (Davison and Wilkie, 1983) and deletion analysis (Mocarski and Roizman, 1982a; Poffenberger *et al.*, 1983; Chou and Roizman, 1985) have provided further evidence that inversion requires a DNA fragment flanked by the '*a* ' sequences and that even the removal of DR_1 elements does not prevent an inversion event to take place, albeit at low frequency (Varmuza and Smiley, 1985). However, two of the four isomers can be generated without an inversion event via the cleavage sites in the concatemeric or circular DNA molecules. Inversion can also occur through inverted copies of other sequences present in an HSV genome (Jenkins *et al.*, 1985; Varmuza and Smiley, 1985; Weber *et al.*, 1987; Harland and Brown, 1989). Moreover, various recombination studies have demonstrated that the genomes of both HSV types 1 and 2 are highly recombinogenic (see for example, Honess *et al.*, 1980; Umene, 1985; Brown *et al.*, 1992; this thesis; see also Section 1F) and that apart from the processes of inversion and isomerization, various other factors, such as, particular viral genes, hot spots of recombination and host factors, either alone or in conjunction with each other may also play important roles during the DNA replication process leading to recombination (the subject of this thesis). Therefore, the involvement of the '*a* ' sequence in a specific inversion mechanism remains open to speculation.

HSV DNA fragments upon denaturation with alkali (Kieff *et al.*, 1971; Wilkie, 1973). This fragmentation may be explained by the presence of short stretches of ribonucleotides on HSV DNA (Biswsal *et al.*, 1974). However, the sedimentation profile in neutral sucrose gradients was found similar for DNA denatured either by formamide or alkali, suggesting that the ribonucleotides could not be entirely responsible for fragmentation of the DNA (Spear and Roizman, 1980). Furthermore, a large number of nicks and gaps have been observed to be randomly distributed on both strands of the viral DNA (Frenkel and Roizman, 1972; Ecker and Hyman, 1981; Wilkie, 1973) which may account for such fragmentation. However, a real functional significance for the presence of these nicks and gaps on the HSV genome remains to be discovered.

1. ORIGINS OF DNA REPLICATION

Early electron microscope studies have revealed the presence of specific regions within the HSV genome, which allow initiation of DNA replication. The position of replicating DNA molecules containing loops and

eyes, termed replicative intermediates, suggested three-origin of DNA replication sites contained within the HSV genome. One of the sites was located at or near the end of the molecules and the remaining at about 10 and 20nm from one end of the DNA molecule (Friedman *et al.*, 1977). Similarly, two origin-specific DNA replication sites were located in the genome of the related alphaherpesvirus, PRV (Jean *et al.*, 1977; Ben-Porat and Veach, 1980). However, definitive mapping of such sites was accomplished by studying the replication of defective interfering genomes (Frenkel, 1975; Frenkel *et al.*, 1975, 1976; Kaerner *et al.*, 1979, 1981) and cloned virus DNA in the presence of non-defective helper genomes (Stow, 1982; Mocarski and Roizman, 1982; Murchie and McGeoch, 1982; Spaete and Frenkel, 1982; Stow and McMonagle, 1983; Weller *et al.*, 1985).

Defective particles exist within an HSV stock. Their genomes are incomplete and require helper functions from non-defective genomes to replicate efficiently. The genetic information required for efficient replication, as well as cleavage and packaging of viral DNA into nascent nucleocapsids, seems to be contained within the *cis*-acting sequences. The defective particles must therefore contain these sequences to be able to maintain themselves in a virus stock. Indeed, the characterisation of defective genomes by restriction endonucleases and Southern blot analysis revealed the presence of reiterated sequences from the short (S) component of the viral genome (Wagner *et al.*, 1974; Frenkel *et al.*, 1976, 1980; Graham *et al.*, 1978; Kaerner *et al.*, 1979, 1981; Denniston *et al.*, 1981). Defective molecules were divided into two classes : class I molecules contained DNA sequences from the short component of the genome plus the '*a* ' sequence (Frenkel *et al.*, 1980; Vlazny and Frenkel, 1981) whereas class II molecules, in addition of containing the '*a* ' sequence, also contained sequences from the middle of the U_L (Schroder *et al.*, 1975/1976; Kaerner *et al.*, 1979, 1981; Frenkel *et al.*, 1980). This has provided firm evidence for the presence of at least two origins of DNA replication in the genome of HSV, one in each component.

The identification of the precise location of *cis*-acting origin regions of replication involved plasmid-based assays. In these assays, cloned viral DNA fragments containing the sequence of interest were transfected into cultured cells to allow *cis*-acting elements to amplify non-HSV sequences in the presence of *trans*-acting helper functions provided by a subsequent infection of cells with non-defective HSV-1 genomes. Thereafter, utilization of Southern blot techniques identified a region within the inverted repeats of the S component. This region was located within a 955 bp fragment in R_S and

termed the short region origin of replication, ori_S (Stow, 1982). Because of its presence in the inverted repeats, ori_S is diploid (see Figure 4). Similarly, origin-specific sequences in the L component of the genome have been identified, located near the middle of the U_L, and termed ori_L (Frenkel *et al.*, 1980; Locker *et al.*, 1982). Thus, the total number of origin regions in the HSV-1 genome is three.

(a) **The ori_S replication origin**

Using a transient replication assay, Stow and McMonagle (1983) identified a 90 bp segment essential for ori_S activity within the 995 bp (see above) fragment from the R_S region. The ori_S specific sequences are sandwiched between the promoter domain of the Vmw175 gene (IE3) and the identical promoters of the IE4 and IE5 genes (Stow, 1982; Stow and McMonagle, 1983). The 90 bp segment contains a 45 bp near-perfect palindromic DNA sequence with a central AT-rich stretch. Insertion and/or deletion of residues from within the palindrome abolishes the ori_S activity, suggesting an essential function for DNA replication (Stow, 1985).

HSV-2 strain HG52 contains four copies of ori_S because of a tandem duplication of a 137 bp element present in both repeats (TR_S, IR_S) (Whitton and Clements, 1984a). HSV-2 ori_S is highly homologous to the corresponding HSV-1 ori_S and includes a 75 bp core origin sequence containing a palindrome (Whitton and Clements, 1984a; Lockshon and Galloway, 1988). The core origin regions of HSV-1 and HSV-2 are almost identical (70 out of 75 residues are the same) and deletion and/or replacement of the central AT residues results in a dramatic loss in DNA replication (Lockshon and Galloway, 1988). The related alphaherpesvirus, VZV, also contains counterpart of HSV-1 ori_S, although an equivalent of ori_L has not been detected in its DNA sequence (Davison and Scott, 1986a; Stow and Davison, 1986; McGeoch *et al.*, 1988b). Stow and Davison (1986) have demonstrated that VZV ori_S can be activated by HSV-1 encoded functions and suggested an 11-bp sequence (CGTTCGCACTT) might play a critical role in origin activity. The 11-bp sequence was found conserved in origin regions of both viruses, although their location with respect to the palindrome differed slightly. The A+T-rich stretch within the palindrome is longer in VZV ori_S (32 residues as compared to 18 in the corresponding part of HSV-1 ori_S) which may facilitate easier unwinding of the DNA duplex at the origin (Stow, 1985; Stow and Davison, 1986; Stow *et al.*, 1990).

Initial studies using gel retardation assays and DNase footprinting have identified the origin-binding protein (OBP) that could

sequence specifically bind to two sites within HSV-1 ori_S (Elias *et al.*, 1986; Elias and Lehman, 1988). Olivo *et al.* (1988) have subsequently recognized the OBP as the protein product of the UL9 gene, an essential viral gene for DNA replication (Wu *et al.*, 1988; McGeoch *et al.*, 1988a). More recent studies using methylation interference (see for example, Koff and Tegtmeyer, 1988) and site-directed mutagenesis (Deb and Deb, 1989, 1991; Weir *et al.*, 1989; Weir and Stow, 1990; Elias *et al.*, 1990; Hernandez *et al.*, 1991; Martin *et al.*, 1991) further demonstrated that the UL9 recognition site is included within the sequence 5'-YGYTCGCACT-3' (where Y represents C or T). Interestingly, one of the two sites is contained within the 11-bp conserved sequence described earlier by Stow and Davison (1986; see above). The two sites are present in opposite orientations and overlap the ends of the DNA palindrome. A third site, located at the left hand end of site one, has also been identified (Weir and Stow, 1990; Hernandez *et al.*, 1991; Martin *et al.*, 1991). All the three origin-binding sites, designated as I, II and III, are required for optimal DNA replication, although conflicting results have been obtained. Mutational analysis within site I have indicated that this site is absolutely essential for origin activity (Lockshon and Galloway, 1988; Deb and Deb, 1989; Weir and Stow, 1990; Stow *et al.*, 1990). However, mutations within site II affects replication efficiency only moderately (Lockshon and Galloway, 1988; Weir and Stow, 1990), suggesting a possibility of its role in the initiation of DNA replication. In contrast, Deb and Doelberg (1988) have reported no affect, whatsoever, on the replication efficiency of the mutant devoid of the complete site II. Moreover, site III has also been shown to be essential for efficient replication and may modulate the binding activity at ori_S (Weir and Stow, 1990; Hernandez *et al.*, 1991; Martin *et al.*, 1991). In addition, a conserved 9-bp region, 5'-CGTTCGCAC-3', has been reported among the HSV-1 ori_S and ori_L, HSV-2 ori_S and ori_L, VZV ori_S and EHV ori (Martin *et al.*, 1991). The conservation corresponds to the critical sequences required for binding activity at site I. Figure 7 represents the 90 bp region of HSV-1 ori_S containing a 45 bp palindrome including the three OBP-binding domains.

The significance for the presence of two copies of ori_S remains obscure. Deletion mutants with only one copy of ori_S have been shown to be viable in tissue culture (Longnecker and Roizman, 1986; Brown and Harland, 1987), suggesting that the second copy is not required at least *in vitro*. However, isolation of deletion variants lacking both copies of ori_S remains unsuccessful (Longnecker and Roizman, 1986; Smith *et al.*, 1989), thus one copy of ori_S seems to be absolutely essential for viral replication (see above).

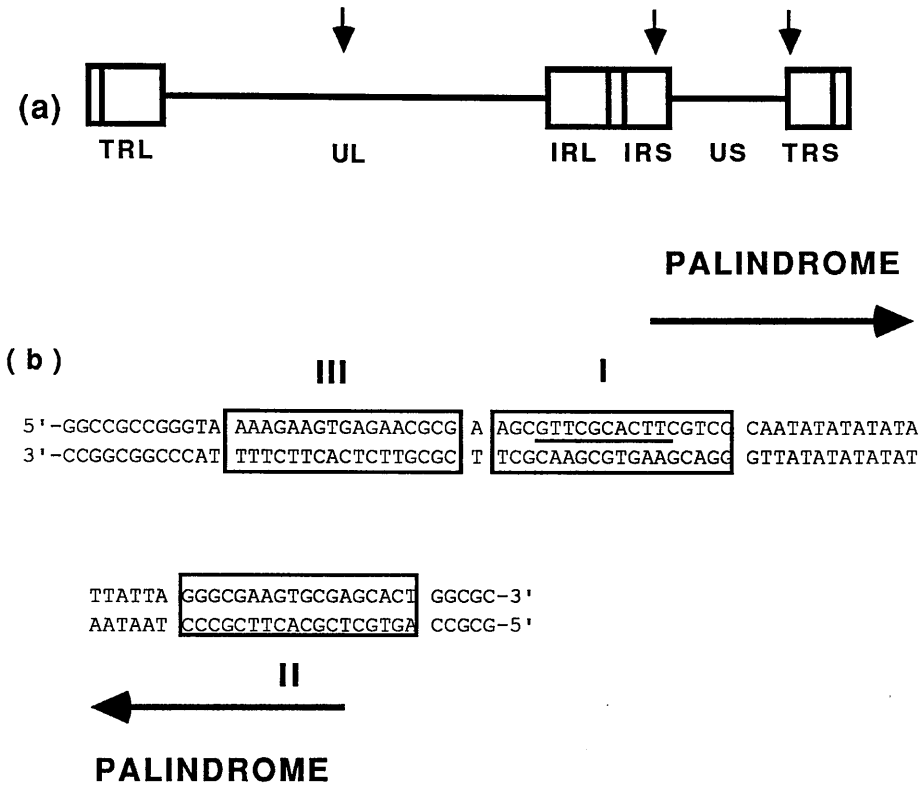

Figure 7. **The 90-bp HSV-1 Ori$_S$ DNA Sequence**

(a) The HSV-1 genome structure showing the locations of the origins of DNA replication.

(b) The 90-bp DNA sequence described for ori$_S$ activity by Stow and McMonagle (1983). The 45-bp palindrome is indicated by arrows and the conserved 11 bp sequence of HSV-1 and VZV origin regions is underlined (Stow and Davison, 1986). The three OBP binding domains are boxed (Elias and Lehman, 1988; Weir and Stow, 1990; Martin *et al.*, 1991).

The regulation of DNA synthesis and transcription of origin regions of replication may involve functional transcripts, although no RNA promoter region has been located within sequences which affect ori_S activity. Two transcripts have been reported to be encoded by the region containing ori_S (Hubenthal-Voss *et al.*, 1987; Hubenthal-Voss and Roizman, 1988). One transcript, termed ori_SRNA2, starts downstream from the transcription initiation sites of IE4/5 genes, extends through the entire ori_S and coterminates at the 3' terminal of the IE3 gene. The second transcript, ori_SRNA1, is 5' coterminal with ori_SRNA2 but the 3' terminal is located at or near the RNA initiation site of IE5 gene. In addition, the second transcript (ori_SRNA1) is detected early in infection whereas ori_SRNA2 is synthesized late in infection (Hubenthal-Voss *et al.*, 1987; Hubenthal-Voss and Roizman, 1988). Recently, the formation of a sequence-specific RNA-protein complex 1 (RPC-1) between a cellular protein and the region containing the transcription initiation sites of ori_SRNAs has also been reported (Roller *et al.*, 1989). However, any role of these transcripts, as well as the formation of such complexes, in regulation of HSV-1 DNA replication has not, as yet, been determined.

(b) **The ori_L replication origin**

As mentioned above, early work with defective genomes has identified the presence of an origin of replication near the centre of U_L, termed ori_L. However, attempts to localize ori_L within a functional specific sequence have failed because of the occurence of spontaneous deletions of about 100-650 bp while cloning the ori_L region into standard bacterial vectors (Spaete and Frenkel, 1982). The first ori_L sequence of HSV-1 strain Angelotti was obtained from a class II defective genome (Gray and Kaerner, 1984) and contained 296 base pairs. Subsequently, *wt* HSV-1 strains KOS and 17 syn^+ ori_L sequences have been determined using non-cloned viral DNA fragments as sequencing substrates (Weller *et al.*, 1985; Quinn and McGeoch, 1985). In addition, successful cloning of a 425 bp undeleted region spanning ori_L sequences has also been reported (Weller *et al.*, 1985). Using a plasmid amplification assay (Stow and McMonagle, 1983), the ori_L-specific sequences were then demonstrated to contain origin activity (Weller *et al.*, 1985).

The long region origin of replication contains a 144 bp perfect palindrome, at the centre of which is located an A+T rich region. The palindromic regions of ori_L and ori_S are highly homologous (85% identical residues) and the homology extends approximately 40 bp to the left of the ori_S palindrome (McGeoch, 1987). The homologous sequences also contain the higher affinity site (site I; see above) essential for OBP-binding (Elias *et al.*,

1986; Elias and Lehman, 1988). Because of the presence of two 72 bp palindromic arms containing the conserved site I sequences, it is likely that ori_L activity is bidirectional as compared to the unidirectional activity of ori_S (Weller *et al.*, 1985; McGeoch, 1987). However, the minimal ori_L signal required for origin activity has not been determined, as yet.

The presence of ori_L in HSV-2 has also been demonstrated and sequence analysis revealed a 136 bp perfect palindrome showing 88% homology to HSV-1 ori_L (Lockshon and Galloway, 1986). In contrast, no ori_L could be located in VZV (Davison and Scott, 1986a; Stow and Davison, 1986).

HSV-1 ori_L is sandwiched between two divergently transcribed genes, UL29 encoding the major DNA-binding protein (MDBP) and UL30 encoding the DNA polymerase (*pol*), both of which are essential components of the DNA replication machinery (Quinn and McGeoch, 1985; McGeoch *et al.*, 1988a, b). A functional ori_L is not essential for virus replication *in vitro*, as viable ori_L-specific-sequence deletion variants have been isolated (Polvino-Bondar *et al.*, 1987). However, whether ori_L plays a role in *in vivo* DNA synthesis or if there is a preferential use of ori_S, remains to be discovered.

2. GENES REQUIRED FOR DNA REPLICATION

In order to identify viral genes required during the replication of HSV DNA, three approaches have been utilized. These include the characterisation of *ts* mutants defective at the NPT (see for example, Preston, 1979; Dixon and Schaffer, 1980), biochemical assays (e.g., Hay *et al.*, 1971; Hay and Subak-Sharpe, 1976; Preston *et al.*, 1984; Purves *et al.*, 1987) and transient replication assays (Challberg, 1986; Wu *et al.*, 1988; McGeoch *et al.*, 1988a; see later).

Mutant viruses containing *ts* lesions have been isolated, characterised and arranged into a series of complementation/functional groups. Their phenotypic and genotypic properties at the NPT have been examined and genetic maps have been constructed on the basis of recombinational analysis of the progeny virus populations (see for example, Brown *et al.*, 1973; Schaffer *et al.*, 1973, 1974, 1976; Timbury and Subak-Sharpe, 1973; Brown and Ritchie, 1975; Schaffer, 1975; Subak-Sharpe and Timbury, 1977; Preston *et al.*, 1978; Chartrand *et al.*, 1979; Wilkie *et al.*, 1979; Halliburton, 1980; Dixon and Schaffer, 1980; Schaffer *et al.*, 1987). The *ts* mutants have particularly been useful in mapping genes encoding IE polypeptides (see Section 1C). However, complementation groups of *ts*

mutants have also been used in the identification of genes directly required for DNA synthesis (see Later).

Using biochemical assays, several enzymes and proteins with possible roles in DNA replication have been identified. For instance, the major DNA binding protein (MDBP) (Bayliss *et al.*, 1975; Purifoy and Powell, 1976; Powell *et al.*, 1981), the DNA polymerase (*pol*) (Keir and Gold, 1963; Hay and Subak-Sharpe, 1976; Honess *et al.*, 1984), an origin-specific DNA binding protein (Elias *et al.*, 1986), helicase-primase (Crute *et al.*, 1989), ribonucleotide reductase (RR) (Cohen, 1972; Dutia, 1983; Preston *et al.*, 1984), an alkaline exonuclease (Morrison and Keir, 1968; Hay *et al.*, 1971a, b; Moss, 1986), thymidine kinase (TK) (Kit and Dubbs, 1963), uracil DNA glycosylase (Caradonna and Cheng, 1981), protein kinase (Blue and Stobbs, 1981; McGeoch *et al.*, 1985; Purves *et al.*, 1987), and deoxyuridine triphosphate nucleotidohydrolase (DUTPase) (Caradonna and Cheng, 1981; Wohlrab *et al.*, 1982) activities have so far been identified. Studies with the DNA negative *ts* mutants have also indicated the importance of some of these proteins, for example, DNA polymerase (Purifoy and Powell, 1981), MDBP (Lee and Knipe, 1983), RR (Preston *et al.*, 1984) and the exonuclease (Moss, 1986).

A set of 7 viral genes involved directly in origin-dependent DNA replication have finally been identified by the use of a transient complementation assay, originally developed by Dr M D Challberg (1986) (Wu *et al.*, 1988). In this approach, cloned viral DNA fragments containing an HSV origin of replication are cotransfected into cultured cells with specific HSV DNA sequences necessary for DNA replication in *trans*. The plasmid amplification is carried out by either ori_S or ori_L in *cis*. Subsequent subcloning of DNA fragments, deletion analysis and inactivation by restriction endonuclease digestion, and in comparison with data from large-scale sequence analysis of the HSV-1 genome (McGeoch *et al.*, 1988a, b), seven genes which are necessary and sufficient to support an HSV origin-dependent DNA replication were identified and characterised (Challberg, 1986; Wu *et al.*, 1988). The genes essential for DNA replication are : UL5, UL8, UL9, UL29, UL30, UL42 and UL52. The identification of these genes correlates well with previous genetic studies and mapping data of *ts* mutants (Purifoy *et al.*, 1977; Dixon and Schaffer, 1980; Chartrand *et al.*, 1980; Purifoy and Powell, 1981; Conley *et al.*, 1981; Weller *et al.*, 1983a, b; Littler *et al.*, 1983; Coen *et al.*, 1984; Weller *et al.*, 1987; Zhu and Weller, 1988; Marchetti *et al.*, 1988). Two of the seven genes (UL29 and UL30 encoding MDBP and *pol* respectively)

were already known to be essential for DNA replication (see above for references) and a third [UL42 encoding a 65K double-stranded DNA binding protein (65KD DBP)] (Marsden *et al.*, 1987) was also known with unknown function. In addition, IE genes 1, 2 and 3 (see Section 1C), and UL39 and UL40 (encoding RR1 and RR2 respectively) were also shown to be required for stimulation of plasmid amplification, although indirectly (Wu *et al.*, 1988). However, it is possible that one or more unidentified viral or host cell genes play important role in viral DNA replication *per se*, as a relationship between origin-dependent plasmid DNA replication and viral replication in their natural environment is unknown. Figure 8 and Table 2 summarise the location and predicted molecular weights with known functions of the 7 essential genes respectively.

More recent approaches to identify and characterize the essential and/or dispensable HSV-1 genes involved the insertion of an *E coli Lac Z* reporter gene encoding ß-galactosidase into specific DNA sequences (see for example, Mullaney *et al.*, 1989; MacLean, C. A. *et al.*, 1991; Johnson *et al.*, 1991). Because of the presence of the *Lac Z*, viral recombinants can easily be detected by their blue plaque morphology in the presence of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal), a chromogenic substrate for ß-galactosidase (Chakrabarti *et al.*, 1985). The second approach involved the expression of HSV genes in recombinant baculoviruses (Smith *et al.*, 1983; Miller, 1988) and subsequently, the functions of various essential genes were investigated (Calder and Stow, 1990; see Later).

(a) **UL29 (MDBP)**

The major DNA binding protein (MDBP; ICP8) is an E gene product encoded by UL29. This protein has been shown to bind preferentially to single-stranded DNA (ssDNA) (Bayliss *et al.*, 1975; Conley *et al.*, 1981; Knipe *et al.*, 1982). The observation that deletions/insertions within the coding DNA sequence for MDBP abolish the viral DNA synthesis has indicated that the protein is absolutely essential for HSV DNA replication (Weller *et al.*, 1983b; Littler *et al.*, 1983). MDBP has also been shown to have a role in transcriptional regulation since *ts* mutants in MDBP overexpress certain L genes at the NPT. Moreover, a functional MDBP is also involved in the repression of IE3 and UL19 (the major capsid protein; MCP; Vmw155) genes (Godowski and Knipe, 1983, 1985, 1986). The interaction of MDBP with viral DNA polymerase to form a DNA synthetic complex has also been reported (Vaughan *et al.*, 1984; Ruyechan and Weir, 1984; Chiou *et al.*, 1985).

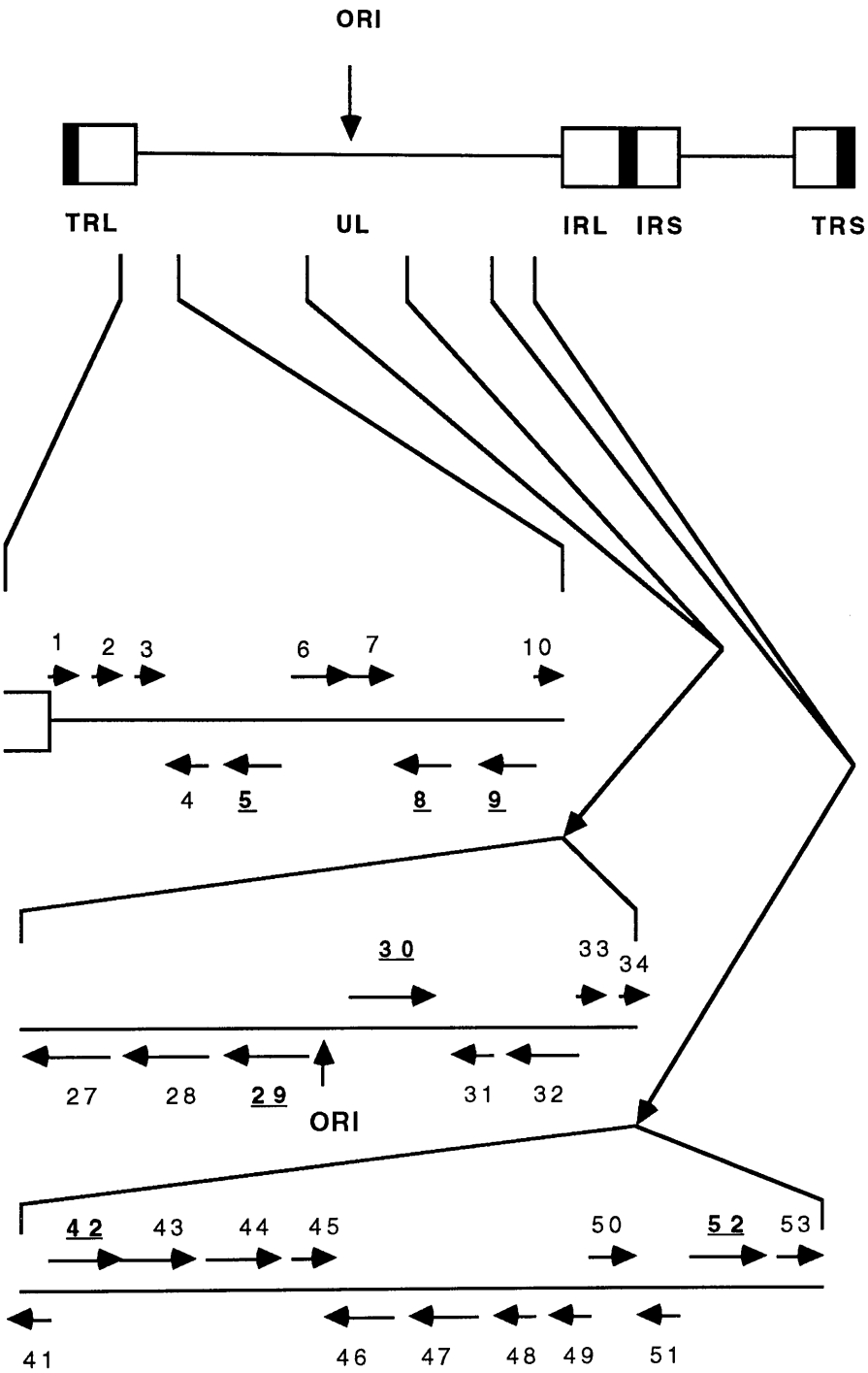

Figure 8. **The Genes Essential For HSV-1 DNA Replication**

Structure of the genome of herpes simplex virus type 1 with expanded regions showing the seven (7) genes essential for DNA replication (bold face and underlined). The genes surrounding UL5, UL8, UL9, UL29, UL30, UL42 and UL52 are also shown with an indication of their relative orientations (arrowheads). See McGeoch *et al.* (1988b), Wu *et al.* (1988) and relevant text for references.

TABLE 2

HSV-1 GENE[1]	PROTEIN (M_r)[2]	FUNCTION[3]
UL5	98,710	Possibly responsible for the DNA helicase activity
UL8	79,921	Not known
UL9	94,246	OBP; binds to Ori_S sequences
UL29	128,342	MDBP; binds preferentially to ssDNA
UL30	136,413	Replicative DNA polymerase
UL42	51,156	$65K_{DBP}$; binds to dsDNA
UL52	114,416	Possibly responsible for the DNA primase activity

Table 2. **HSV-1 genes required for DNA replication**

1. The HSV-1 genes identified using transient complementation assays of Challberg (1986) and Wu *et al.* (1988).
2. M_r : predicted molecular weight; as determined by McGeoch *et al.* (1988b).
3. See relevant text for references.

MDBP is believed to bind tightly to ssDNA at the replication fork, thus stabilising the parental DNA strands for use as templates by the DNA polymerase (Ruyechan, 1983; O'Donnell *et al.*, 1987; Challberg and Kelly, 1989). The use of *ts* and amino acid substitution mutants has indicated the presence of distinct functional DNA binding domains within the polypeptide (Lee and Knipe, 1983; Leinbach *et al.*, 1984; Quinlan *et al.*, 1984; Gao *et al.*, 1988; Gao and Knipe, 1989). It appears that the carboxy terminal region (Leinbach and Heath, 1988) and the amino acid residues 346-450 (Gao *et al.*, 1988) are important for the binding activity and may be used to form intermolecular contacts with the ssDNA.

(b) **UL30 (DNA *pol*)**

The HSV-induced DNA polymerase was first detected by Keir and Gold (1963) and was found to be biochemically distinct from host DNA polymerases (Keir *et al.*, 1966; Weissbach *et al.*, 1973; Powell and Purifoy, 1977). HSV studies using *ts* and drug-resistant mutants have demonstrated that the polymerase is virally encoded and essential for viral DNA synthesis (Hay and Subak-Sharpe, 1976; Purifoy *et al.*, 1977; Dixon and Schaffer, 1980; Chartrand *et al.*, 1979, 1980; Coen *et al.*, 1984). The purified protein product has an apparent molecular weight of 140,000 (Powell and Purifoy, 1977; Knopf, 1979) which is similar to the predicted molecular weight of 136,000 estimated by sequence determination of the UL30 gene (Gibbs *et al.*, 1985; Quinn and McGeoch, 1985). The sequence determination has revealed several homologous regions among the DNA *pol* genes of various herpesviruses (Baer *et al.*, 1984; Gibbs *et al.*, 1985; Quinn and McGeoch, 1985; Davison and Scott, 1986; Kouzarides *et al.*, 1987; Teo *et al.*, 1991) and partial homologies were also found with apparently unrelated polymerases, such as, those of adenoviruses, vaccinia virus and the bacteriophage Ø29 (Gibbs *et al.*, 1985; Quinn and McGeoch, 1985; Davison and Scott, 1986; Argos *et al.*, 1986; Earl *et al.*, 1986; Kouzarides *et al.*, 1987). Moreover, herpesvirus polymerases also posses a 3'-5' exonuclease activity, which may increase fidelity of replication through a proof reading function (Knopf, 1979; Abbotts *et al.*, 1987; O'Donnell *et al.*, 1987). Recently, it has been shown that the *pol* also contains a 5'-3' ribonuclease H activity, which specifically degrades RNA/DNA heteroduplexes or duplex DNA substrates (Crute and Lehman, 1989; Marcy *et al.*, 1990).

The 140KD polymerase product of HSV-2 has been shown to co-purify with a 55KD polypeptide (Vaughan *et al.*, 1984) which is known to be encoded by the UL42 gene. It appears now that the enzyme interacts with the

UL42 gene product to form a heterodimer containing subunits from UL30 and UL42 genes (Gallo *et al.*, 1988; Parris *et al.*, 1988).

(c) **UL42 (65K Double-stranded DNA-binding protein**

The UL42 gene product, $65K_{DBP}$, is a major protein species of HSV-1 and is essential for DNA replication *in trans* (Marsden *et al.*, 1987; Parris *et al.*, 1988; Wu *et al.*, 1988). Gallo *et al.* (1988) have demonstrated that the protein is physically associated with the *pol* product, although it is not required for the catalytic activity of *pol*. The protein binds to double-stranded DNA (dsDNA) in a non-sequence specific manner (Bayliss *et al.*, 1975; Gallo *et al.*, 1988).

Sedimentation analysis through sucrose density gradients indicates that the *pol*-$65K_{DBP}$ complex is composed of a 1:1 ratio of UL42 and UL30 gene products (Crute and Lehman, 1989) and is a heterodimer (Gottlieb *et al.*, 1990). Furthermore, the $65K_{DBP}$ acts as a *pol* accessory protein (Gallo *et al.*, 1989), although DNA polymerase has been shown to have an intrinsic exonuclease activity and apparently does not require any stimulation for its *pol* activity (Dorsky and Crumpacker, 1988; Haffey *et al.*, 1988; Gallo *et al.*, 1989). Recently, a UL42 gene-negative mutant has been constructed (Johnson *et al.*, 1991), which may provide an opportunity to explore the functions of $65K_{DBP}$ during virus DNA replication. UL42 also has a processivity function for DNA *pol* (UL30) (Gottlieb *et al.*, 1990).

(d) **UL9**

UL9 encodes an origin-binding protein (Elias *et al.*, 1986) and has been shown to bind with specific sequences contained within the ori_S regions (see Section 1D : 1a). The UL9 gene possess purine NTP-binding sites, as suggested by its sequence determination (McGeoch *et al.*, 1988a, b) and may have a role in the formation of a multiprotein replication complex (Challberg and Kelly, 1989). However, the exact role of the UL9 gene product in DNA replication remains obscure.

(e) **UL5, UL8 and UL52**

The three remaining DNA replication proteins, encoded by genes UL5, UL8 and UL52 have recently been shown to form a complex in HSV-1 infected cells which exhibits DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase activities (Crute *et al.*, 1988, 1989; Dodson *et al.*, 1989; Calder and Stow, 1990; Crute and Lehman, 1991). Using baculovirus expression system, it was demonstrated that the helicase-primase is a heterotrimer consisting of the products of UL5, UL8 and UL52 genes in a 1:1:1 ratio (Crute and Lehman, 1991). Moreover, the primase activity of the helicase-primase synthesizes oligonucleotides consisting of 10-

12 residues with M13 ssDNA as template whereas the helicase component of the enzyme catalyzes the unwinding of nicked circular plasmid DNA. Therefore, the HSV-1 helicase-primase possibly functions at the viral replication fork. However, no enzymatic activity has so far been associated individually with any of the three gene products. Recently, Calder and Stow (1990) have shown that the UL8 protein is not required for DNA-dependent ATPase or DNA-helicase activity, as UL5 and UL52 products were found sufficient for the production of these activities. In addition, the UL8 product may play a role in efficient entry of the complex into the cell nucleus. More recent work has demonstrated that UL5/UL52 subassembly has all the activities of the complex and that the UL8 protein does not associate stably with either the UL5 or UL52 protein (Dodson and Lehman, 1991), suggesting a separate role for the UL8 gene product. Dodson and Lehman (1991) further suggested that the UL5 protein may be the helicase, and the UL52 protein the primase, but only their association activates the holoenzyme (helicase-primase complex). Further work is required to resolve the individual functions of these proteins.

3. HSV ENZYMES

Herpes simplex virus encodes several enzymes which may have roles in nucleic acid metabolism, thus influencing viral DNA replication indirectly. Apart from the 7 essential genes required for DNA replication and IE genes, the known virally encoded enzymes are : alkaline exonuclease (Keir and Gold, 1963), dUTPase (Wohlrab and Francke, 1980), protease (Pr) (Liu and Roizman, 1991a, b), protein kinase (McGeoch and Davison, 1986), ribonucleotide reductase (Cohen, 1972), thymidine kinase (Kit and Dubbs, 1963) and uracil DNA glycosylase (Caradonna and Cheng, 1981). Many of these enzymes are E proteins and have enzymic activities, which are described briefly in the following sections.

(a) Alkaline exonuclease (UL12; ICP18)

Keir and Gold (1963) have described the activity of a novel enzyme, alkaline nuclease, in cells infected with HSV-1 or HSV-2. The purified enzyme exhibits exo- and endonuclease activities and differs from host cell nucleases in its high pH optimum (Morrison and Keir, 1968; Francke *et al.*, 1978; Hoffman and Cheng, 1978, 1979; Hoffman, 1981). The gene encoding alkaline nuclease was mapped between 0.168 and 0.175 map coordinates (m.c.) on the genomes of HSV-1 and HSV-2 (Preston and Cordingly, 1982; Costa *et al.*, 1983; Banks *et al.*, 1985) and shown to have an

apparent molecular weight of 85,000 (Banks *et al.*, 1983, 1985). Sequence determination has revealed an ORF encoding 626 amino acids (UL12) within a 2.3 Kb mRNA, previously shown to encode the alkaline nuclease (Costa *et al.*, 1983; Draper *et al.*, 1986; McGeoch *et al.*, 1986b, 1988b). Conflicting results have been obtained regarding the role of the alkaline nuclease for viral growth and DNA replication. Early studies with an HSV-2 *ts* mutant have shown that the gene is essential for DNA synthesis and production of infectious virus particles (Francke *et al.*, 1978; Moss *et al.*, 1979; Francke and Garrett, 1982; Moss, 1986). However, the isolation of an HSV-1 *Lac Z* insertion mutant within the UL12 gene has demonstrated that the gene is dispensable in tissue culture (Weller *et al.*, 1990). In addition, the accumulation of a large number of empty capsids (as revealed by electron microscopy) in cells infected with the insertion mutant and a 10^3-fold less yield of infectious virus particles as compared to the *wt* virus suggests that the viral alkaline nuclease is not required for DNA synthesis but may have a role in efficient production of infectious virus particles via packaging of viral DNA (Weller *et al.*, 1990).

(b) **dUTPase (UL50)**

Both HSV-1 and HSV-2 induce the enzyme, dUTPase, which catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate (Wohlrab and Francke, 1980; Preston and Fisher, 1984). The gene encoding the HSV-1-induced dUTPase has been mapped within a genome region (0.69-0.70 m.c.) corresponding to the UL50 gene (Preston and Fisher, 1984; McGeoch *et al.*, 1988b). The gene has been demonstrated to be dispensable for growth in tissue culture (Fisher and Preston, 1986).

(c) **Protease (Pr; UL26)**

A novel HSV-1 protease which catalyzes its own cleavage and that of its substrate (encoded by the UL26.5 gene) has recently been identified (Liu and Roizman, 1991a. b; Preston *et al.*, 1992). The protease is approximately 80,000 in molecular weight and encoded by the UL26 gene. The UL26.5 ORF has been located entirely within the UL26 ORF (Liu and Roizman, 1991a).

(d) **Protein kinases (UL13 and US3)**

HSV induces a protein kinase activity after infection of cells (Blue and Stobbs, 1981). Comparison of eukaryotic protein kinase sequences with the HSV-1 DNA sequence has identified the US3 gene as a putative protein kinase (McGeoch and Davison, 1986). Biochemical and genetic studies have confirmed that the protein kinase is indeed encoded by the US3 gene (Frame

et al., 1987; Purves *et al.*, 1987). Recently, the UL34 gene product has been suggested to act as a substrate of the HSV-1 protein kinase (Purves *et al.*, 1991).

Another HSV-1-induced protein kinase has recently been reported to be encoded by the UL13 gene (Smith and Smith, 1989). Both US3 and UL13 are non-essential for growth in tissue culture (Longnecker and Roizman, 1987; L Coulter, personal communication).

(e) **Ribonucleotide reductase (RR; UL39 and UL40)**

Ribonucleotide reductase (RR) catalyzes the reduction of all four ribonucleoside diphosphates to their corresponding deoxy-ribonucleoside diphosphates and as such plays an essential role in DNA replication in prokaryotic and eukaryotic cells. HSV-1 and HSV-2 induce their own RR activities (Cohen, 1972; Cohen *et al.*, 1974) with distinct properties from their cellular counterpart (Averett *et al.*, 1983, 1984; Frame *et al.*, 1985). The HSV-1 RR is composed of two subunits, RR1 and RR2, with molecular weights of 136,000 and 38,000 respectively (Dutia, 1983; McLauchlan and Clements, 1983a; Preston *et al.*, 1984; Frame *et al.*, 1985; Bacchetti *et al.*, 1986). The RR1 is encoded by the UL39 gene whereas RR2 is encoded by the UL40 gene (McGeoch *et al.*, 1988b). The two subunits interact to form a holoenzyme complex, the formation of which is essential for enzyme activity (Frame *et al.*, 1985; Preston *et al.*, 1984, 1988). Although the enzyme is dispensable for virus growth in dividing cells *in vitro* (Goldstein and Weller, 1988b), evidence exists for its requirement for growth in resting cells or at the NPT (Goldstein and Weller, 1988a; Preston *et al.*, 1988; Jacobson *et al.*, 1989). The enzyme also seems to be essential for growth *in vivo* (Jacobson *et al.*, 1989; Brandt *et al.*, 1991).

(f) **Thymidine kinase (TK; UL23)**

Thymidine kinase (TK) phosphorylates thymidine (Kit and Dubbs, 1963; Dubbs and Kit, 1964), deoxycytidine (Jamieson and Subak-Sharpe, 1974) and thymidylate (Chen and Prusoff, 1978). The HSV-1 TK activity is encoded by the UL23 gene and has been shown to be dispensable for growth in dividing cells in tissue culture (Jamieson *et al.*, 1974; McGeoch *et al.*, 1988b). However, recent studies indicate its involvement in reactivation of the virus from the latent state (Coen *et al.*, 1989; Efstathiou *et al.*, 1989).

(g) **Uracil DNA glycosylase (UL2)**

HSV also encodes a uracil DNA glycosylase (Caradonna and Cheng, 1981), which normally removes uracil residues from DNA. The

enzyme is encoded by the UL2 gene and is non-essential for virus growth in tissue culture (Mullaney *et al.*, 1989).

4. HSV STRUCTURAL PROTEINS

More than 30 structural polypeptides have been detected in HSV-1 virions (Spear and Roizman, 1972; reviewed by Dargan, 1986). Although virion structural proteins are found within all classes (IE, E and L), the majority is encoded by the L genes (Honess and Roizman, 1973, 1974; Morse *et al.*, 1978). These can be classified into three groups : glycoproteins, tegument proteins and capsid proteins.

There are at least 7 distinct glycoproteins encoded by HSV-1 : gB (UL27), gC (UL44), gD (US6), (Spear, 1976), gE (US8) (Baucke and Spear, 1979), gG (US4) (McGeoch *et al.*, 1985; Ackermann *et al.*, 1986a; Richman *et al.*, 1986; Frame *et al.*, 1986a), gH (UL22) (Marsden *et al.*, 1978; Frame *et al.*, 1986a; McGeoch *et al.*, 1987, 1988b) and gI (US7) (Longnecker *et al.*, 1987). In addition, two novel HSV-1 glycoproteins, gK (UL53) (Hutchinson *et al.*, 1992; Ramaswamy and Holland, 1992) and gL (UL1) (Hutchinson *et al.*, 1992) have recently been identified. Moreover, McGeoch *et al.*, (1985) have proposed a small glycoprotein encoded by the gene US5 on the basis of sequence determination, although a protein product has yet to be identified. Three of these glycoproteins, gB, gD and gH are essential for virus infectivity *in vitro* (Little *et al.*, 1981; Weller *et al.*, 1983a; Ligas and Johnson, 1988; Cai *et al.*, 1988) whereas gC, gE, gG and gI have been shown to be dispensable in tissue culture (Longnecker and Roizman, 1987; Weber *et al.*, 1987; Harland and Brown, 1988).

The exact number of the virion tegument proteins is unknown. These proteins can be identified when released from virions in the presence of non-ionic detergents. However, some proteins associate more tightly to capsids than others (Roizman and Furlong, 1974; Lemaster and Roizman, 1980). The major tegument protein is Vmw65 (UL48; VP16), which is required for transactivation of IE genes (see Section 1C). The US9 gene product is a 10K tegument phosphoprotein (Frame *et al.*, 1986b). The product of gene UL47 (VP13/14) may also be a tegument protein (McLean *et al.*, 1990; Whittaker *et al.*, 1991). In addition, another tegument protein, VP22 (Spear and Roizman, 1972), has recently been shown to be encoded by the UL49 gene (Elliott and Meredith, 1992). Other tegument proteins include the UL36 gene product, Vmw273 (Batterson *et al.*, 1983; McGeoch *et al.*, 1988b).

Three classes of capsids are isolated from HSV-1 infected cells : full, intermediate and empty (Schaffer *et al.*, 1974; Atkinson *et al.*, 1978). Full capsids contain DNA and have a densely staining core, the intermediate capsids are devoid of DNA but have an electron-translucent core whereas empty capsids contain neither DNA nor the core (Gibson and Roizman, 1972; Furlong *et al.*, 1972). The empty capsids are made up of at least 5 proteins, the major capsid protein (MCP; ICP5; Vmw155; VP5; NC1; p155; UL19), VP19c (Vmw53; NC2; p50; UL38), VP23 (Vmw36; NC5; p32; UL18), VP24 (Vmw24; NC6; p25) and 12K (Vmw12; NC7; p12) (Gibson and Roizman, 1972; Heine *et al.*, 1974; Heilman *et al.*, 1979; Cohen *et al.*, 1980; McGeoch *et al.*, 1988b; Rixon *et al.*, 1990). The mature (full) capsids contain 2 additional proteins, VP22a (Vmw40; ICP35; NC4; p40; UL26) and VP21 (NC3; a component of the cylindrical protein plug which forms the virion core in conjunction with the viral DNA) (Gibson and Roizman, 1972; Preston *et al.*, 1983; Rixon *et al.*, 1988). Other viral proteins, including UL6, UL25, UL28, UL32 and UL33 gene products, have also been shown to be important for the formation of full capsids (Addison *et al.*, 1984, 1990; Sherman and Bachenheimer, 1987; Al-Kobaisi *et al.*, 1991).

Recently, Barker and Roizman (1992) identified an additional HSV-1 (strain F) gene designated UL49.5. The putative UL49.5 ORF was located between the UL49 and UL50 coding sequences and consisted of 91 amino acids (aa). The expression of UL49.5 was demonstrated by inserting in frame an oligonucleotide encoding a 15aa epitope known to react with an MAb, cloning of the resultant construct into the TK gene of *wt* virus and detection of the predicted chimaeric gene in infected cells by the MAb. As attempts to isolate the deletion/insertion mutants of UL49.5 were unsuccessful, the authors suggested that the gene may be essential for DNA replication *in vitro*. They further suggested that the UL49.5 protein product might be the small capsid protein known as NC7 (12K; Vmw12; p12; Heilman *et al.*, 1979; Cohen *et al.*, 1980) on the basis of their nucleotide sequence. On the other hand, comparative sequence analysis of HSV-1 (strain 17^+) and HSV-2 (strain HG52) revealed a small ORF (termed UL49A) of 91 and 87 codons respectively between the coding sequences of the UL49 and UL50 genes (Barnett *et al.*, 1992 : submitted for publication). Counterparts of UL49A were found in most of the sequenced herpesviruses such as VZV, EHV-1, HCMV and EBV, suggesting that the gene is conserved among alpha-, beta- and gamma-herpesviruses. However, Barnett *et al.* (1992) predicted that the UL49A gene product bears the characteristics of a membrane-inserted protein

and not a capsid protein as proposed by Barker and Roizman (1992). In addition, amino acid sequence comparison of all sequenced herpesviruses further revealed that the UL49A protein might be glycosylated thereby exhibiting the properties of a glycoprotein. Interestingly, a more recent work (McNabb and Courtney, 1992) using immunoprecipitation and immunoblotting techniques demonstrated that the NC7 or p12 capsid protein is encoded by the UL35 gene. Therefore, further studies are required to find out the precise nature of the UL49A/UL49.5 gene product. Discovery of this gene has now risen the total number of HSV-1 ORFs to 76 (73 distinct genes).

5. CLEAVAGE AND PACKAGING OF VIRAL DNA

Following replication, HSV DNA undergoes two processes : the cleavage of genomic-length DNA from mature circles or concatemers and packaging of the unit-length molecules into capsids. Experiments using *ts* mutants have demonstrated that these two processes are tightly linked as mutants which fail to encapsidate DNA are also unable to cleave the high molecular weight viral DNA into unit length molecules (Ladin *et al.*, 1980; Preston *et al.*, 1983; Addison *et al.*, 1984; Sherman and Bachenheimer, 1987, 1988). Cleavage of DNA molecules occurs at the *'a'* sequence present at the termini of standard molecules. Insertion of an additional *'a'* sequence into the viral genome results in the generation of novel termini corresponding to the position of the inserted *'a'* sequence (Smiley *et al.*, 1981; Mocarski and Roizman, 1982). The *cis*-acting signals required for encapsidation of viral DNA into particles have also been located within the *'a'* sequence (Stow *et al.*, 1983, 1986; Varmuza and Smiley, 1985; Spaete and Frenkel, 1985; Deiss and Frenkel, 1986; Nasseri and Mocarski, 1988).

The *'a'* sequence is present as a direct repeat at both termini of the HSV genome and in an inverted orientation at the L-S junction (Wadsworth *et al.*, 1975, 1976). It consists of a short (17-21 bp) terminal repeat designated as DR1, a unique region termed U_b, a 12-bp repeat sequence (1 to 22 copies) designated direct repeat 2 (DR2), a 37-bp sequence (present in 0 to 3 copies) called direct repeat 4 (DR4), a unique sequence termed U_c and a second copy of DR1 (Mocarski and Roizman, 1981, 1982; Mocarski *et al.*, 1985; see Figure 9). The terminal DR1 of the L component of HSV-1 strain 17 possesses 20 bp plus a single nucleotide extended 3' whereas the S component contains a single nucleotide at the terminus. Hence in concatemeric DNA molecules, adjacent *'a'* sequences share an intervening DR1 element. Similar observations have been reported

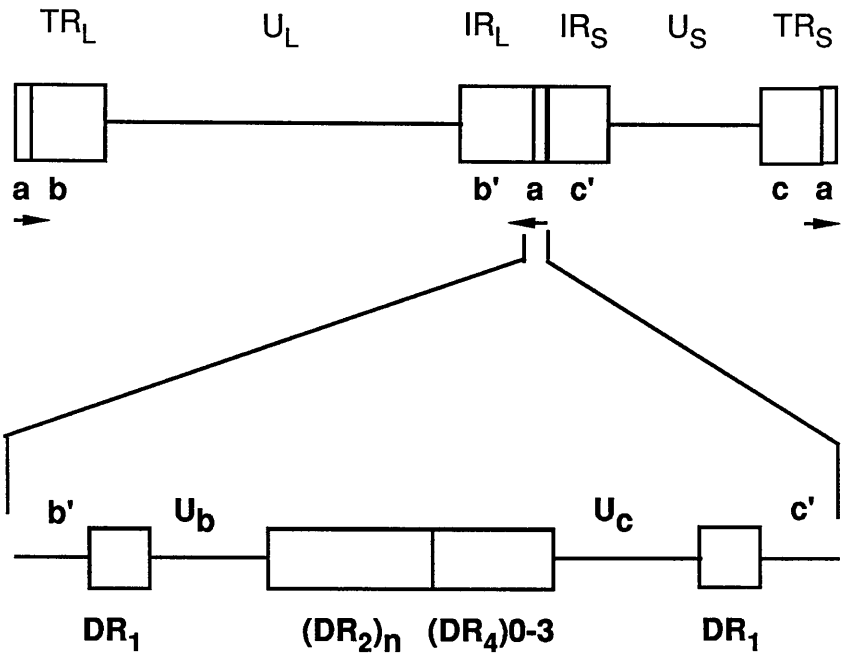

Figure 9. **Structure Of The HSV-1 *"a"* Sequence**

The genome of HSV-1 is shown at the top with an expanded region below showing the structure of the *"a"* sequence in the inverted orientation found at the L-S junction.

DR_1	a 17-21 bp element present as a direct repeat at the *"a"* sequence termini
DR_2	a 12 bp repeat element present in 1 to 22 copies
DR_4	a 37 bp element present in 0-3 copies
U_b	a unique sequence present towards the b' sequence
U_c	a unique sequence present towards the c' sequence

in other strains of HSV-1, as well as other herpesviruses (Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982; Davison and Rixon, 1985). The number of copies of DR2 and DR4 vary from strain to strain, thus accounting for the observed *'a'* sequence variations (Locker and Frenkel, 1979; Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982; Mocarski *et al.*, 1985; Varmuza and Smiley, 1985; Deiss *et al.*, 1986). Despite the presence of the variation in the nucleotide composition of the *'a'* sequence, two highly conserved sequences are located in U_C and U_b called *pac 2* and *pac 1* respectively (Davison and Wilkie, 1981; Davison, 1984; Varmuza and Smiley, 1985; Spaete and Mocarski, 1985; Mocarski *et al.*, 1985, 1987; Deiss *et al.*, 1986; Hammerschmidt *et al.*, 1988; Marks and Spector, 1988; see Figure 9).

Because of the observation that the terminal DR1 elements at the L and S termini of the genome contain a single nucleotide, it was concluded that the cleavage of concatemeric DNA occurs within the DR1 element to produce unit-length genomes. However, the analysis of HSV-1 strain Justin defective genomes has revealed the presence of only 4 bp of the DR1 element with no defect in cleavage and packaging, suggesting that the cleavage/packaging signals are located outside the DR1 element (Mocarski *et al.*, 1985). Further deletion/insertion analysis of the *'a'* sequence has demonstrated that the signals required for cleavage and encapsidation of the viral DNA are located within U_b and U_C (Varmuza and Smiley, 1985; Deiss and Frenkel, 1986; Deiss *et al.*, 1986). These signals, *pac 1* and *pac 2,* are sequence-independent and are located within a 179 bp fragment from the junction of two tandem *'a'* sequences (containing U_b and U_C elements) (Nasseri and Mocarski, 1988).

In order to understand the mechanisms involved in the amplification of *'a'* sequences and their role in cleavage and packaging of the viral DNA, several models have been proposed. If HSV DNA replicates by a rolling circle mechanism to form head-to-tail concatemers, initial circularization of linear genomes containing an *'a'* sequence at each end will generate a novel joint fragment consisting of tandem *'a'* sequences sharing a DR1 element. Consequently, the concatemeric DNA will possess a reiterated novel joint fragment at intervals corresponding to a single genome length. A cleavage event at the shared DR1 element of the joint fragment is then followed after recognition of the packaging signal by a putative cleavage-packaging protein complex. Subsequent similar cleavage events will result as packaging of unit length genomes with an *'a'* sequence at each terminus. A recent study using gel retardation analysis of protein-DNA interactions has

demonstrated the formation of complexes involving *pac 2* and DR1 sequences but not with DNA fragments containing *pac 1* and DR1 sequences, either alone or in combination (Chou and Roizman, 1989). The proteins which formed complexes include an HSV DNase, probably the viral alkaline exonuclease (UL12); the structural protein, VP1 (UL36; ICP1) (Lemaster and Roizman, 1980) and an unidentified protein with an apparent molecular weight of 140,000.

Other cleavage/packaging models include a single-strand nick-repair model, the 'theft' model (Varmuza and Smiley, 1985), the directional cleavage model and the double strand break and gap repair model (Szostak *et al.*, 1983; Deiss *et al.*, 1986). A recent recombination study supports the double strand break and gap repair model (Umene, 1991).

6. ASSEMBLY OF VIRIONS

The actual mechanisms leading to the assembly of nucleocapsids are still obscure. However, studies using *ts* mutants have identified several structural proteins essential for the formation and assembly of capsids. The HSV-1 UL6, UL18, UL19, UL25, UL26, UL32, UL33, UL38 and UL54 gene products are probably required for capsid formation (Weller *et al.*, 1983, 1987; Preston *et al.*, 1983, 1984; Addison *et al.*, 1984, 1990; Sherman and Bachenheimer, 1987, 1988; Rixon *et al.*, 1988, 1990; Al-Kobaisi *et al.*, 1991). The individual roles played by these proteins are still not fully understood.

The herpesvirus envelope appears to be acquired from the inner lamella of the nuclear membrane (Darlington and Moss, 1968; Nii *et al.*, 1968; Roizman and Furlong, 1974). Although viral DNAs less than unit-length may also encapsidate, only capsids containing unit-length genomes are transported to the cytoplasm forming mature virions (Vlazny *et al.*, 1982). The release of virus particles from infected cells has been proposed to occur via the Golgi apparatus to the cytoplasmic membrane (Johnson and Spear, 1982). Another process termed, reverse phagocytosis, has also been implicated in the virus release (Nii *et al.*, 1968; Katsumoto *et al.*, 1981). In addition, a recent report indicates that the UL20 gene product is required for the efficient egress of virus particles in atleast some cell lines (Baines *et al.*, 1991).

SECTION 1E : GENETICS OF HSV

The use of various types of mutants clearly constitutes a powerful approach in understanding the genetics of animal viruses. A large number of HSV studies utilized mutants and provided useful information regarding functional organization of the genomes. In particular, the analysis of conditional lethal mutants with *ts* lesions has identified several genes essential for virus growth both in tissue culture and *in vivo*. On the other hand, the isolation and characterization of deletion/insertion mutants has indicated the non-essential nature of various HSV genes in tissue culture. The various types of HSV mutants are briefly described in the following sections.

1. DELETION/INSERTION MUTANTS

The isolation of either spontaneous deletion and/or insertion mutants (see for example, Harland and Brown, 1985, 1989; Brown and Harland, 1987; MacLean and Brown, 1987b, c; MacLean, A R *et al.*, 1991; this thesis) or deliberately constructed deletion/insertion mutants (see for example, Post and Roizman, 1981; Weber *et al.*, 1987; Mullaney *et al.*, 1989; MacLean, C A *et al.*, 1991) has facilitated the identification of a number of genes non-essential for HSV DNA replication *in vitro*. The removal of an entire ORF or deletion/insertion within the sequence of an ORF precisely determines the essential/non-essential nature of a particular gene. To date, the following genes within the HSV genomes have been identified to be non-essential for growth in tissue culture : RL1 (ICP34.5; Taha *et al.*, 1989a, b, 1990; Chou *et al.*, 1990; MacLean, A R *et al.*, 1991; McGeoch *et al.*, 1991; Dolan *et al.*, 1992; this thesis), RL2 (IE110; Stow and Stow, 1986; McGeoch, 1989), UL2 (Mullaney *et al.*, 1989), UL3, UL4 (Baines and Roizman, 1991), UL10 (MacLean, C A *et al.*, 1991; Baines and Roizman, 1991), UL11 (MacLean, C. A. *et al.*, 1992), UL12 (Weller *et al.*, 1990), UL13 (L Coulter, personal communication), UL16 (Baines and Roizman, 1991), UL20 (Baines *et al.*, 1991), UL23, UL24 (Post *et al.*, 1981; McGeoch, 1989), UL39 (Goldstein and Weller, 1988b), UL41 (Fenwick and Everett, 1990), UL43 (MacLean, C A *et al.*, 1991), UL44 (McGeoch, 1989),UL45 (Visalli and Brandt, 1991), UL46, UL47 (Barker and Roizman, 1990), UL50 (Fisher and Preston, 1986), UL51 (Barker and Roizman, 1990), UL55, UL56 (MacLean and Brown, 1987b, c; Meignier *et al.*, 1988a; Junejo *et al.*, 1991), US1 to US5, US7 to US12 (Post and Roizman, 1981; Sears *et al.*, 1985; Longnecker and Roizman, 1986, 1987; Umene, 1986, 1987; Brown and Harland, 1987; Longnecker *et al.*, 1987; Weber *et al.*,

1987; Meignier *et al.*, 1988b; Harland and Brown, 1988) and most of the repeat regions including RL1, RL2, the 'a' sequence, LATs, and one copy of Vmw175 (Poffenberger *et al.*, 1983; Brown *et al.*, 1984; Poffenberger and Roizman, 1985; Harland and Brown, 1985, 1988, 1989; Stow and Stow, 1986; Umene, 1986, 1987; Jenkins and Roizman, 1986; Brown and Harland, 1987; MacLean and Brown, 1987b, c; McGeoch *et al.*, 1988b; McGeoch, 1989; Steiner *et al.*, 1989; Mitchell *et al.*, 1990; Junejo *et al.*, 1991; MacLean, A R *et al.*, 1991a; this thesis).

Although more than 30 of the HSV encoded genes are dispensable (as above) in tissue culture, many of these are probably required for viral replication and pathogenesis in experimental animal systems. For example, the deletion variants of ICP34.5 in HSV-1, as well as HSV-2, grow normally *in vitro* but are completely avirulent following intracranial inoculation of mice (Taha *et al.*, 1989a, b, 1990; MacLean, A. R. *et al.*, 1991a, b; Robertson *et al.*, 1992; this thesis). Other examples include the alkaline nuclease (UL12), TK (UL23), RR (UL39), the glycoproteins, etc. (see relevant sections).

2. DRUG-RESISTANT MUTANTS

Mutations within the coding sequences of genes required (either directly or indirectly) for HSV replication confer resistance to various antivirals. Most of the drug-resistant mutants isolated so far have lesions within the viral-encoded TK or *pol* (Honess *et al.*, 1984; Hall, 1986; Larder *et al.*, 1987). The majority of antiviral drugs, such as, phosphonoacetic acid (PAA), phosphonoformic acid (PFA), acyclovir (ACV), 5-bromo-2'-deoxyuridine (BUdR), adenosine arabinoside (Ara-A), 5-bromo-2'-deoxycytidine (BCdR), (R)-9-(3,4-dihydroxybutyl)guanine (Buciclovir; BCV), 2'-nor-2'-deoxyguanosine (Bw759U or DHPG) and several others have an inhibitory effect on HSV replication via the virally encoded enzymes. The isolation and analysis of drug-resistant mutants have proved useful in elucidation of the mechanism of action of antiviral drugs, as well as the identification of genes which mutate to confer drug resistance (Kit and Dubbs, 1963; Brown and Jamieson, 1978; Larder *et al.*, 1986; Coen *et al.*, 1985; Field and Coen, 1986; Chiou *et al.*, 1986; Coen, 1986).

The antiviral agents (for example, ACV and DHPG) are phosphorylated by HSV-induced TK to their triphosphates, the active form of the drug. The phosphorylated drug then interacts with the virus coded enzyme (DNA *pol*) or incorporates directly with the viral DNA to inhibit virus

replication. Mutants resistant to an antiviral agent are often deficient in TK activity and, therefore, use the cellular TK (Kit and Dubbs, 1963; Dubbs and Kit, 1964; Elion *et al.*, 1977; Stow *et al.*, 1978; Field *et al.*, 1980) and do not phosphorylate the drug. However, ACV-resistant mutants have also been found to have lesions within the DNA *pol* instead of TK and thus, the phosphorylated drug is not recognized by the enzyme (Crumpacker *et al.*, 1980; Larder and Darby, 1985; Larder *et al.*, 1987). On the other hand, drugs like PAA act directly on the DNA *pol* and bind to the pyrophosphate binding site (Leinbach *et al.*, 1976). PAA^R mutants have been isolated and shown to have altered DNA pol (Hay and Subak-Sharpe, 1976; Purifoy and Powell, 1977; Lee *et al.*, 1978).

3. HOST-RANGE MUTANTS

These mutants show ability to grow in some cell lines but not in other. An HSV-1 (strain MP) mutant was shown to grow in dog kidney cells in contrast to the parent *wt* strain (Aurelian and Roizman, 1964; Spring *et al.*, 1968). Recently, transformed cell lines providing essential viral gene functions have been developed. HSV mutants with defects in specific essential genes can be grown in cells expressing the required viral functions. Thus, detailed analysis of an essential gene, such as, Vmw175 (Davidson and Stow, 1985), gB (Cai *et al.*, 1987) or UL8 (Carmichael and Weller, 1989) has been carried out using such cell lines.

4. IMMUNE CYTOLYSIS-RESISTANT MUTANTS

Immune cytolysis-resistant mutants (*icr*) show altered conformation of glycoproteins, aberrant glycosylation and/or failure to incorporate the glycoproteins into infected cell membranes (Machtiger *et al.*, 1980; Glorioso *et al.*, 1980; Pancake *et al.*, 1983). Hence, mutant-infected cells become resistant to antibody and complement mediated immune cytolysis. Using such mutants, novel genes controlling the synthesis and processing of glycoproteins have been identified. The structural and functional organization of glycoproteins can also be studied by using these mutants. Monoclonal antibody-resistant (*mar*) mutants also show resistance to neutralization by monoclonal antibodies because of the presence of mutations affecting the antigenic sites of glycoproteins exposed on the virion envelope (Holland *et al.*, 1983; Marlin *et al.*, 1985). The *icr* locus has been identified within the gene UL32 (Coen *et al.*, 1984; McGeoch *et al.*, 1988b).

5. PLAQUE MORPHOLOGY MUTANTS

The normal cytopathic effect in HSV-infected cells is characterized by rounding up of cells (syn$^+$ plaques). However, variants containing mutations in certain genes have been isolated which, in contrast, induce cell fusion or syncytium (syn) formation (Hoggan and Roizman, 1959; Ejercito *et al.*, 1968; Brown *et al.*, 1973; Timbury *et al.*, 1974). These plaque morphology mutants have been used as unselected markers in three-factor reciprocal crosses (Brown *et al.*, 1973) along with *ts* markers. In addition, mixed plaques (syn + syn$^+$) have also been reported, and considered as internal heterozygotes arising as a result of genetic recombination (Brown and Ritchie, 1975) which may segregate to form homozygous molecules (offsprings) (Ritchie *et al.*, 1977). Mutants with syn phenotype have been isolated in several strains of HSV-1 and HSV-2 and their loci have been mapped at distinct genome locations (reviewed by Spear, 1985, 1987; Marsden, 1987). These loci include ORFs encoding gD (US6; Noble *et al.*, 1983; Gompels and Minson, 1986), gB (UL27; Bzik *et al.*, 1984; DebRoy *et al.*, 1985; Cai *et al.*, 1988), gK (UL53; Ruyechan *et al.*, 1979; Bond and Person, 1984; Pogue-Geile and Spear, 1987), gL (UL1; Little and Schaffer, 1981), probably gC (UL44; syn 2 locus; Ruyechan *et al.*, 1979; Little and Schaffer, 1981; Machuca *et al.*, 1986), TK (UL23; Sharpe *et al.*, 1983; McGeoch and Davison, 1986) and the UL24 gene (Sanders *et al.*, 1982; Tognon *et al.*, 1988, 1991; Jacobson *et al.*, 1990). Another syn locus has recently been mapped between the coding sequences of the RL1 and RL2 genes (Romanelli *et al.*, 1991). Although the involvement of several glycoproteins in cell fusion is not surprising, it is possible that the same locus has been mapped to different adjacent genes (for example, the UL22, UL23 and UL24 genes). Two types of cell fusion have been described : the fusion (as above) has been named fusion from within (FFWI) because of its induction during the course of virus replication and fusion from without (FFWO) (Falke *et al.*, 1985). FFWI is detected 3-4 hour post infection (pi) whereas FFWO is produced 30-45 min pi. In addition, FFWI can be inhibited by inhibitors of transcription or translation which is contrary to FFWO. However, FFWO is induced only by certain strains of HSV but this property may possibly be useful to study early virus-cell interactions (Walev *et al.*, 1991a, b).

6. RESTRICTION ENDONUCLEASE SITE-DELETION MUTANTS

Restriction endonuclease (RE) site-deletion mutants of HSV-1 and HSV-2 have recently been isolated (Brown *et al.*, 1984; Harland and Brown, 1985, 1988; Brown and Harland, 1987; MacLean and Brown, 1987a; this thesis). Two methods have been employed to isolate these variants : the enrichment selection of genomes devoid of particular RE sites and site-directed mutagenesis (see Section 2B : Methods). The serial selection enrichment procedure involves cleavage of viral DNA using 2-5 fold excess of the relevant enzyme, transfection and subsequent RE analysis of the resultant progeny population. The surviving probability of DNA molecules containing fewer RE sites is expected to be higher as compared to the *wt* population containing the standard number of RE sites. Using this selective pressure, HSV-1 and HSV-2 *Xba I* RE negative variants have been isolated (see above for references). The second approach utilized an oligonucleotide containing a conserved single base change which destroys the RE site without altering the amino acid sequence. This oligonucleotide is then used in site-directed mutagenesis to isolate a cloned HSV fragment devoid of a specific RE site. Subsequently, the cloned fragment is marker rescued into the viral genome to obtain a RE site-deletion variant. Mutants devoid of RE sites have proved useful in recombination studies (MacLean, 1988; Harland, 1990; Brown *et al.*, 1992; this thesis), in superinfection experiments to study latency (Cook and Brown, 1987) and in vector systems to allow the expression of foreign genes at a high frequency in eukaryotic cells (Rixon and McLauchlan, 1990).

7. TEMPERATURE-SENSITIVE MUTANTS

Ts mutants have been used extensively to study the genomic organisation of HSV. These mutants can replicate at the permissive temperature (PT: 31°-34°C) but not at the NPT (usually 38°-39.5°C) and are rarely isolated spontaneously. However, *ts* lesions have been introduced in the HSV genome artificially using a variety of mutagenic agents, such as, BUdR, 5-methyl-N'-nitro-N-nitrosoguanidine (NTG), UV, nitrous acid (NA) and hydroxylamine (HA). Thus, producing a missense substitution of an amino acid in a protein-coding sequence and as a consequence, the resultant polypeptide becomes non-functional at the NPT (Schaffer *et al.*, 1970, 1973; Timbury, 1971; Brown *et al.*, 1973; Manservigi, 1974; Esparza *et al.*, 1974). Although *ts* mutations can theoretically be introduced in any virus gene, the screening procedure results in the isolation of only those mutants which contain defects in essential genes. Using *ts* mutants, over 30 cistrons in HSV-1 and 20 in HSV-2 have been identified by complementation studies,

suggesting the presence of a probable minimum number of essential genes within HSV genomes (Schaffer *et al.*, 1978, 1987; Timbury *et al.*, 1976).

In order to map *ts* lesions precisely, marker rescue of *ts* mutations or *wt* HSV DNA RE fragments have been carried out (Wilkie *et al.*, 1974, 1978; Stow *et al.*, 1978; Chartrand *et al.*, 1981). However, the correlation of physical map locations of *ts* lesions and recombinational linkage map of HSV-1 strain 17 was found poor whereas those of HSV-2 were in good agreement (Stow *et al.*, 1978; Wilkie *et al.*, 1978; Parris *et al.*, 1980).

The characterization of *ts* mutants has provided useful information regarding virus penetration and uncoating (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Batterson *et al.*, 1983; Addison *et al.*, 1984), regulation of virus gene expression (Preston, 1979b; Sacks *et al.*, 1985), virus DNA synthesis (Schaffer *et al.*, 1973) and encapsidation (Preston *et al.*, 1983).

SECTION 1F : RECOMBINATION

1. GENERAL RECOMBINATION

Recombination can simply be defined as swapping of genetic information among DNA molecules or chromosomes. This reshuffling may involve either intrachromatid interactions between linked sequences on a DNA molecule or sister chromatid interactions between unequally paired homologous sequences on sister DNA strands. The transfer or exchange of genetic information between homologous sequences may occur reciprocally, i.e., for a given pair of distinguishable alleles, *A* and *a*, half of the products of a meiotic event should contain allele *A* and half should contain allele *a* (Mendelian segregation) or non-reciprocally, in which aberrant or unequal segregation takes place. The non-reciprocal transfer of information with segregation ratios of 3:1, 5:3 or 6:2 apparently show an increase in copy number of one allele whereas there is a decrease in copy number of another allele and has been termed as 'gene conversion'.

Both homologous and nonhomologous recombination events among DNA molecules have been described to occur in a number of prokaryotic and eukaryotic systems. However, the precise definitions of the terms 'homologous' and 'nonhomologous' are not clear in the literature. Various recombination studies have demonstrated that homologous sequences are required to pair DNA molecules (an indication of a recombination event), although the minimum amount of the required

homology shown in different studies varied from 07 bp to more than 200 bp (Singer *et al.*, 1982; Gonda and Radding, 1983; Rubnitz and Subramani, 1984; Landy, 1989). Moreover, the term 'homologous' does not mean identical and the homologous DNA molecules may carry genes which may differ in one or more nucleotides. In addition, comparison of homologous and nonhomologous recombination processes in intramolecular assays has revealed that both mechanisms have similar frequencies (Roth and Wilson, 1985; Chakrabarti *et al.*, 1985). However, the rate of nonhomologous recombination was found to be 2-3 times higher than the rate of homologous recombination (Roth and Wilson, 1985). In contrast, Brouillette and Chartrand (1987) have shown that the frequency of nonhomologous recombination was at least 7 times more than that of homologous recombination in an intermolecular recombination assay.

Intra- and intermolecular gene conversions have been demonstrated between closely linked sequences (Bollag *et al.*, 1989; Wysocki and Gefter, 1989) leading to genetic rearrangements, such as deletions, gene amplifications or sequence inversions (Lin and Sternberg, 1984; Subramani and Rubnitz, 1985; Schimke, 1988; Bollag and Liskay, 1988). Moreover, a recent study has shown that intermolecular gene conversion is the dominant form of homologous intrachromosomal interactions and may provide a means for gene amplification via reciprocal exchanges between unequal sister chromatids (Bollag and Liskay, 1991).

The exact mechanism(s) of gene conversion is still not known. However, extensive experimental studies using bacterial, fungal, bacteriophage and yeast systems have led to the proposal of a number of models, most of which involve single strand invasions between interacting homologous sequences, resulting in strand displacement and the formation of heteroduplex DNA. The mismatch repair of heteroduplex DNA, in turn, results in conversions (see reviews by Stahl, 1979; Szostak *et al.*, 1983; Landy, 1989; Wysocki and Gefter, 1989). Robin Holliday's (1964) model for single-strand exchange or crossing-over in gene conversion was the first to explain the molecular pathway leading to the formation and resolution of heteroduplex DNA. According to this model, during the course of recombination the process of exchange occurs through only one strand of each homologous duplex. The strands are nicked and exchanged on one side to form a symmetric heteroduplex DNA including the formation of a 'crossed-strand structure', a

Holliday junction or a Holliday intermediate. Resolving the intermediate into separate duplexes will require another cleavage and subsequent ligation. The cleavage can occur either on the original crossed strands or on the complementary strands. The former event would lead to no crossover whereas the latter will involve a reciprocal exchange of strands between the two duplexes. The physical events of the Holliday model are illustrated in Figure 10.

The Holliday model explains the various types of segregation in gene conversion (ratios 3:1, 5:3, 6:2). However, experiments after the proposal of this model have demonstrated the existence of asymmetric heteroduplex DNA as well (Meselson and Radding, 1975; Fogel *et al.*, 1978). In addition, asymmetric heteroduplex DNA is assumed to make a much greater contribution to the observed segregation than does symmetric heteroduplex DNA. Moreover, mismatch repair of heteroduplex DNA is required to form a 6:2 segregation (Szostak *et al.*, 1983).

The finding that asymmetric heteroduplexes also exist has led to the proposal of several other models. The Meselson-Radding model (1975) has accounted for asymmetric and symmetric heteroduplexes as well as crossing-over. However, more recent experimental data from yeast and Ascobolus suggest that the model may not be completely correct. Therefore, the double-strand break repair model has been proposed by Szostak *et al.* (1983) to provide a simpler explanation of the observed data. The observation that double-strand breaks increase illegitimate recombination and that the double-strand gaps are repaired by the nonreciprocal exchange of genetic information (Orr-Weaver *et al.*, 1981; Orr-Weaver and Szostak, 1983) has led to the proposal of this model. Figure 11 represents the various steps of the molecular model for double-strand-break and -gap repair. A double-strand endonuclease activity initiates the process of recombination by cutting one of the paired duplexes, termed the recipient chromatid. An exonuclease activity is followed, resulting in the enlargement of the cut into a gap with the formation of 3' single-stranded termini. One of the free 3' termini then invades a homologous region of the other duplex, termed the donor chromatid, displacing a small D loop. This D loop is enlarged by repair synthesis primed from the invading 3' terminus. Subsequently, the D loop will contain sequences complementary to the 3' end from the other side of the gap and the complementary single-stranded regions will anneal. A second round of repair synthesis is initiated by the other 3' terminus and the gap is completely repaired. The resultant heteroduplex DNA will contain two Holliday junctions,

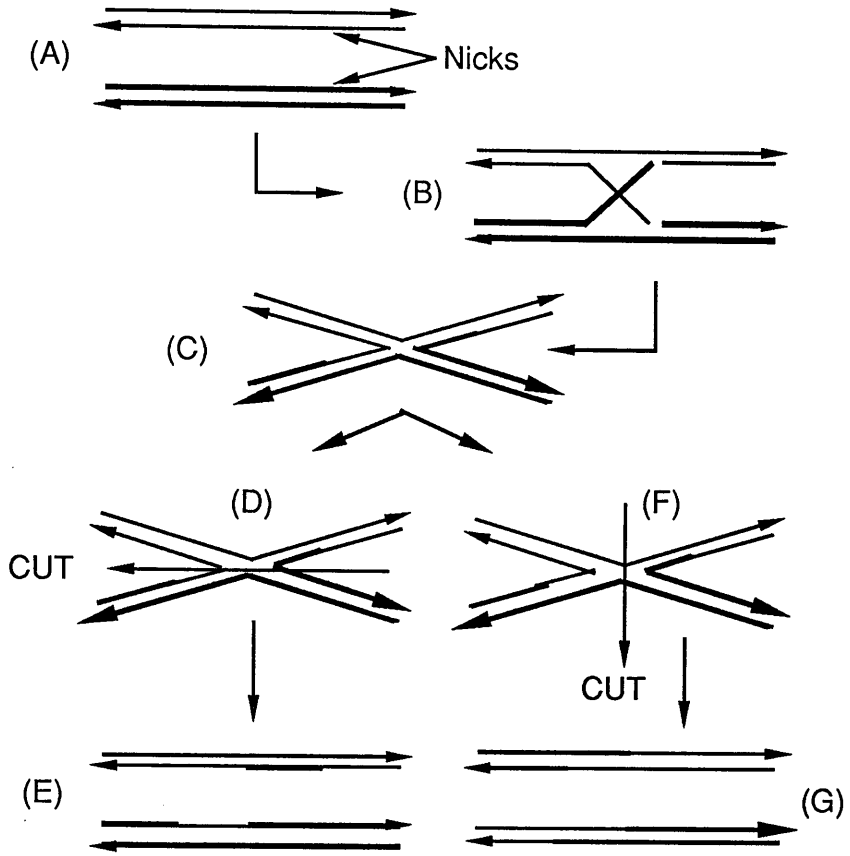

Figure 10. **The Holliday Model**

(A) The two aligned homologous duplexes are nicked on one strand (each) only. One duplex is represented by thin lines whereas the other by thick lines.

(B) The nicked strands intersect at right angles to form a Holliday junction and a heteroduplex DNA.

(C) The subsequent ligation of ends and isomerization can take place to form a symmeterical intermediate.

(D & E) Cutting the intersection to generate noncrossed strands except a short length of ssDNA segment.

(F & G) Cutting the intersection to generate crossed strands.

Figure 11. **The Molecular Model For Double-Strand-Break And -Gap Repair**

(A) A double-strand endonuclease activity initiates the recombination process by cutting one of the paired duplexes, termed the recipient chromatid (RC).

(B) The endonuclease activity is followed by an exonuclease activity, resulting in the enlargement of the cut into a gap and the formation of 3' single-stranded termini.

(C) One of the 3' termini then invades a homologous region of the other duplex, termed the donor chromatid (DC) displacing a D loop.

(D) The D loop is enlarged by repair synthesis primed from the invading 3' terminus until the other 3' terminus can anneal to complementary single-stranded sequences.

(E) A second round of repair synthesis is initiated by the other 3' terminus and the gap is completely repaired. As a result, two Holliday junctions are produced, branch migration of which would then generate a symmetry in the heteroduplex. Resolution of the two Holliday junctions by cutting either inner or outer strands leads to:

(F) two possible noncrossover, and

(G) two possible crossover configurations.

In the example given in this figure, the righthand junction was resolved by cutting the inner, crossed strands.

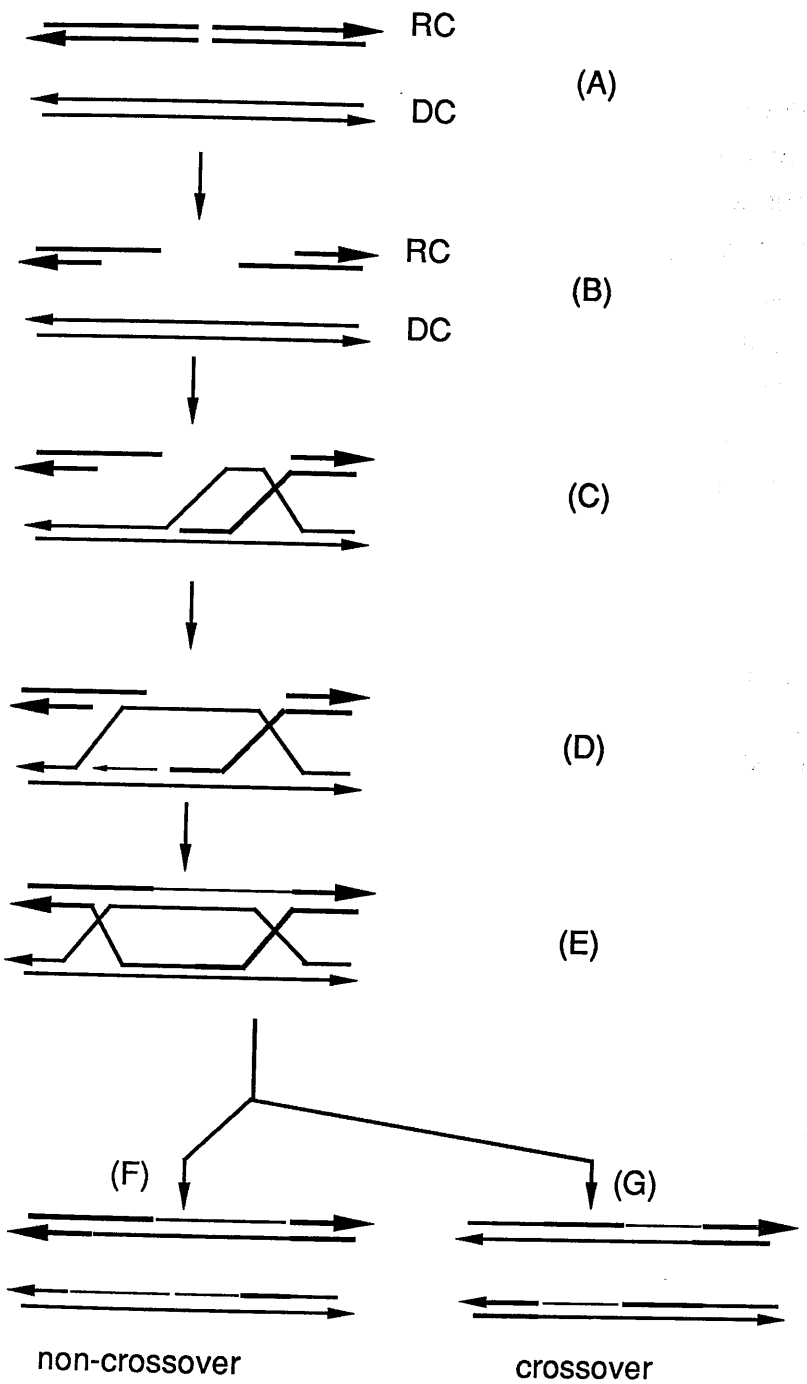

Figure 11. **The Double-Strand-Break And -Gap Repair Model**

branch migration of which would then generate a symmetry in the heteroduplex. Resolution of the two Holliday junctions in the same sense (cutting inner or outer strands in both cases) will lead to noncross-over configuration of flanking markers whereas resolution in the opposite sense would result as cross-over.

The double-strand break repair model has been widely accepted because it accounts for the observed gene conversion in two ways : if a marked site falls within a double-strand gap, it will be converted by a double-strand transfer of information, without involving a heteroduplex DNA. However, if the marked site falls within either of the flanking regions of asymmetric heteroduplex DNA, mismatch repair will lead to 6:2 segregation. Moreover, if a mismatch repair does not takes place then the heteroduplex DNA will result in postmeiotic segregation (Szostak *et al.*, 1983).

Electron microscopic studies have revealed the presence of physical connections between DNA molecules (Chi structures; chiasmata). These physical connections occur between homologous sequences and are usually observed as single or multiple fused regions of DNA molecules. Such molecules are closely correlated with the recombination intermediates containing one or more Holliday junctions (Zickler and Olson, 1975; Bell and Byers, 1983; Jones, 1984; Padmore *et al.*, 1991). The occurrence of site-specific double-strand breaks at or near the hot spots of recombination have also been demonstrated (Nicolas *et al.*, 1989; Cao *et al.*, 1990; Padmore *et al.*, 1991) in some recombination systems. These observed double strand breaks lead to the formation of both reciprocal and non-crossover recombinants (Nicolas *et al.*, 1989; Padmore *et al.*, 1991). Therefore, the above models may represent, at least to some extent, the actual mechanism(s) of genetic recombination in at least some of the recombination systems.

2. RECOMBINATION IN HIGHER EUKARYOTES

In order to understand the processes involved in cellular recombination, virus- and plasmid-based gene transfer methods have extensively been used. It has been demonstrated that mammalian cells provide the enzymatic processes necessary for both homologous and nonhomologous recombination of introduced DNA (Winocour and Keshet, 1980; Folger *et al.*, 1982; Pomerantz *et al.*, 1983; Subramani and Berg, 1983). Similar to the observations made in bacteria, fungi and yeast (see above; Section 1F:1), the viral studies also indicate that recombination between viral DNAs can involve 'Holliday structures' (Wolgemuth and Hsu, 1981). In

addition, the transfected DNA molecules are also thought to undergo breakage or cleavage followed by end joining (Wilson *et al.*, 1982; Calos *et al.*, 1983; Razzaque *et al.*, 1984; Roth and Wilson, 1986).

It has been shown that a number of viruses including HSV (see later; Section 1F:3), polyoma virus (Miller and Fried, 1976) and adenovirus (Ginsberg and Young, 1977) undergo extensive recombination during the course of a productive mixed infection in tissue culture. Recombination studies using different strains of adenovirus (for example, Ad2 and Ad 5) have demonstrated that only homologous regions of the genomes take part in recombination (Young and Silverstein, 1980; Boursnell and Mautner, 1981; Mautner and Boursnell, 1983). Moreover, the recombination frequency has been observed to increase throughout the course of virus replication (Williams *et al.*, 1974) suggesting multiple rounds of mating during the period of DNA synthesis. Using two *ts*$^+$ adenovirus strains, Young and Silverstein (1980) have shown an increase in the number of crossovers within the *ts*$^+$ intertypic recombinants isolated at late times as compared to early isolates. These observations suggest that both parental and progeny molecules take part in adenovirus recombination. In addition, recombination does not occur if DNA replication is blocked by an antiviral agent, thus indicating a direct link between DNA replication and recombination (Young *et al.*, 1984). However, the actual factors responsible for such a link are not known. Specific genes essential for DNA replication, formation of single-stranded DNA templates during viral replication and certain cellular polypeptides may also play a role either directly or indirectly in the recombination process (Flint *et al.*, 1976; Young *et al.*, 1984). The role of temperature in adenovirus recombination has also been demonstrated, as delayed recombination at 40.9 °C occurred despite the occurrence of normal DNA replication (Young *et al.*, 1984).

3. RECOMBINATION BETWEEN HSV VARIANTS

Recombination between strains of HSV was first shown by Wildy (1955) and the subsequent work by Subak-Sharpe (1969) has confirmed the recombinogenicity of HSV genomes by isolating *ts*$^+$ recombinants from crosses between pairs of HSV *ts* mutants. Further recombination studies using HSV-1 *ts* mutants have led to the construction of linear genetic linkage maps (Brown *et al.*, 1973; Schaffer *et al.*, 1974). Similar linkage maps for HSV-2 (Timbury and Calder, 1976) and PRV (Pringle *et al.*, 1973) have also been constructed. On the other hand, circular linkage maps have been proposed by Honess *et al.* (1980) on the basis of a variety of selected and

unselected markers. A circular genetic map can simply be explained by considering the occurrence of generalised recombination between circular molecules or concatemeric DNA. Additional support of this model came from a PRV recombination study using kinetics of the appearance of recombinants during a productive infection (Ben-Porat *et al.*, 1982). It has been shown that parental rather than progeny molecules take part in PRV recombination. However, contrasting results have been obtained in HSV (see below). In addition to the genetic linkage maps, physical maps showing physical locations of genetic markers have also been constructed (Stow *et al.*, 1978; Wilkie *et al.*, 1978, 1979; Chartrand *et al.*, 1979; Parris *et al.*, 1980). Comparison of the physical and genetic maps of HSV-2 were found in good agreement. However, in the case of HSV-1 poor correlation was found, possibly because of multiple crossovers between distant markers.

Intertypic recombination between HSV-1 and HSV-2 was first demonstrated by Timbury and Subak-Sharpe (1973) by isolating recombinants containing *ts* lesions from both parental viruses, HSV-1 strain 17 and HSV-2 strain HG52 *ts* mutants. Similar recombinants were then isolated by several other groups using various types of mutations, such as *ts* , plaque morphology, drug resistant, etc (Esparza *et al.*, 1976; Morse *et al.*, 1977, 1978; Halliburton *et al.*, 1977; Knipe *et al.*, 1978; Preston *et al.*, 1978; Stow and Wilkie, 1978; Wilkie *et al.*, 1978). Because of the presence of limited homology between the genomes of HSV-1 and HSV-2, intertypic recombination has not provided much information about the factors involved in the recombination process. However, it has proved useful in mapping viral polypeptides (Marsden *et al.*, 1978; see review by Halliburton, 1980).

Like the adenovirus recombination process (see above; Section 1F:2), recombination in HSV also appears to be linked with DNA replication. It has been shown that recombination is not detected until after the onset of viral replication (Ritchie *et al.*, 1977). Further evidence regarding an inter-relationship between replication and recombination has been provided by Batra and Brown (1989). These workers have demonstrated the occurrence of preferential recombination between intact HSV-1 strain McKrae genomes and HSV-2 strain HG52 RE fragments containing an origin of replication. Moreover, even the large fragments which recombined always included the Ori region of the genome. Recently, an HSV-1 strain 17 variant 1719 with a DNA duplication of 7.5 Kb of sequences around ori_L including a complete copy of UL29 (DBP) and a partial copy of UL30 *(pol)* has been isolated and characterised (MacLean, A R., 1992; submitted for publication). It is

speculated that the variant has probably been produced as a result of recombination between partially replicated genomes during the process of DNA replication, therefore, supporting a link between the two processes.

The presence of hot spots (specific DNA sequences containing a recombinase function) of recombination on the genomes of HSV cannot be ruled out. Because of the possible lack of total homology between strains of either HSV-1 or HSV-2 and the presence of nonhomologous sequences between HSV-1 and HSV-2 strains, it would be desirable to study homotypic recombination in order to investigate the possible presence of such sequences. To this end, Brown *et al.* (1984) have suggested the use of RE sites as unselected markers in homotypic recombination studies. Umene (1985), using two HSV-1 strains differing in eight RE sites has investigated the intermolecular recombination events but found no hot spots. Recently, Brown *et al.* (1992) have reported the use of only one strain of HSV-1 (17^+) and one of HSV-2 (HG52) with variants devoid of *Xba I* sites in intrastrain recombination studies. However, they could not detect any hot spots, possibly because of distant as well as few (only 5 or 7) markers. Therefore, a large number of closely located markers throughout the genome of HSV might reveal the possible recombination 'hot spots', if any. To this end, an HSV-1 variant lacking 1 *Bgl II*, 7 *Hind III* and 4 *Xba I* sites along with a variant containing an additional *Hind III* and an additional *Xba I* site have been isolated and used in intrastrain recombination studies (this thesis).

As mentioned above, only parental genomes take part in PRV recombination. However, recombination studies in HSV indicate that both parental and progeny molecules are involved. Earlier studies (Brown *et al.*, 1973; Ritchie *et al.*, 1977) using time course experiments and tri-parental crosses have demonstrated that the recombination frequency increases with time. The observation that both parental and progeny DNA molecules take part in HSV recombination (Ritchie *et al.*, 1977) has now been confirmed (Brown *et al.*, 1992). In addition, an increase of more than 45-fold in recombination frequencies has been observed when compared in the presence and absence of PAA (PAA restricts recombination to parental genomes) (Brown *et al.*, 1992).

The presence of four genomic isomers of HSV make recombination analysis more difficult. To determine the precise contribution of all the four isomers, extensive recombination studies including a large number of markers in both components (L and S) of the genome are required. The recombination data presented by Umene (1985) indicates that both of two

arrangements of the L component of parental DNA (P or I_S AND I_L or I_{SL}) contribute in the generation of recombinants. However, the data of Brown *et al.* (1992) suggests that all four isomers probably participate in the recombination process.

Site-specific recombinational events involving reiterated sequences including the *"a"* sequence have also been described in HSV. However, the roles, if any, played by such sequences in HSV recombination remains to be determined [see Section 1D (5)].

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CHAPTER 2:

MATERIALS AND METHODS

MATERIALS AND METHODS

SECTION 2A : MATERIALS

1. CELLS

Baby hamster kidney 21 clone 13 (BHK21/C13) cells (MacPherson and Stoker, 1962) were routinely used for the growth of virus and for other experimental work during this study.

2. VIRUSES

Herpes simplex virus type 1 (HSV-1), Glasgow strain 17+ (Brown *et al.*, 1973) was the wild type (*wt*) virus used in most experiments. The HSV-1 restriction endonuclease (RE) site deletion variants B1/2, B9/6, X2 (Brown *et al.*, 1984), 1701 and 1702 (previously designated as X3 and X4 respectively; MacLean and Brown, 1987a) were also used. Another HSV-1 variant 1708 [with an additional *Xba I* site around 0.74 map coordinates (m.c.); MacLean and Brown, 1987c] was used in recombination experiments.

3. TISSUE CULTURE MEDIA

BHK21/C13 cells were routinely grown in Glasgow modified Eagle's medium (GMEM; Busby *et al.*, 1964) supplemented with 10% calf serum, 10% tryptose phosphate broth, 100 units/ml penicillin, 100µg/ml streptomycin and 0.002% (w/v) phenol red (ETC10).

The following modified media were also used:

ETC5	GMEM containing 5% calf serum
EHu5	GMEM containing 5% human serum
Emet/5C2	GMEM containing 1/5 of the normal concentration of methionine and 2% calf serum
Freezing Mix	20% glycerol, 20% calf serum and 60% GMEM
MC	GMEM containing 1.5% methyl cellulose, 2-5% calf serum, and 5% tryptose phosphate broth
PIC	Phosphate-free GMEM containing 1% calf serum

4. BACTERIA

The bacterial strains of *Escherichia coli* used were:

DH5 [F^-, endA1,hsdR17 (r_K^-,m_K^+), supE44, thi-1, λ^-, recA1, gyrA96, relA1]; DH5α [F^-, endA1,hsdR17 (r_K^-,m_K^+), supE44, thi-1, λ^-, recA1, gyrA96, relA1, Δ(argF-lacZya)U169, Ø80dlacZ ΔM15]; NM522 [recA+, supE, thi, Δ(lac-proAB), hsd5, (F',proAB, $lacI^Q$, lacZ ΔM15]; and BW313 (dut-, ung-, thi-, relA1, spoT1, HfrkL16).

The bacterial strains DH5 and DH5α were obtained from BRL, England; BW313 and the bacteriophage M13 R408 (Russel *et al.*, 1986) were provided by Dr R Thompson; NM522 (Mead *et al.*, 1985) was supplied by Dr R M Elliott; and the bacteriophage M13 K07 (Zoller and Smith, 1987) was provided by Dr A R MacLean.

5. BACTERIAL CULTURE MEDIA

The following media were used:

L-Broth	10g/l NaCl, 10g/l bactotryptone and 5g/l yeast extract
L-Broth agar	L-Broth containing 1.5% w/v agar
2XYT broth	5g/l NaCl, 16g/l bactotryptone and 10g/l yeast extract
Top agar	0.6% Difco bactoagar w/v in water
Noble agar	3.2% Noble agar in water
S.O.C.	2% bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose; make 100ml with distilled water

6. PLASMIDS

(a). Cloning vectors

The plasmids used for cloning and production of single-stranded DNA for site-directed mutagenesis were: Plasmid pAT153 (Twigg and Sherratt, 1980) was provided by Dr R Thompson; the recombinant plasmid pGEM-1 and pGEM-2 (Riboprobe Gemini Transcription Plasmids; Melton *et al.*, 1984) were supplied by Dr Joan Dolan; the pTZ range of phagemid expression vectors pTZ18U, pTZ18R, pTZ19U and pTZ19R (Mead *et al.*, 1986) were obtained from Dr R M Elliott and the plasmid pFJ5ΔHind (Rixon and McLauchlan, 1990) was supplied by Drs F J Rixon and J McLauchlan. The plasmids pTZ18U/19U and pFJ5ΔHind are diagramatically represented in Figures 12 and 13.

(b) HSV-1 cloned fragments

The following plasmids containing HSV-1 fragments were used during the course of this work. These were provided by Drs A J Davison, F J Rixon and V G Preston:

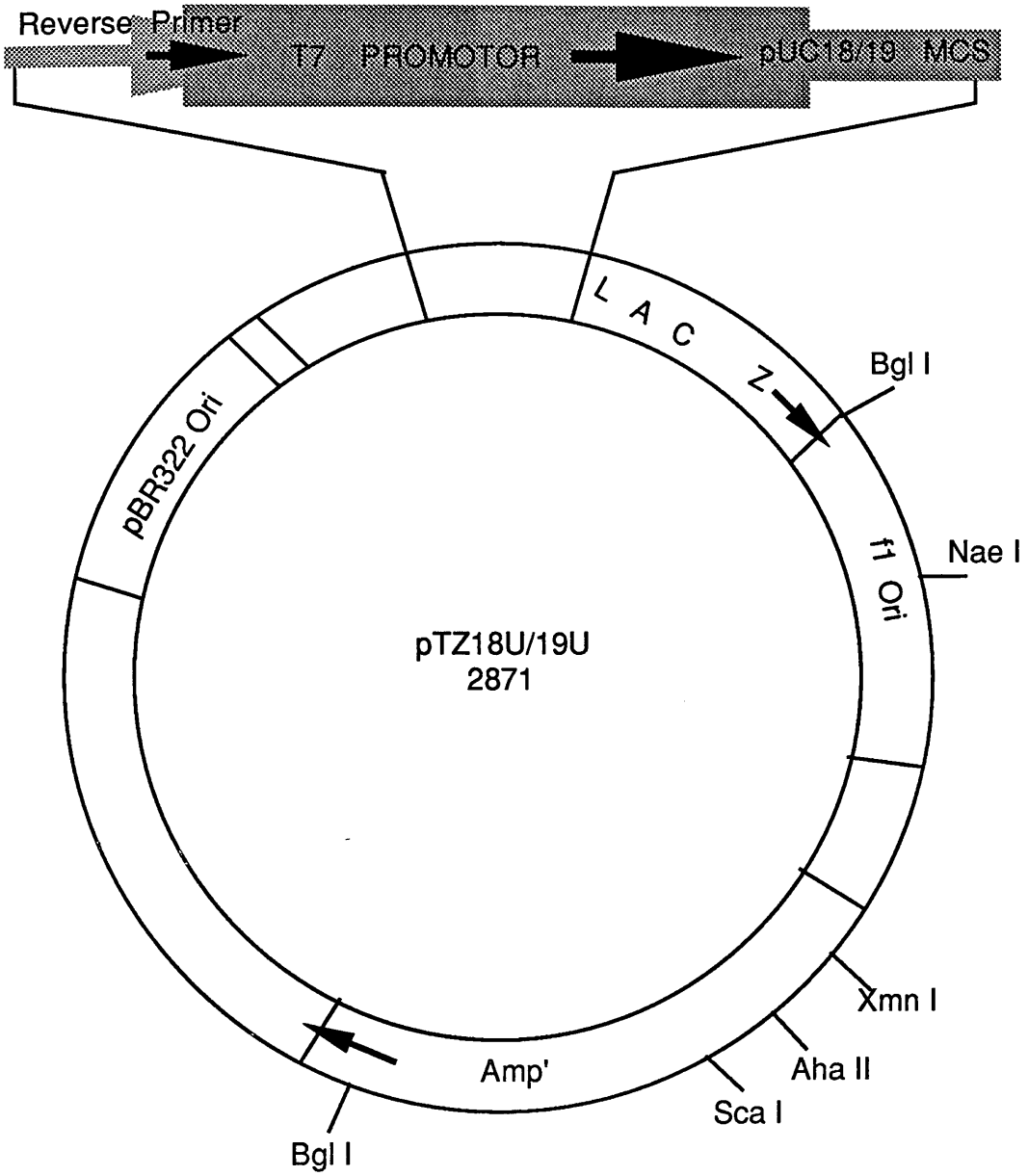


Figure 12. **The Multifunctional Phagemid Expression Vectors pTZ18U And pTZ19U**

The main characteristics of these plasmids include a multiple cloning site (MCS) from pUC18/19, an ampicillin resistance gene (Amp), the ß-galactosidase coding sequences (Lac Z), an f1 origin of replication for the production of ssDNA and a T7 polymerase promoter. Plasmids pTZ18U and pTZ19U are identical except that pTZ19U containing the MCS in reverse orientation. The vector plasmids pTZ18R and pTZ19R (not shown) are identical except that the f1 Ori is in the reverse orientation.

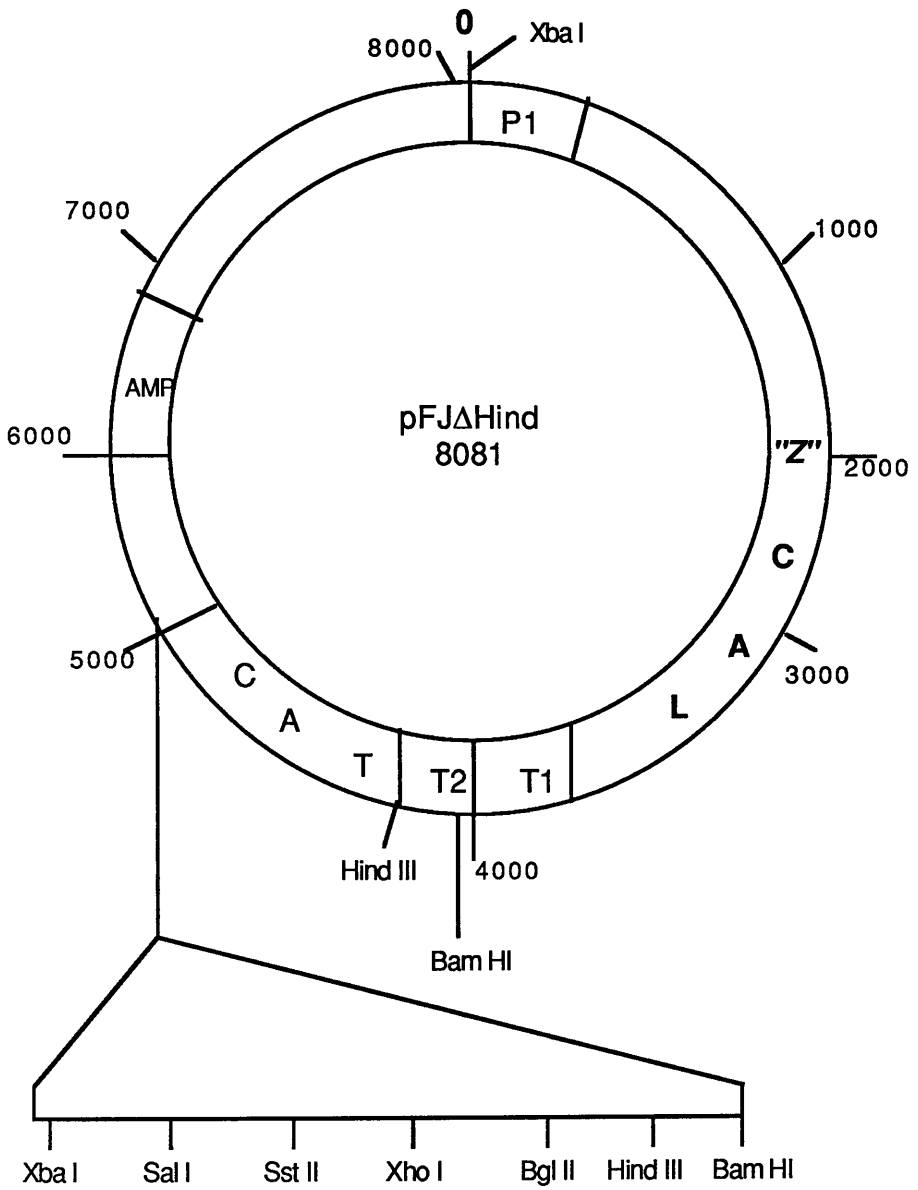


Figure 13. **The Plasmid pFJΔHind**
(Rixon and McLauchlan, 1990)

The main features of this plasmid include a multiple restriction endonuclease cleavage site linker, an SV40 early promoter (P1), the SV40 polyadenylation sequences (T1), the HSV-2 IE5 polyadenylation sequences (T2), the β-galactosidase coding sequences *(Lac Z)* and the chloramphenicol acetyltransferase coding sequences (CAT).

	HSV-1 fragment	nucleotides	vector
1.	Bam HI *a*	21655 to 34129	pAT153
2.	Bam HI *h*	79441 to 86980	pBR322
3.	Bam HI *j*	136289 to 142746	pAT153
4.	Bam HI *n*	131399 to 136289	pAT153
5.	Bam HI *o*	87744 to 91610	pAT153
6.	Eco RI *a*	74916 to 96751	pACYC184
7.	Kpn I *b*	3663 to 16273	pAT153
8.	Kpn I *d*	90383 to 103012	pAT153
9.	Kpn I *h*	134792 to 144897	pAT153
10.	Kpn I *i*	34192 to 43624	pAT153

7. SYNTHETIC OLIGONUCLEOTIDES

These were synthesised by Dr J McLauchlan of this Institute using a model 8600 Biosearch multiple column DNA synthesiser. A list of the oligonucleotides used during the course of this study is given in Table 3.

8. CHEMICALS

Chemicals were mainly supplied by either BDH Chemicals Ltd., Poole, England or Sigma Chemical Co. Ltd. except for the following:

Amersham International plc., U.K.: Hybond-N (nylon blotting membrane).

Beecham Research Laboratories, Brentford, England: Penbritin (ampicillin sodium B.P.).

Bio-Rad Laboratories, Ltd. : TEMED (N'N'N'N'tetramethylenediamine).

Boehringer Mannheim Gmbh, W Germany: Proteinase K and Tris (hydroxymethyl) aminomethane.

DuPont UK Ltd.: En^{3}Hance autoradiography enhancer.

Formachem (Research International) Ltd., Strathanen, Scotland: Boric acid (orthoboric acid).

Koch-Light Limited, Suffolk, England: Acrylamide, caesium chloride and dimethyl sulphoxide.

Leo Laboratories Ltd., Buckinghamshire: Sodium heparin 1,000 units/ml.

May and Baker Ltd., Dagenham, England: Acetic acid glacial.

Medicell International Ltd.: Dialysis membrane.

Pharmacia LKB Biotechnology AB, Uppsala, Sweden: DEAE-sephacel, ficoll 400, gene 32 protein and ultra-pure dNTP set.

Schleicher and Schuell, Dassel, W Germany: Nitrocellulose sheets and nytran (modified nylon-66 membrane).

TABLE 3

01.	18-MER :	5'-CATGGCAAGTTTGGAGCT-3'	**0.08**
02.	18-MER :	5'-CCCGTCAAGTTTCCCCAA-3'	**0.1**
03.	18-MER :	5'-AACGGCAAGTTTATGTTC-3'	**0.18**
04.	18-MER :	5'-CCCCCCGAAACTTCGGGC-3'	**0.18**
05.	18-MER :	5'-AAGCGAAGTTTCTGAGAG-3'	**0.26**
06.	18-MER :	5'-AATAAAACTTTATTGGTA-3'	**0.52**
07.	18-MER :	5'-ACGTCTCCAGGCTTTCCG-3'	**0.58**
08.	18-MER :	5'-GCGTTTAAGTTTGGTCAG-3'	**0.64**
09.	18-MER :	5'-GTGTGCAAACTTCCTTGT-3'	**0.88**
10.	18-MER :	5'-GTGTGCAAGCTCCCTTGT-3'	**0.88**
11.	18-MER :	5'-GGTCATAAGCTACAGCGC-3'	**0.91**
12.	24-MER :	5'-ATCAGCTCCAAGCTCGCCATGACC-3'	**0.08**
13.	27-MER :	5'-GATATATTTGGGGAAACTTGACGGGCC-3'	**0.1**

Continued.....

TABLE 3 (continued)

14. 90-MER : 5'-TATGGTCTTAACGGCAAGTTTATGTTCGCG
AATCAGTTGGACGTCACCGTATCCCCCAGA
CCCCCCGAAACTTCGGGCCCCGGGGATCTC-3'
0.18

15. 96-MER : 5'-CTCGAGATCCCCGGGGCCCGAAGTTTCGGG
GGGTCTGGGGGATACGGTGACGTCCAACTGATTCGC
GAACATAAGGTCGCCGTTAAGACCATAAAG-3'
0.18

16. 99-MER : 5'ACCCTCGAGATCCCCGGGGCCCGAAGTTTCGGG
GGGTCTGGGGGATACGGTGACGTCCAACTGATTCGC
GAACATAAGCTCGCCGTTAAGACCATAAAG-3'
0.18

17. 21-MER : 5'-GTAAAGCGAAGTTTCTGCGAG-3' **0.26**

18. 27-MER : 5'-GAGCTAAAGCGAAGTTTCTGCGAGACG-3'
0.26

19. 27-MER : 5'-AGTTACCAATAAAGTTTTATTATGTTA-3' **0.52**

20. 24-MER : 5'-GCCCTCGGAAAGTTTGGAGACGTC-3' **0.58**

21. 24-MER : 5'-CTCCTGACCAAGTTTAAACGCGAA-3' **0.64**

Continued.....

TABLE 3 (continued)

22. 24-MER : 5'-CAAACAAGGAAG**T**TTGCACACGGG-3' **0.88**

.....

Table 3. **Synthetic Oligonucleotides**

Synthetic oligonucleotides used to mutate *Hind III* sites. The *Hind III* site-specific sequence (AAGCTT) is underlined. The bold letter within the *Hind III* specific sequences represent third base mutation of the respective codon, thus destroying the *Hind III* site without altering the amino acid. The figures (0.08, 0.1, 0.18, 0.26, 0.52, 0.58, 0.64, 0.88 and 0.91) on the right hand side of oligonucleotides sequences represent map coordinates of the respective *Hind III* site on the HSV-1 genome.

CODON USAGE

The single base mutation within the above cited oligonucleotides was created in the third base position of the respective codon, thus altering the *Hind III* recognition sequence (AAGCTT) without affecting the coding potential. The amino acid and DNA sequence of the 24-mer oligonucleotide (no. 12) used to destroy the 0.08 m.c. *Hind III* site (located within the UL5 gene) is hereby given as an example:

amino acid sequence:		I	S	S	K	L	A	M	T
wild-type DNA sequence:	5'-ATC	AGC	TCC	AAG	CTT	GCC	ATG	ACC-3'	
mutated DNA sequence:	5'-ATC	AGC	TCC	AAG	CT**C**	GCC	ATG	ACC-3'	

Serva Feinbiochemica, Heidelberg: Ammonium persulphate.

9. RADIOCHEMICALS

Radiochemicals were supplied by Amersham International plc., UK :

	Radioisotope	Specific activity
(a)	5'[α-^{32}P]-deoxyribonucleoside triphosphate	3000 Ci/mmol (10 μCi/μl)
(b)	5'[α-^{32}P]-adenosine triphosphate	5000 Ci/mmol (10 μCi/μl)
(c)	5'[α-^{32}P]-deoxyadenosine thiotriphosphate	>1000 Ci/mmol (10 μCi/μl)
(d)	[^{35}-S]-L-methionine	800 Ci/mmol (15 μCi/μl)
(e)	[^{32}P]-orthophosphate	200 Ci/mmol (10 μCi/μl)

10. ENZYMES

Most of the restriction endonucleases were obtained either from Bethesda Research Laboratories (GIBCO Ltd.), Paisley, Renfrewshire, Scotland or Boehringer Mannheim Corporation, W Germany. The restriction enzymes *Bsm I*, *Nhe I*, *Sca I*, *Sfi I* and *Sph I*, *Kpn I* linker and polynucleotide kinase were supplied by New England Biolabs, Beverly, MA, USA. *E coli* DNA polymerase I was purchased from Nbi (Northumbria Biologicals Limited) enzymes Ltd., Northumberland, England. BSA, lysozyme, ribonuclease T1 grade v and ribonuclease type IIA were obtained from Sigma Chemical Company Ltd., Poole, Dorset, England.

11. COMMONLY USED BUFFERS AND SOLUTIONS

Acrylamide gel tank buffer :	53mM glycine, 3.5mM SDS and 52mM Tris
Alkaline lysis solution I :	10mM EDTA, 50mM glucose, 25mM Tris-HCl pH 8.0 and 5 mg/ml lysozyme added prior to use
Alkaline lysis solution II :	0.2M NaOH and 1% (w/v) SDS
Alkaline lysis solution III :	5M potassium acetate pH 4.8
Cell lysis buffer :	2mM EDTA, 0.5% (w/v) SDS, 20mM Tris-HCl pH 7.5

Chloroform:isoamyl alcohol :	A mixture of chloroform and isoamyl alcohol (24:1 v/v) was made for use in the extraction of DNA
50X Denhardt's buffer :	1% BSA, 1% Ficoll, 1% polyvinyl-pyrrolidone
Dissolve buffer :	1mM EDTA, 50mM NaCl, 10mM Tris-HCl pH 7.4
Electrophoresis buffer :	1mM EDTA, 30mM NaH_2PO_4, 36mM Tris-HCl pH 7.8
Elution buffer :	0.5M ammonium acetate, 1mM EDTA, 0.1% (w/v) SDS
Gel soak I :	600 mM NaCl, 200mM NaOH
Gel soak II :	600 mM NaCl, 1M Tris-HCl pH adjusted to 8.0 with HCl
Giemsa stain :	1.5% (w/v) Giemsa in glycerol heated at 56 °C for 2 hours and diluted with an equal volume of methanol
HEBS :	9mM D-glucose, 21mM Hepes-NaOH pH 7.05, 137mM NaCl, 5mM KCl, 0.2mM Na_2HPO_4
Hybridisation buffer I :	5X Denhardt's solution containing 100 µg/ml salmon sperm DNA, 3X SSC and10mM Tris-HCl pH 7.5
Hybridisation buffer II :	7% (w/v) SDS, 0.5M Na_2HPO_4 pH 7.0
10X Ligase buffer :	200mM DTT, 100mM $MgCl_2$, 0.5M Tris-HCl pH 7.8
10X Kinase buffer :	100mM DTT, 1mM EDTA, 100mM $MgCl_2$,10mM spermidine, 0.5M Tris-HCl pH 7.6
NT (Nick Translation) buffer :	50 µg/ml BSA, 1mM DTT, 5mM $MgCl_2$, 50mM Tris-HCl pH 7.5
NTE :	1mM EDTA, 100mM NaCl, 10mM Tris-HCl pH 7.4
PBS :	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4,1.8mM KH_2PO_4 pH 7.2
PBS-A :	PBS supplemented with 6.8mM $CaCl_2$ and 4.9mM $MgCl_2$
PBS/Calf serum :	PBS containing 5% calf serum

Phenol : Chloroform :	Equal volumes of chloroform:isoamyl alcohol (24:1) and saturated phenol
RE Stop :	100mM EDTA, 10% Ficoll 400, 0.25% bromophenol blue, 0.1% xylene cyanol, 5X TBE or E buffer
Resolving gel buffer :	0.4% (w/v) SDS, 1.5M Tris-HCl pH 8.9
Restriction enzyme buffers :	These buffers were used as recommended by BRL, Boehringer Mannheim or Maniatis *et al.* (1982)
RSB :	1.5mM $MgCl_2$, 10mM KCl, 10mM Tris-HCl pH 7.4
Saturated phenol :	Phenol was saturated by mixing 2:1 with phenol buffer (10mM EDTA, 100mM NaCl, 10mM Tris-HCl pH 7.5)
Spacer gel buffer :	14mM SDS, 487mM Tris-HCl pH 6.7
20X SSC :	3M NaCl, 0.3M trisodium citrate pH 7.5
Stacking gel buffer :	0.11mM Tris-HCl pH 6.7, 0.1% (w/v) SDS
TBE :	3mM EDTA, 89mM boric acid, 89mM Tris-HCl pH 8.0
TE :	1mM EDTA, 10mM Tris-HCl pH 8.0
Tris-Saline :	1 mg/ml glucose, 140mM NaCl, 280mM Na_2HPO_4, 30mM KCl, 0.0015% (w/v) phenol red, 25mM Tris-HCl pH 7.4, 100 units/ml penicillin, 100 µg/ml streptomycin
Trypsin :	0.25% Difco trypsin powder dissolved in Tris-saline
Versene :	0.6mM EDTA dissolved in PBS containing 0.002% (w/v) phenol red
Trypsin-Versene :	This is a 1:4 mixture of trypsin and versene

12. MISCELLANEOUS MATERIALS

Automatic pipette tips	Gordon Keeble Ltd.
Kapton tape	Scotch Brand tape, St. Paul, MN, USA
Pi pump	Glasfirn Giessen, W Germany
Polaroid films	Polaroid Corporation, Cambridge, MA. USA
Tissue culture plastics	Sterilin Ltd., Feltham

15ml and 50ml sterile tubes	Becton Dickinson and Co., Oxnard, CA, USA
0.5ml, 1.5ml and 15ml tubes	Sarstedt, W Germany
2 ml white soda screw neck vials	FBG Trident, Bristol, Avon
XS1 X-Omat S X-ray film	Kodak Ltd.

SECTION 2B : METHODS

1. CELL GROWTH

BHK21/C13 cells were grown in ETC10 medium at 37 °C in an atmosphere of 5% CO_2 (MacPherson and Stoker, 1962). Confluent cells from 80 oz roller bottles were harvested in 20 ml ETC10 after two washes of monolayers with trypsin-versene and remained viable at 4 °C for up to 7 days. A confluent roller bottle contains approximately 3 x 10^8 cells which are sufficient to seed a further 10 roller bottles. 50mm and 35mm diameter plastic petri dishes were seeded at 2 x 10^6 cells per dish in 4 ml ETC10 and 1 x 10^6 cells per dish in 2 ml ETC10 respectively. 24-well Linbro trays were seeded at 5 x 10^5 cells per well in 500µl ETC10.

2. CELL STORAGE

Cells were harvested from a roller bottle as described under cell growth, pelleted and resuspended in 10 ml freezing mix. Cells were transferred to 2 ml screw neck vials and frozen slowly to -140 °C. These were recovered by thawing quickly and growing in ETC10.

3. VIRUS STOCKS

(a) Growth of virus stocks

Virus stocks of wild type (*wt*) HSV-1 and its variants were propagated in BHK21/C13 cells. Confluent cells in 80 oz roller bottles were infected with virus at a multiplicity of infection (moi) of 0.003 plaque forming units (pfu) per cell in 20 ml ETC10. The roller bottles were incubated at 31 °C for 3-5 days or until the appearance of extensive cytopathic effect (cpe). The cells were harvested into the medium by vigorous shaking and pelleted at 2000 rpm (revolutions per minute) for 10 minutes at 4 °C in a Beckman GPR Tabletop centrifuge. The cell pellet was resuspended in 5 ml supernatant, sonicated in an ultrasonic bath to release the virus and centrifuged at 2000 rpm for 10 min at 4 °C. The supernatant was transferred to a universal and stored at 4 °C whereas the pellet was resuspended in 5 ml of the original supernatant, sonicated and centrifuged as above. The two supernates were mixed to give the cell-associated virus (CAV) and stored at -70 °C in aliquots. The original supernatant from the infected cells was centrifuged at 12000 rpm for 2 hours at 4 °C in a Sorval GSA rotor and the pellet of cell-released virus (CRV) resuspended in 5 ml of supernatant, sonicated and stored in 1 ml

aliquots at -70 °C. Sterility checks on virus stocks were carried out as described in section 3 (b).

(b) **Sterility checks on virus and cell stocks**

All virus and cell stocks were checked for bacterial or fungal contamination by streaking on blood agar or brain heart infusion agar plates. The blood agar plates were sealed with parafilm (Nescofilm, Bando Chemical Ind. Ltd., Japan) and incubated at 37 °C for up to 7 days. Similarly, brain heart infusion agar plates were incubated at room temperture (r.t). Stocks were considered sterile, if no contamination was found within the time specified.

(c) **Titration of virus stocks**

Virus stocks were titrated on BHK21/C13 cell monolayers grown in 50mm plastic petri dishes. Stocks were serially diluted 10-fold in PBS-calf serum and 0.1 ml of each dilution was added onto cell monolayers from which the growth medium had been removed. The virus was allowed to adsorb at 37 °C for 1 hour, 4 ml EHu5 or MC added and incubation continued at the appropriate temperature (2 days at 37 °C, 3 days at 31 °C). Monolayers were fixed and stained by adding approximately 4 ml Giemsa stain per petri dish at r.t for at least 30 minutes. The stain was washed off with water and the viral plaques counted under a dissecting microscope.

(d) **Plaque purification of virus**

Virus was titrated as described in section 2 (c) and cell monolayers washed four or five times with PBS-calf serum. Single, well separated plaques were picked into 0.5 ml PBS-calf serum in a screw neck vial, sonicated for 45 seconds and stored at -70 °C. 200 µl of a plaque- pick sonicate was used to infect BHK21/C13 cells grown in a 35mm plastic petri dish and incubated at the appropriate temperature to grow a plate stock.

The ^{32}P-labelled DNA [see section 4 (f)] of plate stocks were prepared and analysed with various restriction endonucleases and their profiles compared with those of *wt*. On finding a variant virus, it was plaque purified an additional 3 times prior to the production of a roller bottle virus stock for further analysis.

(e) **Virus growth properties**

Single cycle growth experiments were carried out as described by Brown and Harland (1987). BHK21/C13 cell monolayers were grown in 35mm plastic petri dishes at 37 °C and infected at an moi of 5 pfu/cell. Following virus adsorption at 37 °C for 45 minutes, the cell monolayers were washed twice with PBS-calf serum, overlaid with 2 ml ETC10 and incubated at

37 °C. Samples were harvested at 0, 2, 4, 6, 8, 10, 12, 16, 24 and 30 h post infection (pi). Virus was sonicated and titrated as described [section 3 (c)].

Multiple cycle growth experiments involved infection of cell monolayers at an moi of 0.001 pfu/cell, harvesting samples at 0, 4, 12, 24, 36, 48, 60, 72 and 96 h pi, ultra-sonication and titration.

4. VIRUS DNA

(a) Preparation of HSV-1 DNA

HSV-1 DNA was prepared as described by Wilkie (1973). Infected cells were harvested as described in section 3 (a). The cell pellet was resuspended in RSB containing 0.5% NP40 and placed on ice for 10 minutes. The nuclei were extracted by centrifugation at 2000 rpm for 3 min at 4 °C in a Beckman GPR tabletop centrifuge. The supernatant was added to the original cell supernatant and the pellet re-extracted as before. The supernatant from the final spin was also added to the original cell supernatant and the pooled supernates were centrifuged at 12000 rpm for 2 h at 4 °C in a Sorval GSA rotor. The virus pellet containing CAV and CRV was resuspended in 8 ml NTE and EDTA and SDS added to a final concentration of 10mM and 2% respectively for lysis of virus. Viral DNA was then sequentially extracted by gentle mixing, twice with saturated phenol, once with phenol:chloroform (1:1) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated by the addition of 2 volumes of ethanol and 1/10 volume of 5M NaCl, pelleted at 2000 rpm for 10 min, washed with 70% ethanol (aqueous), dried in a vacuum desiccator and resuspended in water.

(b) Preparation of infected cell DNA

Infected cell DNA was prepared as described by Stow *et al.* (1983). BHK21/C13 cell monolayers were grown in 24-well Linbro trays, infected at an moi of 5 pfu/cell and incubated at 31 °C for 48 hours. The supernates were transferred to screw neck vials and stored at -70 °C, and the cells lysed by the addition of 200 µl per well of cell lysis buffer containing 0.25 mg per ml proteinase K with incubation at 37 °C for 3 hours. The mixtures transferred to 1.5 ml tubes and the DNA extracted twice with an equal volume of saturated phenol and once with chloroform, precipitated by the addition of 1/20 vol of 5M NaCl and 2.5 vols of ethanol, dried and dissolved in 20 µl TE. Restriction endonuclease analysis was usually carried out on 10% of the total sample (2 µl).

(c) Quantitation of viral DNA

The viral DNA was quantified by agarose gel electrophoresis [see Section 7 (a)]. Various amounts of DNA (in volumes) to be quantitated were resolved on an ethidium bromide containing 0.9% agarose gel with a standard DNA of known concentration. DNA bands were visualised under UV light and photographed using Polaroid film. The intensities of the bands were compared to the intensity of the standard and the concentration of the DNA estimated.

(d) **Transfection of viral DNA**

Transfection was carried out as described by Graham and Van der Eb (1973) and modified by Stow and Wilkie (1976). BHK21/C13 cell monolayers in 50mm petri dishes were transfected with 2 µg of intact or partially digested virus DNA in a mixture containing 10 µg/ml calf thymus carrier DNA and 130mm $CaCl_2$ in HEBS (pH 7.05). The plates were incubated at 37 °C for 45 min, overlaid with 4 ml ETC5 or ETC10 and incubation continued at 37 °C. At 4 h post transfection, the cell monolayers were washed with ETC5 and given a dimethyl sulphoxide (DMSO) boost by adding 1 ml HEBS containing 25% (v/v) DMSO per plate at r.t for exactly 4 min. Following removal of DMSO, the cell monolayers were washed twice with ETC5 and overlaid with 4 ml ETC5 or MC. The plates were then incubated at either 37 °C (2-3 days) or 31 °C (3-5 days) and observed daily until the development of plaques. The plaques were picked directly from the MC overlaid plates and plate stocks grown from single plaque-pick sonicates for further analysis. For plates receiving ETC5 overlay, cells were scraped into the medium, pelleted at 2000 rpm for 10 min at 4 °C, resuspended in 1 ml medium, sonicated and titrated out. Single plaques were picked from the titrated transfection plate stocks and treated as described in section 3 (d).

For marker rescue of restriction endonuclease site-deleted variant in a cloned HSV-1 fragment, the transfection mixtures also contained 5, 10 and 20-fold molar excess of linearized plasmid or purified HSV-1 fragment over the intact viral genome. The procedure carried out for the isolation and analysis of individual plaques has already been described [Section 3 (d)].

(e) **Selection enrichment of virus DNA**

One microgramme of HSV-1 DNA was digested with 5 units of *Hind III* restriction endonuclease at 37 °C and samples were removed at 1/4, 1/2, 1, 2, 4 and 16 h. Digested DNA samples along with a sample of undigested HSV-1 DNA were transfected as described above [section 4 (d)] except that about 1 µg of viral DNA per plate was used. Single plaques were

isolated from the plate receiving digested DNA for the longest time and showing cpe. The transfected cells were harvested into the medium, sonicated and stored at -70 °C. Individual plaque isolates were analysed as described in sections 3 (d) and 4 (f) and the transfected plate stock was used to produce virus DNA [section 4 (a)]. This procedure was carried out for several rounds of selection enrichment (Jones and Shenk, 1978; Brown *et al.*, 1984).

(f) Preparation and isolation of ^{32}-P-labelled virus DNA

This is a modification of the method described by Lonsdale (1979). BHK21/C13 cell monolayers grown in 24-well Linbro trays in PIC medium were infected at an moi of 10 pfu/cell. The virus was adsorbed at 37 °C for 45 min and the medium removed using a fresh sterile tip for each well. The cells washed with 800 µl PIC, overlaid with 450 µl of PIC and incubated at 31 °C for 2 h. Fifty microlitre of PIC containing 1-5 µCi of ^{32}-P-orthophosphate was added per well and incubation continued at 31 °C for 48 h or until extensive cpe was observed.

^{32}-P-labelled virus DNA was harvested by the addition of SDS at a final concentration of 2.5% (v/v; for cell lysis) and incubation at 37 °C for 10 min. The DNA was extracted once with an equal volume of saturated phenol and precipitated with two volumes of ethanol. The DNA was dried by inverting the tubes upside down and incubating at 37 °C for 30 min and redissolved in 200 µl water. 20 µl per sample of DNA suspension was subjected to digestion with appropriate restriction endonucleases and run on appropriate concentration of agarose gels [see section 7 (a)] for 14-18 hours. The gels were dried at 85 °C for 4 h and set up for autoradiography using X-Omat XS1 film.

5. PLASMID DNA

(a) Large scale preparation and purification of plasmid DNA

Large scale preparation of plasmid DNA was carried out as described by Birnboim and Doly (1979). An overnight culture was grown by inoculating 5 ml L-broth containing the appropriate antibiotic with a bacterial colony from an L-broth agar plate or 25 µl from a bacterial stock [see section 6 (b)] at 37 °C in an orbital shaker. This culture was transferred into 500 ml L-broth containing the appropriate antibiotic in a 2 litre flask and shaken at 37 °C overnight.

The bacterial cells were pelleted by centrifugation at 8000 rpm (Sorval GSA rotor) for 10 min at 4 °C. The pellet was resuspended in 5 ml

alkaline lysis solution I (10mM EDTA, 50mM glucose, 25mM Tris-HCl pH 8.0 and 5 mg/ml lysozyme added prior to use) and incubated at r.t for 5 min. 10 ml of freshly prepared alkaline lysis solution II (0.2M NaOH and 1% SDS) was added, mixed by gentle inversion of the tube and incubated on ice for 10 min. 7.5 ml of an ice-cold alkaline lysis solution III (5M KAc, pH 4.8) was added, the contents mixed by inverting the tube sharply several times and incubation continued on ice for a further 10 min. The cell debris was pelleted at 15000 rpm (Sorval SS34 rotor) for 30 min at 4 °C and the supernatant transferred to a 50 ml sterile tube. The DNA was extracted several times with an equal volume of saturated phenol:chloro-form (1:1 v/v), once with an equal volume of chloroform and precipitated by the addition of two volumes of ethanol or 0.6 volumes of isopropanol at r.t for 15 min, followed by centrifugation (Sorval SS34 rotor) at 12000 rpm for 30 min at r.t. The DNA pellet was washed with 70% ethanol, dried in a vacuum desiccator and dissolved in 1-1.5 ml water or TE.

The DNA was further purified by caesium chloride/ethidium bromide gradients as described by Davison and Wilkie (1981). The volume of DNA suspension was adjusted to 10 ml with TE, 10.0g of caesium chloride were added to give a final density of 1.55 to 1.60 g/ml and ethidium bromide was added to a final concentration of 0.5 mg/ml. The samples were centrifuged in sealed plastic tubes at 40,000 rpm (Beckman type 65 rotor) for 36-48 h at 15 °C and DNA bands visualised under long wave-length UV light. The lower band containing the supercoiled plasmid DNA was collected with a syringe and the ethidium bromide removed by 4 successive extractions with an equal volume of isoamyl alcohol. The DNA was dialysed overnight against TE, precipitated with 2 volumes of ethanol and 0.1 volume of 3M sodium acetate, dried and redissolved in water.

The plasmid DNA was quantitated by running a small sample on an agarose gel along with a DNA sample of standard DNA of known concentration. DNA samples were visualised under UV light, photographed using Polaroid film and their intensities compared.

(b) **Small scale preparation of plasmid DNA**

Transformed bacterial colonies [Section 6 (c & e)] were picked using a cocktail stick and resuspended in 5 ml L-broth containing 100 µg/ml ampicillin and shaken at 37 °C overnight. 1.5 ml samples were pelleted in Eppendorf tubes at 13000 rpm for 1 min in a microfuge, resuspended in 100 µl alkaline lysis solution I (see above) and incubated at r.t for 5 min. 200 µl alkaline lysis solution II was added and mixed briefly by inverting the tubes

several times before incubating on ice for 5 min. 150 µl ice-cold alkaline lysis solution III was added, mixed by vortexing briefly and incubated on ice for a further 5 min. The cell debris was removed by centrifugation in a microfuge and the supernatant containing the plasmid DNA was extracted once with an equal volume of phenol:chloroform (1:1) and once with an equal volume of chloroform:isoamyl alcohol (24:1). Two volumes of ethanol were added and the DNA precipitated at r.t for 2 min. The DNA was pelleted in a microfuge for 5 min, washed with 70% ethanol, dried in a vacuum desiccator and redissolved in 50 µl water. 10 µl of DNA samples were used to set up restriction endonuclease digestion at the appropriate temperature.

6. BACTERIA

(a) Growth of bacterial cultures

Bacterial cultures were grown overnight at 37 °C in L-broth or 2X YT broth containing 100 µg/ml ampicillin, where appropriate. The cultures were set up using either a single colony picked from an agar plate or 25 µl of a bacterial stock (see below) and allowed to stand or shake.

(b) Glycerol/DMSO stocks of bacteria

For glycerol stocks of bacteria, 10 ml of overnight grown cultures were centrifuged at 2800 rpm for 10 min and pellets were resuspended in 5 ml of 2% bactopeptone. 5 ml of 80% glycerol was added to the suspension, mixed by gentle inversion of the tube and stored at both -20 °C and -70 °C. DMSO stocks were prepared using the same procedure except that the pellets were resuspended in L-broth containing 10% (v/v) DMSO. The DMSO stocks were stored only at -70 °C.

(c) Preparation of ampicillin/tetracycline/kanamycin plates

L-broth agar, in a 350 ml bottle was melted and allowed to cool gradually at r.t until hand hot. Ampicillin (100 µg/ml), kanamycin (70 µg/ml) or tetracyclin (12.5 µg/ml) was added and the agar poured into 90mm sterile petri dishes and allowed to set at r.t for approximately 30 min. The plates were dried for 4-16 h at 37 °C in an inverted position and stored at 4 °C for up to one month before being used.

(d) Preparation of competent bacteria

A 5 ml bacterial culture was set up as described [section 6 (a)]. One ml of this culture was used to inoculate 40 ml L-broth in a 250 ml flask and shaken at 37 °C until an optical density (OD) of 0.2 at 630nm (approximately 2 - 2 &1/2 h). The bacterial cells were pelleted at 9000 rpm for 1 min in a Sorval SS34 rotor at 4 °C. The cell pellet was resuspended in 20

ml of 10mM $CaCl_2$, vortexed briefly and pelleted again before being resuspended in 20 ml of 75mM $CaCl_2$. The cell suspension was placed on ice for 20 min and centrifuged again at 9000 rpm for 1 min at 4 °C. The pellet was resuspended in 1-2 ml of 75mM $CaCl_2$ and divided into 100 µl aliquots. 25 µl of 80% glycerol was added to each aliquot, mixed and frozen in a -70 °C dry ice/ethanol bath for 5 min followed by storage at -70 °C (Maniatis *et al.*, 1982).

(e) **Transformation of bacterial cells**

An aliquot of competent bacterial cells was thawed on ice and divided into 20 µl aliquots in chilled sterile Eppendorf tubes. One microlitre of ligation mix or plasmid DNA (10-50 ng) were added to the cells, shaken gently and incubated on ice for 30 min. The cells were then heat shocked in a 42 °C water bath for 40-60 seconds, placed back on ice for 60 seconds before adding 80 µl S.O.C. The Eppendorf tubes were shaken at 225 rpm at 37 °C for 1 h and the cells were spread onto agar plates containing appropriate antibiotic [see section 6 (c)]. The plates were incubated overnight at 37 °C in an inverted position, individual bacterial colonies were picked from the plates and analysed [section 5 (b) and Hanahan, 1983].

(f) **Preparation of bacteriophages M13 R408 and M13 K07 stocks**

Bacteriophages M13 R408 and M13 K07 were diluted 10-fold in 2X YT broth. 200 µl of overnight grown bacterial culture (for example, *E coli* strain NM522) and 100 µl of each dilution of bacteriophage were added to 3 ml of top agar (TA; maintained at 45 °C) and poured onto L-broth agar plates containing kanamycin (for M13 K07) or no antibiotic (for M13 R408). The plates were allowed to set at r.t for 15 min and incubated at 37 °C overnight in an inverted position. Single colonies (M13 K07) or plaques (M13 R408) were picked to grow 5 ml cultures in 2X YT broth overnight. These cultures were then used to inoculate 250 ml 2X YT broth and shaken at 37 °C overnight. The bacterial cells were pelleted at 12000 rpm (Sorval GSA rotor) for 15 min at 4 °C, the supernates transferred to fresh GSA bottles and centrifuged again at 12000 rpm for 15 min (to remove the remaining bacterial cells). The supernates containing bacteriophages were transferred to sterile bottles and placed at 55 °C for 30 min (to kill bacterial cells, if any). These stocks were then titrated out (colonies or plaques on plates containing various bacteriophage dilutions were counted to calculate the phage titre) and stored at 4 °C.

7. GEL ELECTROPHORESIS

(a) **Agarose gel electrophoresis**

Restriction endonuclease analysis used 0.5-1.8% (w/v) agarose gels boiled in 250 ml 1X TBE or 1X E buffer. Once hand hot, the solutions were poured onto glass plates (16.5 x 26.5 cm) whose edges had been sealed with gel tape and onto which 15-tooth well-forming combs had been placed. For non-radioactive samples, 1.5 µg/ml ethidium bromide were added to the agarose solution immediately before pouring. The gels were allowed to set at r.t for 1 h and then transferred to horizontal tanks containing 2-3 l of 1X TBE or E buffer. DNA samples were mixed with one-fourth volume of RE stop and loaded into the gel tracks. The gels were electrophoresed at either 45-90V overnight (for HSV-1 DNA) or 30-120V for 3-16 (for plasmid DNA). For non-radioactive samples, DNA bands were visualised by exposure to UV light and photographed on Polaroid film [see section 4 (f) for radioactive samples].

(b) **Polyacrylamide gel electrophoresis**

For purification or analysis of oligonucleotides, vertical slab gels were poured using 16% sequencing gel mix (80 ml of 29:1 acrylamide : N,N'-methylene bisacrylamide stock solution and 8.3M urea in 1X TBE). The final solution (60 ml) containing 9.6% polyacrylamide in TBE was polymerized with 600 µl of 10% ammonium persulphate and 25 µl of TEMED. The gels were formed between two 16 x 19 cm glass plates separated by 1.5mm spacers and sealed with kapton tape. 8-tooth teflon combs were used to make approximately 1 cm wide wells for loading DNA samples. The gels were transferred to vertical tanks containing 1X TBE, samples loaded into the wells and electrophoresed at 30 mA for 3-4 h.

(c) **SDS-PAGE polyacrylamide gel electrophoresis**

This procedure was carried out essentially as described by Studier (1973) and modified by Marsden *et al.* (1976, 1978). Gels were set up in a sandwich of glass plates (20 x 22 cm) separated by 1.5mm thick perspex spacers and sealed with kapton tape. Two types of resolving gel were used:

(i) Single concentration gel solutions were prepared with a ratio of acrylamide to N,N'-bisacrylamide (cross-linking reagent) of 29.25:0.75 in 375mM Tris-HCl pH 8.9 and 0.1% (w/v) SDS.

(ii) Gradient gels consist of a 5%-12.5% linear gradient of acrylamide cross-linked with 5% (w/v) of N,N'-bisacrylamide in resolving gel buffer.

For polymerisation, 0.006% (w/v) of ammonium persulphate (APS) and 0.004% (w/v) of N,N,N',N'-tetramethylenediamine (TEMED) were added prior to pouring the gels into the sandwich of glass plates. Buton-2-ol was gently layered on top of the gels to ensure a smooth interface on

polymerisation and was rinsed off using either unpolymerised stacking gel solution or deionized water prior to addition of the stacking gel. The stacking gels were prepared using a solution of 5% acrylamide (prepared as above) in stacking gel buffer, to which had been added APS and TEMED as above. This was then layered on top of the resolving gel and a teflon comb was inserted for the formation of the wells. Protein samples were boiled for 5-10 min in a boiling water bath in sample buffer (50mM Tris-HCl pH 6.7, 2% SDS, 700mM 2-mercaptoethanol, 10% glycerol and a small amount of bromophenol blue), loaded and the gels electrophoresed in acrylamide tank buffer at 10-12 mA overnight. The gels were fixed/stained for 1 h in methanol:acetic acid:water (50:7:43), destained in methanol:water:acetic acid (5:88:7) for 1-2 h and heat-dried under vacuum onto Whatman filter paper. The gels were then exposed to autoradiography films at either r.t or -70 °C.

8. RESTRICTION ENZYME DIGESTION OF DNA

Restriction endonuclease digestion of plasmid and viral DNA was carried out in the presence of manufacturer's recommended conditions. DNA were usually digested in a final volume of 30 µl per sample containing the appropriate concentration of commercial restriction endonuclease buffer (BRL or Boehringer Mannheim) and the restriction enzyme (2-5 units/µg DNA) at 37 °C for 4 h. The reaction was stopped either by the addition of 10 µl of RE stop (see Materials) or by phenol : chloroform (1:1) extraction [see sections 4(f), 5(b) and 7(a)]. For *Xba I*, *Xba I / Hind III* and *Xba I / Bgl II* restriction enzyme digestions of viral DNA, the following buffer was also used:
100mM NaCl, 6mM $MgCl_2$, 6mM Tris-HCl pH7.4 and 0.01% BSA.

9. DNA CLONING

(a) Elution of DNA fragments from agarose gels

Plasmid or HSV-1 DNA were cleaved with the appropriate restriction enzyme and electrophoresed on an agarose gel containing 1.5 µg/ml ethidium bromide. The DNA was visualised under long wave-length UV light and the relevant fragment excised from the gel. The gel slice was placed in a pre-boiled (10 min in 1X TBE) dialysis tubing with 5 ml of 1X TBE and placed in an electrophoresis tank containing 1X TBE. Electroelution was carried out at 200V for 2-3 h to transfer the DNA fragment into the dialysis tubing. The current was briefly reversed to remove the DNA from the side of the tubing and the buffer containing the DNA transferred to a sterile tube. The DNA was extracted once with an equal volume of phenol: chloroform (1:1),

once with an equal volume of chloroform and then precipitated with 2 volumes of ethanol and 0.1 volume of 5M NaCl. The DNA was washed with 70% ethanol, lyophilised and resuspended in H_2O.

In some instances, the DNA was further purified over a DEAE-sephacel column. An empty DEAE-sephacel column was washed sequentially with sterile water (sd H_2O), 1M NaOH, 1M Tris-HCl pH 7.5 and sd H_2O before packing the column with sephacells [sephacells were kept at 4 °C in an equilibrated (v/v) solution of 10mM Tris-HCl pH 7.6, 1mM EDTA and 60mM NaCl] up to 0.5 ml mark. The sephacells were washed with 1.5 ml of NTE (TE + 0.1M NaCl) and then the buffer (TBE) containing the DNA fragment was loaded into the column. The flow through was collected and re-applied to the column. The DNA bound to the sephacells was washed with 2.5 ml of NTE (as above) and eluted with two aliquots of 250 µl of NTE. The eluted DNA was precipitated with 2 volumes of ethanol overnight at -20 °C or -70 °C, washed with 70% ethanol, dried in a vacuum desiccator and redissolved in 20 µl water. The DNA was quantitated by running a small sample onto an agarose gel along with standard of known concentration.

(b) **Filling in 5' overhangs with Klenow polymerase**

Linearised plasmid DNA, containing 5' single strand overhangs, was purified by phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1) extractions, ethanol precipitated, dried and resuspended in water. To 1 µg of this DNA, 4 µl of 10X NT (50 µg/ml BSA, 1mM DTT, 5mM $MgCl_2$, 50mM Tris-HCl pH 7.8), 10 µl of dNTPs (cold; 2mM each), 1 µl (2 units) of Klenow polymerase (large fragment of *E coli* DNA polymerase I, which carries the 5'---->3' polymerase activity and the 3'---->5' exonuclease activity of intact DNA polymerase I but lacks the 5'----->3'exonuclease) were added in a final volume of 40 µl and the mixture incubated at r.t for 1 h. The reaction was stopped with phenol:chloroform extraction followed by ethanol precipitation and lyophilization. The DNA pellet was resuspended in an appropriate volume of water (Maniatis *et al.*, 1982).

(c) **Ligation of linkers**

Plasmid vector (for example, pAt153 or pGEM-1) was digested with an appropriate restriction endonuclease in the presence of 1 unit of calf intestinal phosphatase to minimise re-ligation of plasmid molecules. The recessed ends created by restriction enzyme digestion were filled with klenow polymerase (as above) and one microgramme of this DNA was incubated at 15 °C overnight in ligase buffer (50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 1mM ATP, 20mM DTT, 50 µg/ml BSA) containing 2 units of T4 ligase and 100-fold

molar excess of commercial linker. The ligation mixture was extracted once each with phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1), ethanol precipitated, lyophilised and resuspended in water. 10-50 ng of ligated DNA were transformed into bacteria [see section 6 (e)] and the resultant colonies analysed [section 5 (b)].

(d) Cloning of DNA fragments

HSV-1 DNA fragments were cloned into the appropriate restriction endonuclease sites of the plasmid vector pAT153 or in the polylinker of pTZ18U or pTZ19U [see Materials 6 (a)]. The linearised vector was treated with calf intestinal phosphatase (CIP; 1 unit per μg of plasmid DNA) in CIP buffer (50mM Tris-HCl pH 9.0, 1mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1mM spermidine) at 37 °C for 1 h. The DNA was then extracted twice with phenol:chloroform (1:1) and once with chloroform, ethanol precipitated, lyophilised and resuspended in water. The purified HSV-1 fragments were ligated at a ratio of 5:2 insert:vector in ligase buffer (see above) containing 2 units of T4 ligase at 15 °C overnight.

10. HYBRIDISATION

(a) Transfer of DNA to nitrocellulose

Restriction endonuclease digested DNA samples were run on an agarose gel in 1X TBE or 1X E buffer for Southern transfer (Southern, 1975). Following electrophoresis, the gel was visualized under UV light and photographed using a Polaroid film. The gel was sequentially placed in gel soak I for 45 min (for denaturation) and gel soak II for 45 min (for neutralisation). The DNA was then transferred overnight onto a wet Hybond blotting membrane using 10X SSC, a wick of Whatman 3 mm paper and a weighted capillary stack of absorbent paper towels. The Hybond blotting membrane was air dried and UV cross-linked using Stratalinker UV crosslinker (Stratagene, La Jolla, CA 92037, USA) prior to hybridisation.

In some instances, the capillary transfer of DNA from a single agarose gel was carried out simultaneously on to two Hybond blotting membranes. After neutralization, the gel was soaked in 20X SSC for 30-60 min and was covered from above and below with a presoaked blotting filter. Presoaked 3 mm papers were placed at the bottom and the top of the sandwich of blotting filters and gel and then the entire sandwich was transferred onto a stack of paper towels. The sandwich was covered with a second stack of paper towels onto which a heavy weight was placed

(Sambrook *et al.*, 1989). Following transfer of DNA, the filters were treated as described above.

(b) ***In vitro*** **^{32}P-labelling of DNA by nick-translation**

The procedure followed for nick-translation of DNA was exactly as described by Rigby *et al.*, (1977). 200-300 ng of DNA was radiolabelled in a reaction mixture containing 1X NT buffer (50mM Tris-HCl pH 7.8, 5mM $MgCl_2$, 1mM DTT), 50µg/ml BSA, 2 units of DNA polymerase I, 2µCi [α-^{32}P] dCTP or 2µCi [α-^{32}P] dGTP, 0.2mM each of cold dNTPs (dATP, dTTP, dGTP or dCTP) and 2.5µl of the diluted DNAase I (10ng/ml) in a final volume of 25µl. The reaction mix was incubated at 16 °C for 60 min and then the DNA was precipitated with 0.6 volumes of isopropanol and 0.1 volume of 3M sodium acetate on dry ice for 15 min. The precipitation of DNA was repeated and the resultant pellet was resuspended in 25µl of 80% aqueous formamide solution. The probe was denatured by boiling for 10 min prior to use.

(c) **5' end-labelling of oligonucleotides**

Oligonucleotides (50-100ng) were dissolved in kinase buffer (0.1mM EDTA, 10mM DTT, 10mM $MgCl_2$, 1mM spermidine, 50mM Tris-HCl pH 7.6) containing 20µCi of [γ-^{32}P]-ATP and 2 units of T4 polynucleotide kinase. The mixture was incubated at 37 °C for 1 h and the DNA extracted once each with an equal volume of phenol, phenol:chloroform (1:1) and chloroform and precipitated with 2 volumes of ethanol and 0.1 volume of 5M NaOH on dry ice. The DNA was pelleted in a high speed microfuge, dried in a vacuum desiccator and resuspended in 80% aqueous formamide solution.

(d) **DNA/DNA Hybridisation**

The Hybond blotting membrane containing separate DNA fragments [see above (a)] was incubated at 65 °C - 75 °C for 2-3 h in 20ml of prehybridisation buffer (6X SSC, 0.1% Ficoll 400, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1 mg/ml single-stranded salmon sperm DNA and 10mM Tris-HCl pH 7.5) in a heat-sealed polythene bag. The prehybridisation buffer was replaced by hybridisation buffer I or II (see Materials) containing the denatured labelled DNA and incubation continued at 65-75 °C for 16-48 h with constant agitation. The membrane was then removed and washed, three times for 1 h each, in wash buffer (1X SSC, 0.1% SDS) at 65 °C. The Hybond membrane was air dried and autoradiographed.

With oligonucleotide probes, the hybridisation was usually carried out at 37 °C - 45 °C and the membranes were washed briefly at room temperature.

11. PREPARATION OF CALF THYMUS DNA

Calf thymus (CT) carrier DNA was prepared by dissolving 100mg of CT DNA in 10ml of water at 37 °C. The DNA was sonicated three times for 30 seconds each, with 30 seconds intervals for incubation on ice, in a probe sonicator. It was then sequentially extracted, 3 times each, with phenol and ether and dialysed against water overnight. The OD_{260nm} was measured and the concentration of DNA adjusted to 1 mg/ml before being stored at -20 °C.

12. OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS

(a) Synthesis and purification of synthetic oligonucleotides

Oligonucleotides were prepared by Dr J McLauchlan using a Biosearch 8600 DNA synthesizer. The DNA was eluted from the column by resuspending the beads in 1ml of ammonia. The ammonia solution was incubated at 55 °C for 5 h, aliquoted into two halves and the DNA was dried overnight in a vacuum desiccator. One-half of the DNA was stored at -20 °C and the remainder was redissolved in 30 µl of 90% (v/v) deionized formamide in 1X TBE. Oligonucleotides were then loaded onto a denaturing polyacrylamide gel [16%; Section 7 (b)] for purification. The gel was run with a separate dye track (2µl of formamide-dye mix) to monitor progress and the electrophoresis stopped after the dye had migrated 2/3 through the gel. The gel was removed from the plates, wrapped in cling film and the DNA bands visualized by the shadow casting technique, in which the gel was placed on a fluorescent thin layer chromatography plate with an angled long wave-length UV light lamp. The oligonucleotide appears as a dark, predominant band along with a few lower molecular weight bands. The top band was excised, diced and incubated at 42 °C overnight in 1-1.5 ml elution buffer (0.5M ammonium acetate, 1mM EDTA. 0.1% SDS). The sample was then filtered through siliconised glass wool in a small Eppendorf tube (0.5ml) punctured 3-4 times with a medium gauge needle, and flow through was collected into larger Eppendorf tube (1.5ml). The DNA was extracted, precipitated, lyophilised and resuspended in 50 µl water. The concentration of DNA was determined by reading OD at 260nm following the conversion factor for synthetic oligonucleotides of 1 OD unit = 20 µg/ml. DNA solution volumes were re-adjusted to give a concentration of 0.1 µg/µl.

(b) Adding 5' phosphate to non-phosphorylated DNA

DNAs were 5' phosphorylated by incubating 100-500 ng in a mixture containing 1X kinase buffer (0.1mM EDTA, 10mM DTT, 10mM $MgCl_2$, 1mM spermidine, 50mM Tris-HCl pH 7.6), 4 µl of 10mM ATP, 2 units of

polynucleotide kinase in a final volume of 40 µl, at 37 °C for 30 min. These were extracted once each with phenol:chloroform and chloroform, ethanol precipitated, washed with 70% ethanol, lyophilized and resuspended in water.

(c) **Preparation and purification of uracil-rich single-stranded DNA**

Recombinant plasmid DNAs (HSV-1 fragments cloned into pTZ range of phagmid expression vectors) were transformed into *E coli* strain BW313 (ung^-, dut^-), bacterial colonies picked and 10 ml cultures grown overnight in 2X YT broth containing 100 µg/ml ampicillin. 250 ml of 2X YT broth containing 0.25 µg/ml uridine was inoculated with 5 ml of the overnight culture and shaken at 37 °C for 30 min or until an OD of 0.3 at 260nm. The culture was then infected with helper phage M13 KO7 or M13 R408 at a multiplicity of infection of approximately 20 and shaken again at 37 °C for 30 min. Thereafter, kanamycin (75 µg/ml; depending on the helper phage used) was added to the culture and incubation continued overnight. The supernatant was harvested by pelleting the cells at 12000 rpm (Sorval GSA rotor) for 15 min at 4 °C and the centrifugation was repeated using a fresh 250 ml centrifuge-bottle before the phage were precipitated by the addition of 0.25 volume of 3.5M ammonium acetate/20% (w/v) polyethylene glycol 6000 solution. The contents were mixed by swirling the bottle, incubated on ice for 30 min and then centrifuged at 12000 rpm for 15 min at 4 °C. The bottle was thoroughly drained and the pellet was resuspended in 500 µl of TE (10mM Tris-HCl pH 8.0, 1mM EDTA). An equal volume of phenol:chloroform (1:1) was added, the tube vortexed for 1 min and centrifuged in a high speed microfuge for 5 min. The top aqueous phase containing ssDNA was transferred to a fresh sterile Eppendorf tube and the DNA extraction repeated until there was only a slight interphase left (approximately 5-6 times). The ssDNA was then extracted with an equal volume of chloroform, precipitated with 0.1 volume of 3M sodium acetate and 2 volumes of ethanol at -70 °C for 30 min and pelleted in a high speed microfuge for 5 min. The pellet was washed with 95% ethanol, dried in a vacuum desiccator and redissolved in 100 µl H_2O. The suspension was transferred to a fresh Eppendorf tube and the OD at 260nm determined (1 OD_{260nm} = 40 µg/ml).

(d) **Site-directed mutagenesis**

Specific mutations were introduced into the plasmids containing HSV-1 fragments using the following two methods:

(i) **The method using a gapped, heteroduplex molecule of DNA**

The method used was essentially as described by Oostra *et al.* (1983) and Liang *et al.* (1986). The HSV-1 DNA fragment carrying the target sequence was cloned into plasmid vector pAT153. The plasmid DNA suspension was divided into two equal volumes (usually 5 µg per 10 µl each). One batch of DNA was cleaved with an enzyme whose only site was located outwith the HSV-1 fragment. The second was cleaved with one or two enzymes whose only sites (two sites in total) surrounding the sequence to be mutated. Following restriction enzyme digestion, both batches of plasmid DNA were extracted once with phenol:chloroform (1:1), ethanol precipitated, washed in 70% ethanol, lyophilized and resuspended in 10 µl H_2O. The plasmid populations were then mixed and denatured by incubating with NaOH to a final concentration of 0.2M at room temperature for 20 min. The DNA was reannealed (to form 'heteroduplexes') by sequential addition of 200 µl water, 25 µl Tris-HCl pH 8.0 and 50 µl 0.1M HCl, followed by incubation at 63 °C for 3 h. The gapped duplex DNA was precipitated with ethanol, dried and resuspended in 25 µl of buffer containing 10mM Tris-HCl pH 7.4, 50mM NaCl and 1mM EDATA. 200 pmoles of the appropriate (a 100-fold excess) phosphorylated mutagenic oligonucleotide (with a single base change) was added to the DNA suspension and incubation was carried out at r.t for 2 h to allow annealing of the oligonucleotide with the cloned HSV-1 fragment. After 2 h incubation, 6 µl H_2O, 10 µl 10X ligase buffer (0.5M Tris-HCl pH 7.8, 100mM $MgCl_2$, 200mM DTT), 2 µl 50mM ATP, 20 µl dNTPs (containing 10 mmoles of all four deoxynucleotide triphosphates), 5 µl (5 units) Klenow polymerase, and 2 µl (2 units) T4 DNA ligase were sequentially added and the mixture was incubated overnight at r.t or 15 °C. The DNA was extracted once with phenol:chloroform (1:1), precipitated with ethanol, dried and redissolved in 25 µl H_2O. 10 µl of DNA was digested with *Hind III* (site to be mutated) to enrich those molecules lacking *Hind III* sites, followed by transformation into *E coli* along with the undigested DNA. The resultant colonies were screened by restriction enzyme digestion analysis [section 5 (b)]. This method is diagramatically represented in Figure 14.

(ii) **The Kunkel method**

This method (Kunkel, 1985; Kunkel *et al.*, 1987) employs a single-stranded (ss) DNA template containing uracil-rich residues. These templates are generated in *E coli* strains carrying the dut$^-$ ung$^-$ mutations. *E coli* dut$^-$ mutants lack the enzyme dUTPase (normally converts dUTP to dUMP) and therefore contain elevated concentrations of dUTP which effectively competes with TTP for incorporation into DNA. *E coli* ung$^-$ mutants

Figure 14. **The Site-Directed Mutagenesis Method Using A Gapped, Heteroduplex Molecule Of DNA**
(Oostra *et al.*, 1983; Liang *et al.*, 1986)

(a) One batch of plasmid (containing the HSV-1 fragment) DNA is cleaved with a restriction endonuclease (RE) whose only site is located outwith the cloned HSV-1 fragment.

(b) Second batch of DNA is cleaved with one or more restriction endonucleases whose only sites (two sites in total) are located around the target sequence (*Hind III* site in the example given).

(c) The two batches of cleaved DNA are then mixed, denatured and reannealed to form gapped heteroduplex molecules, including the example shown. The mutagenic oligonucleotide is then hybridized to the DNA molecule.

(d) The single-stranded gaps are filled-in with ***Klenow*** polymerase and dNTPs and ligated with T4 DNA ligase. Following transformation into *E. Coli*, the resultant bacterial colonies are screened for plasmids containing the mutagenic oligonucleotide.

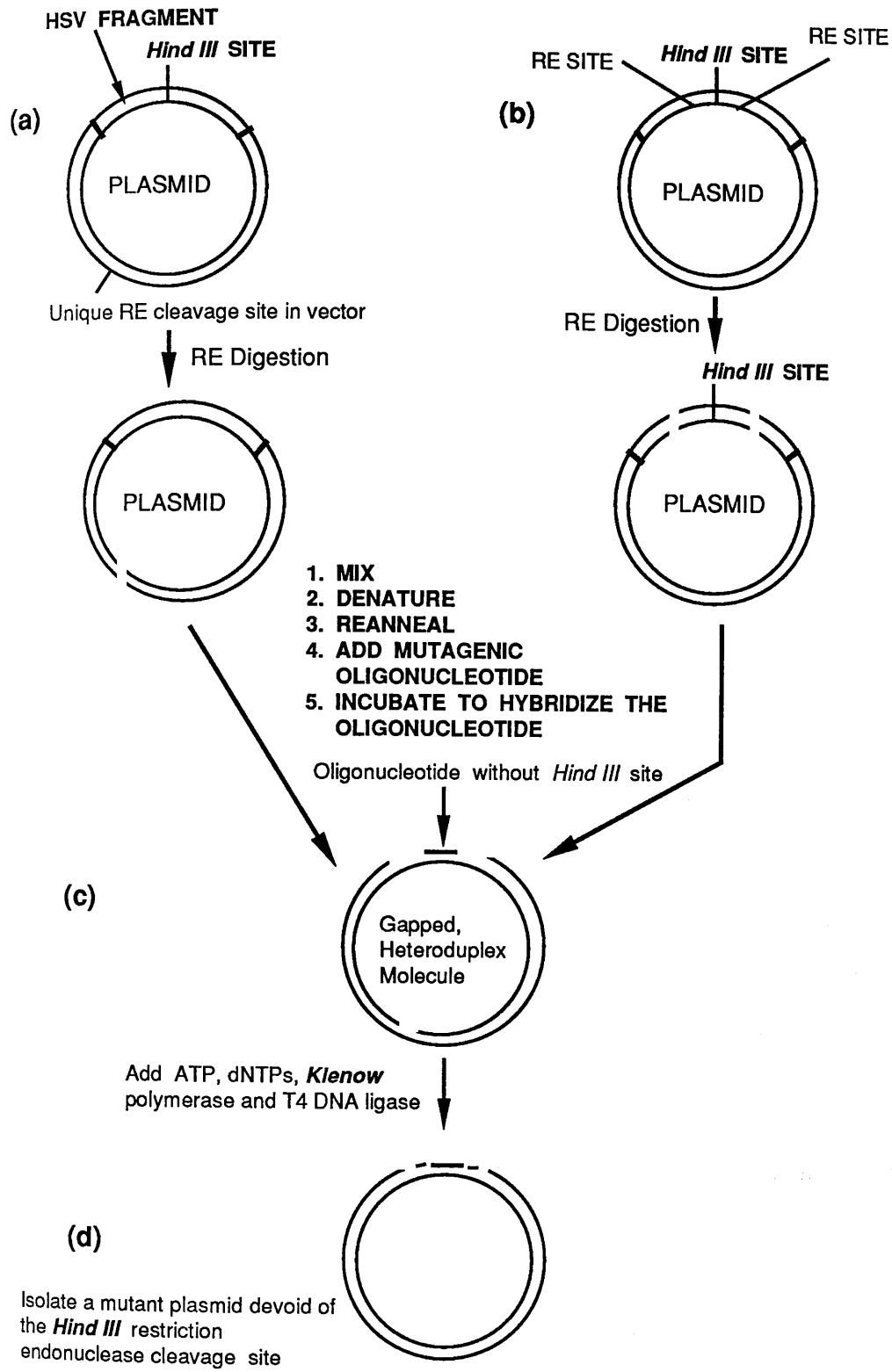


Figure 14. **Schematic Illustration Of The Site-Directed Mutagenesis Method Using A Gapped, Heteroduplex Molecule Of DNA**

lack the enzyme uracil N-glycosylase which normally removes uracil from DNA. When the combined dut$^-$ ung$^-$ mutant bacteria are transformed with pTZ phagemid vectors [see above (c)] and grown with an infection of a helper phage, uracil is incorporated into ssDNA in place of thymine and is not removed. This ssDNA is then used as the template in a mutagenesis procedure, in which a mutagenic oligonucleotide is annealed with the template to generate hybrid molecules and the mixture is then allowed to synthesize the complementary strand containing the desired mutation and thymine in an *in vitro* reaction. The DNA molecules are transformed into an ung$^+$ dut$^+$ strain (for example, DH5 or NM522) in which the uracil-rich template strand is destroyed along with suppression of the production of wild-type molecules. As a result, a majority of the progeny molecules are produced from the replication of the transfected uracil-free strand, many of which carry the specific mutation. This is outlined diagramatically in Figure 15.

The oligonucleotide carrying specific mutation was 5'phosphorylated [see above (b)] and annealed to the appropriate uracil-rich ssDNA [see above (c)] at a molar ratio of 20:1 by incubating at 65 °C for 10 min, then r.t for 5 min. The primer extension reaction was carried out at r.t for 4-16 h in the presence of 1mM ATP, 100 µM each of dATP, dCTP, dGTP and dTTP, 100 µg/ml gene 32 protein, 2 units of Klenow polymerase, 4 units of T4 DNA ligase and 1X ligase buffer (20mM DTT, 10mM $MgCl_2$, 50mM Tris-HCl pH 7.8). The reaction mix was transformed into *E coli* DH5 cells (dut$^+$ ung$^+$) and plated onto L-broth agar containing 100 µg/ml ampicillin [see section 6 (c & e)]. Individual colonies were picked and the mutants identified by preparing DNA samples on a small scale [section 5 (b)] and analysing the DNAs with appropriate restriction enzymes.

13. COMPUTING

Computer analysis of various plasmid vectors and HSV-1 DNA sequences was carried out on the MicroVAX 11 and PDP11/14 computers in this institute, using the University of Wisconsin Genetics Computer Group software package.

14. IMMUNOPRECIPITATION

The immunoprecipitation experiments were kindly performed by Dr C A MacLean.

Figure 15. **Site-Directed Mutagenesis Using The Kunkel Method**

(A) Grow the pTZ phagemid containing the cloned HSV-1 fragment in dut^-, ung^- strain of *E. Coli* to generate uracil-rich DNA molecules.

(B) Infect the bacterial culture with helper phage to generate uracil-rich single-stranded (ss) DNA.

(C) Carry out the *in vitro* mutagenesis reaction using a mutagenic oligonucleotide followed by second-strand synthesis using ***Klenow*** polymerase and dNTPs.

(D) Transform into dut^+, ung^+ *E. Coli* strain.

(E) Screen the resultant bacterial colonies and isolate the mutant plasmids.

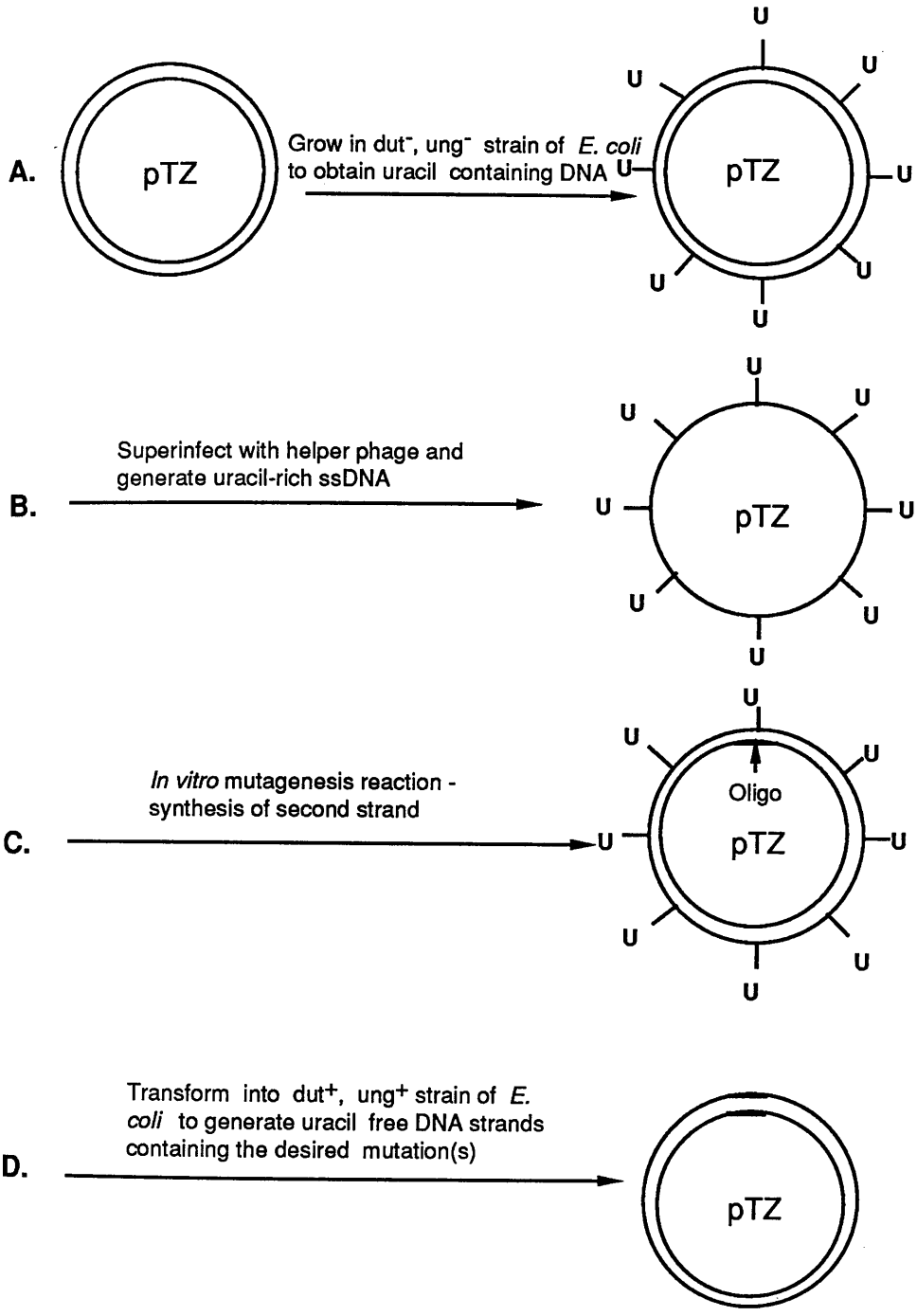
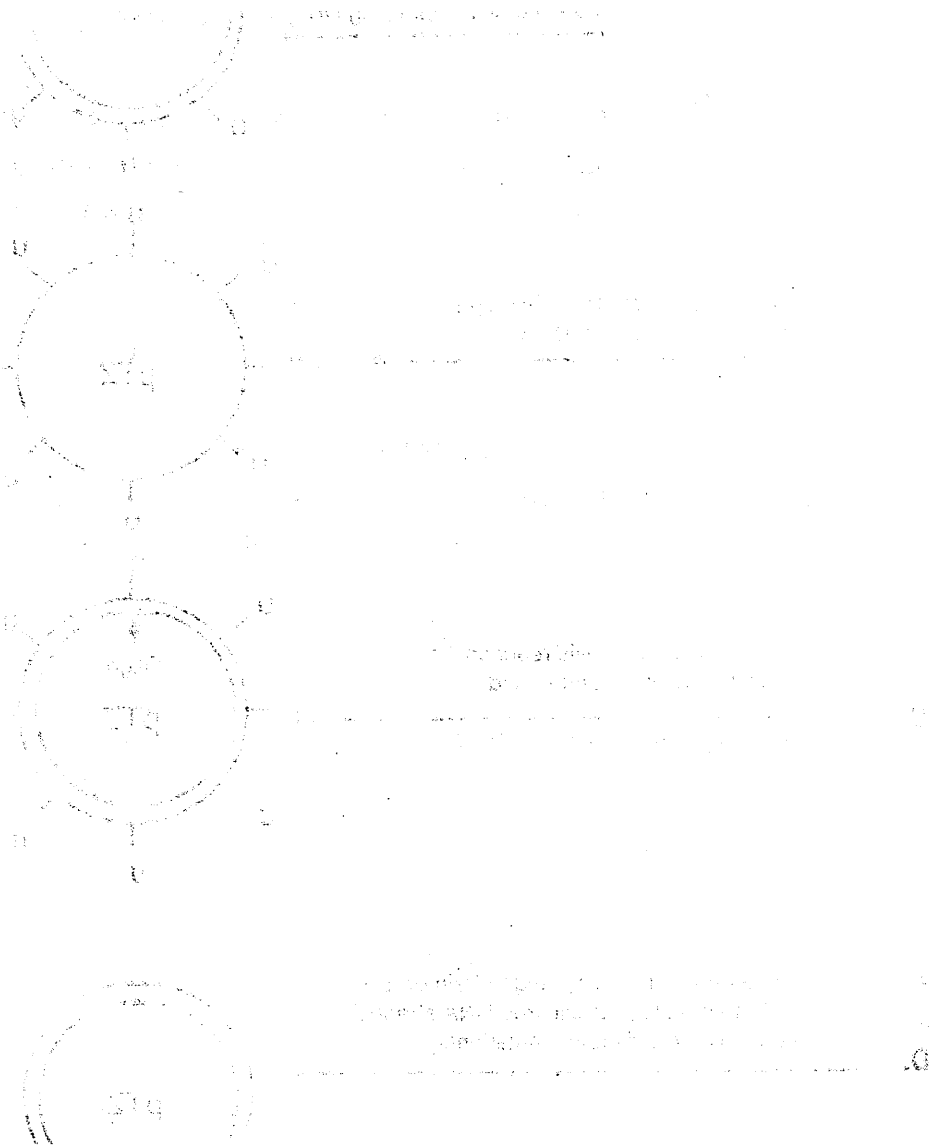


Figure 15. **The Kunkel Method For Site-Directed Mutagenesis**



* Three viruses were only used once in an attempt to construct the variant virus 1733 by coinfecting the variants 1722, 1726 and 1728 [see Section 3B:1 (a) (vii)] whereas in experiments to analyse recombination frequency, only two viruses were used.

adsorbed at 37 °C for 45 min and the cell monolayers were washed with PBS and incubated in ETC10 at 37 °C. At 24 h post infection, the cells were harvested, sonicated and titrated out at 37 °C or 31 °C. Individual plaques were picked, their Linbro-well stocks prepared and stored at -70 °C. ^{32}P-labelled DNA of the isolates were then prepared and subjected to RE analysis to determine the presence or absence of *Bgl II*, *Hind III* and *Xba I* RE sites [see section 4 (f)].

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CHAPTER 3:

RESULTS

RESULTS

SECTION 3A : RECOMBINANT PLASMIDS

1. WILD TYPE PLASMIDS

Apart from the recombinant plasmids [containing cloned wild-type *(wt)* HSV-1 DNA fragments] listed in Section 2A:6 (b) (see Materials), the following *wt* plasmids were constructed during the course of the research presented in this thesis. The nucleotide numbers (n.) used throughout the work described here represent the numbering system of the HSV-1 strain 17+ DNA sequence (McGeoch *et al.*, 1988b). The nucleotide numbers given in the following sections represent the first base of the respective restriction endonuclease (RE) site of HSV-1 DNA.

(a) Construction of the plasmid pMF1

To construct the plasmid pMF1, the plasmid vector pAT153 (Twigg and Sherratt, 1980) was completely digested with *Hind III* (pAT153 contains a single *Hind III* site at position 29; see Fig. 16) and the resulting 5' termini were filled-in with ***Klenow*** fragment of DNA polymerase I [see Section 2B:9 (b); Maniatis *et al.*, 1982]. A *Bgl II* linker was then ligated [see Section 2B:9 (c)] to generate a plasmid pF101, devoid of the *Hind III* site.

The HSV-1 *Bgl II **n*** (n. 35729 to 41448) fragment was eluted out [see Section 2B:9 (a)], purified and cloned into the *Bgl II* site of pF101 to generate the plasmid pMF1. The construction of pMF1 is diagramatically represented in Figure 16.

(b) Construction of the plasmid pMF2

This plasmid was constructed following an identical procedure used for the construction of pMF1 (see above). The HSV-1 *Bgl II **o*** (n. 25380 to 30671) fragment was ligated into the *Bgl II* site of pF101 and a clone containing the *Bgl II **o*** fragment was isolated and analysed with various restriction endonucleases (data not shown).

(c) Construction of the plasmid pMF3

To construct pMF3, the cloned HSV-1 *Bam HI **n*** (n. 131399 to 136289) fragment in pAT153 [see Section 2A:6 (b)] was cleaved with *Eco RI* and *Bam HI* restriction endonucleases and run on an agarose gel. The resulting HSV-1 (n. 131534 to 136289) and pAT153 (3284 bp) *Eco RI-Bam HI* fragments were eluted out, purified, ligated and transformed into *E. coli* DH5

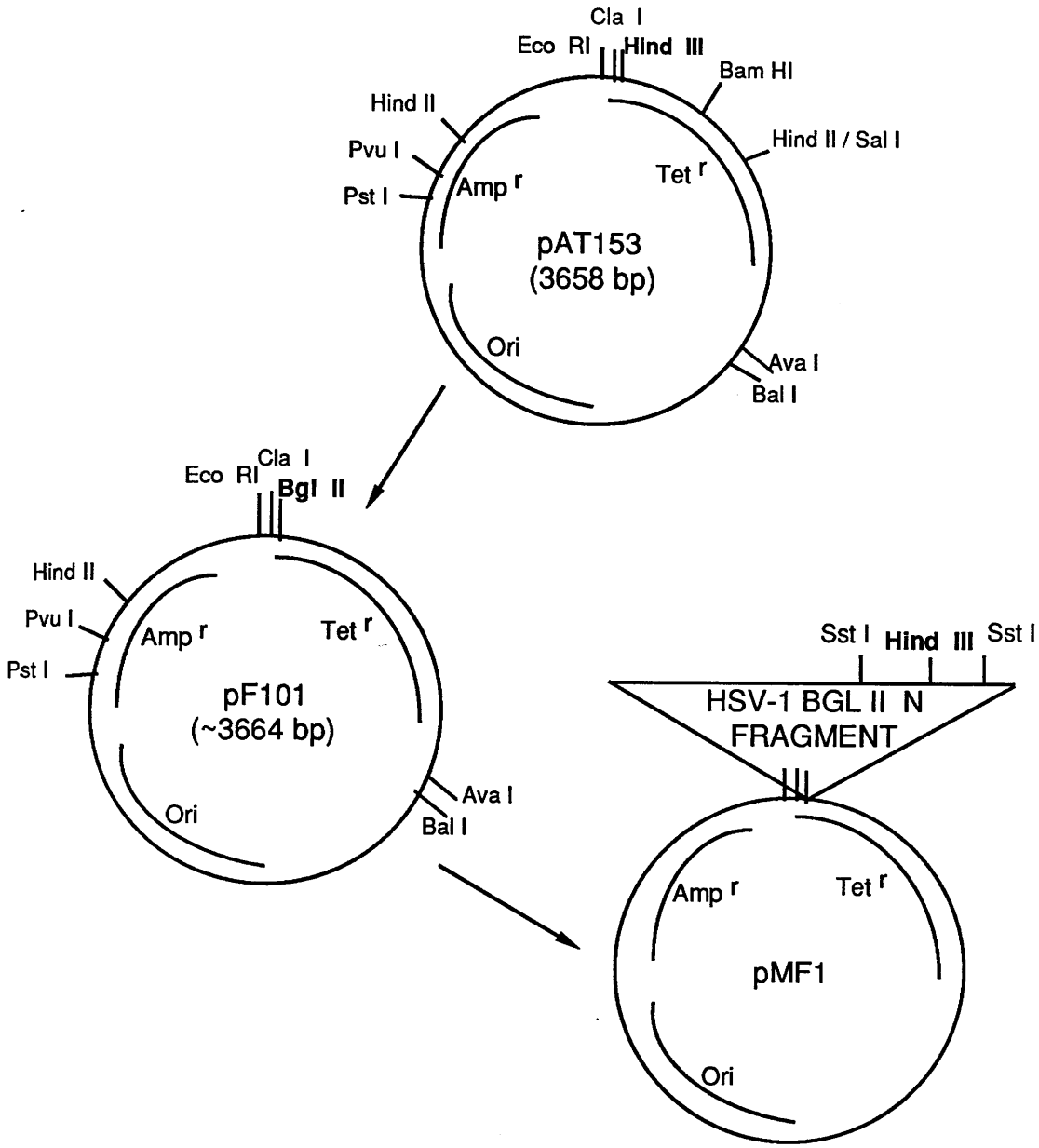

Figure 16. **Construction of plasmid pMF1**

To make pF101, the *Hind III* site at position 29 in pAT153 was converted to a *Bgl II* site. The *wt* (HSV-1 strain 17+) *Bgl II* ***n*** fragment was then cloned into the *Bgl II* site of pF101 to generate the plasmid pMF1. The *Hind III* and surrounding *Sst I* sites in the HSV-1 fragment are shown.

cells (see Section 2B:9). Bacterial colonies were then screened and a desired plasmid isolated [data not shown; see Sections 2B:6 (e) and 9 (d)]. This procedure was carried out to remove the *Hind III* site in pAT153. Construction of pMF3 is schematically illustrated in Figure 17.

(d) **Construction of the plasmid pMF4**

The HSV-1 *Bam HI j* [see Section 2A:6 (b)] fragment was recloned into the *Eco RI-Bam HI* sites of pAT153 to generate the plasmid pMF4. This involved the complete digestion of the recombinant plasmid containing the HSV-1 *Bam HI j* fragment with *Eco RI* and *Bam HI* restriction endonucleases, elution and purification of *Bam HI j* and pAT153 (3284 bp) DNA fragments and ligation of these fragments. As a result, a plasmid (pMF4) was generated with the removal of a 374 bp fragment from pAT153 containing the *Hind III* site. The construction of this plasmid is shown in Figure 18.

(e) **Construction of the plasmid pMF5**

In order to remove the *Hind III* site in pAT153, plasmid DNA was digested with *Hind III* to completion, 5' overhangs were filled-in with ***Klenow*** fragment of DNA polymerase I (Maniatis *et al.*, 1982) and a *Bam HI* linker was ligated. The resulting plasmid, pF102, was then isolated and its DNA analysed (data not shown). The HSV-1 *Bam HI **o*** (n. 87744 to 91610) fragment was then cloned into the *Bam HI* site(s) of pF102 to generate the plasmid pMF5. As a consequence, a 346 bp DNA fragment from the vector plasmid (pF102) was deleted out. This is diagramatically represented in Figure 19.

(f) **Construction of the plasmids pMF6, pMF7, pMF8, PMF9, PMF10, pMF11 and pMF12**

To construct plasmids pMF6 and pMF7, the HSV-1 *Eco RI-Kpn I* (n. 12593 to 16273) fragment was cloned into the respective RE sites within the cloning regions of pTZ18U and pTZ19U respectively (see Fig. 12 in Section 2A).

Similarly, the *Sac I-Sal I* (n. 27945 to 30093) fragment was cloned into the cloning region of pTZ18U to generate the plasmid pMF8; the *Sph I-Kpn I* (n. 39733 to 43624) fragment was cloned into pTZ19U to generate the plasmid pMF9; the *Xba I-Pst I* (97669 to 100549), the *Bam HI-Kpn I* (n. 87744 to 90383) and the *Bam HI **o*** (n. 87744 to 91610) fragments were cloned into the multiple restriction endonuclease linker region of pTZ18U to generate the plasmids pMF10, pMF11 and pMF12 respectively.

(g) **Construction of the plasmid pMF13**

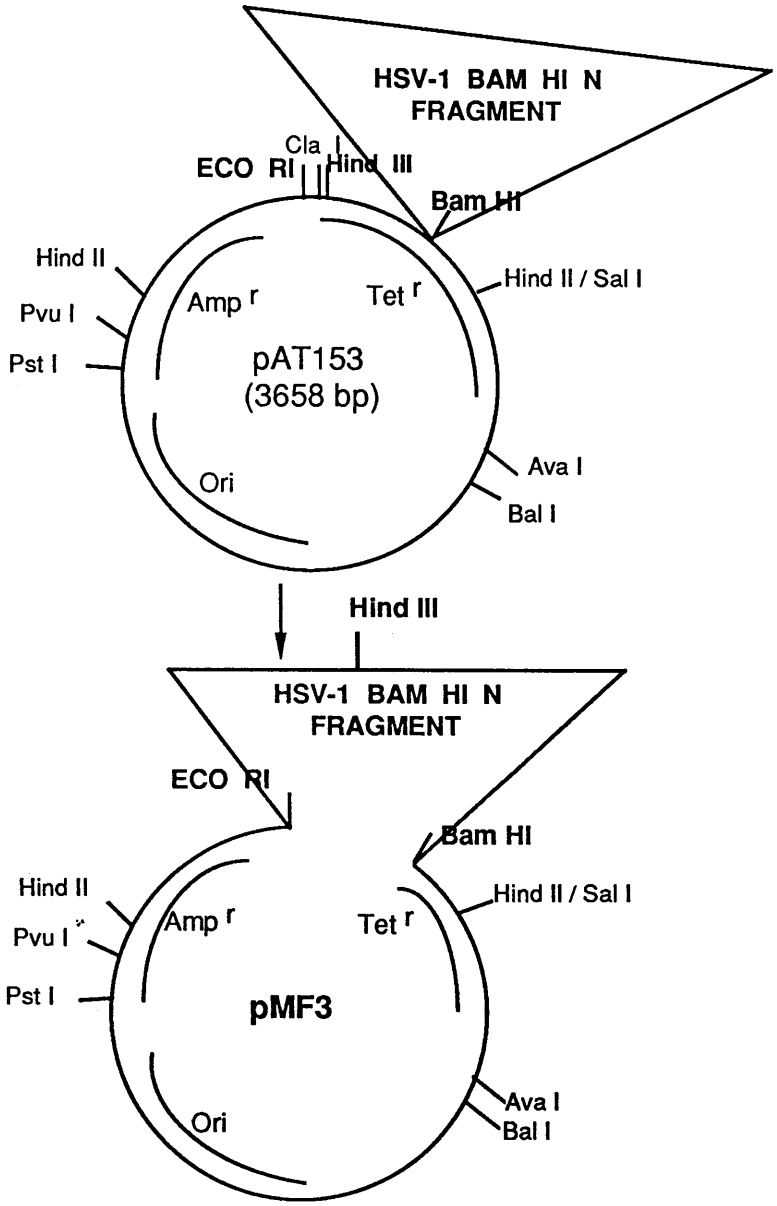

Figure 17. **Generation of plasmid pMF3**

Construction of pMF3 involved recloning of the HSV-1 *Bam HI* ***n*** fragment into the *Eco RI-Bam HI* sites of pAT153. A 374 bp DNA fragment containing the *Hind III* site of pAT153 has been removed during the process. The *Hind III* site contained in the *Bam HI* ***n*** fragment is also indicated.

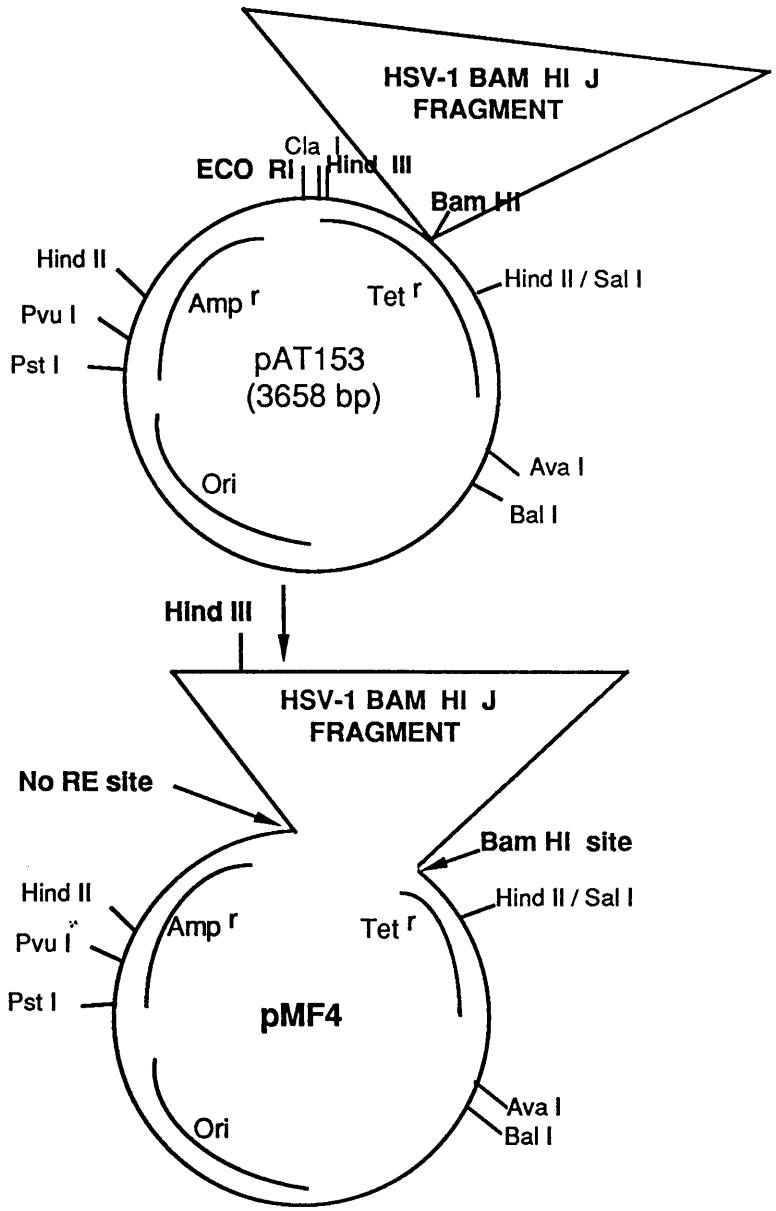

Figure 18. **Generation of plasmid pMF4**

Construction of pMF4 was carried out by recloning the HSV-1 *Bam HI j* fragment into the *Eco RI-Bam HI* sites of pAT153. A 374 bp DNA fragment containing the *Hind III* site of pAT153 has been removed during the process. The *Eco RI* site of pAT153 was also destroyed because of the ligation of incompatable termini. The *Hind III* site within the *Bam HI j* fragment is also indicated.

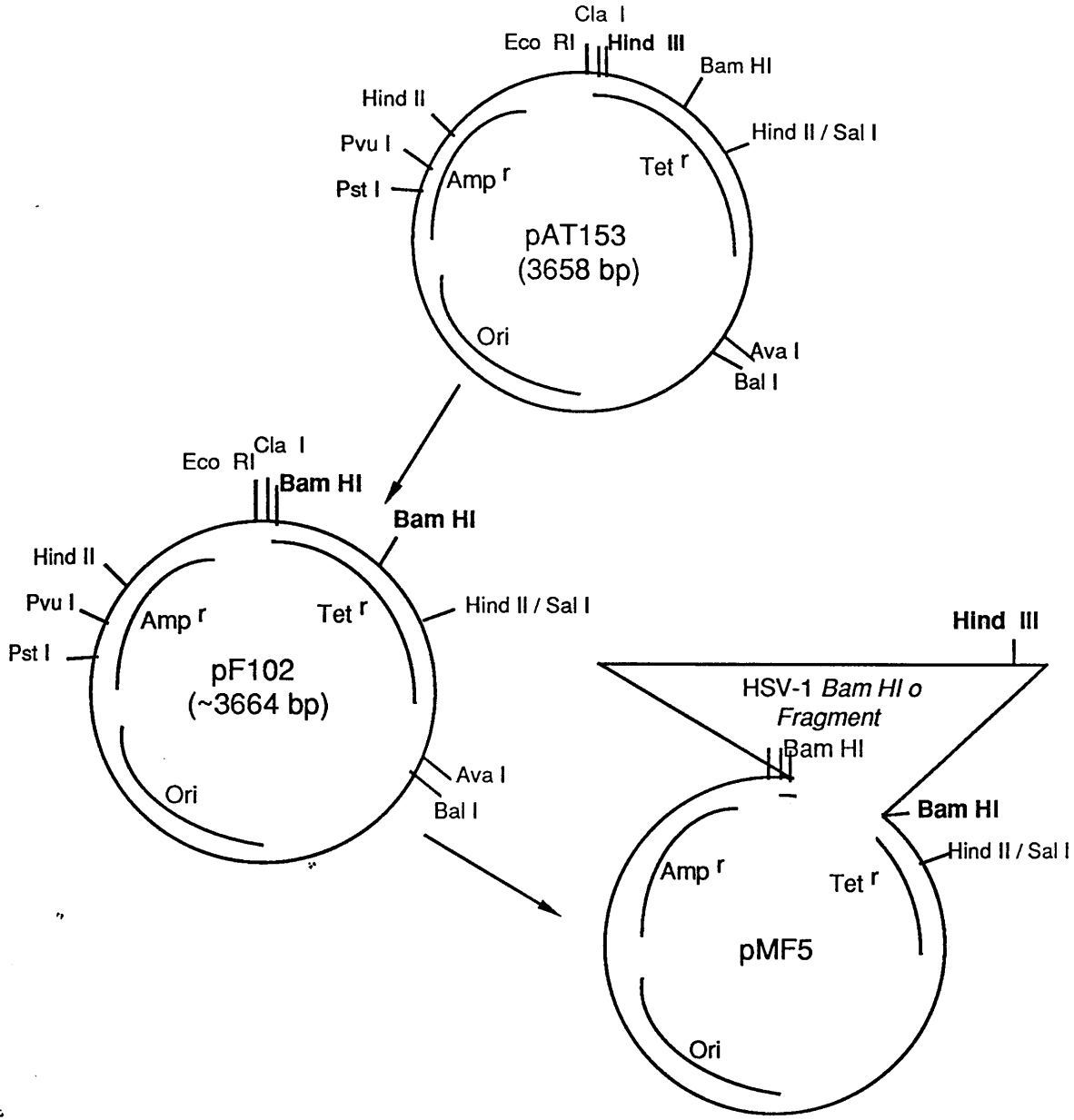

Figure 19. **Construction of plasmid pMF5**

To make pF102, the *Hind III* site at position 29 in pAT153 was converted to a *Bam HI* site. The *wt* (HSV-1 strain 17+) *Bam HI* ***o*** fragment was then cloned into the *Bam HI* sites of pF102 to generate the plasmid pMF5. The *Hind III* site within the *Bam HI* ***o*** fragment is indicated. The 346 bp *Bam HI* fragment is deleted from pF102.

This plasmid was constructed in an identical manner to pMF5 (see above). The HSV-1 *Bam HI* ***n*** (n. 131399 to 136289) fragment was eluted out, purified and cloned into the *Bam HI* site(s) of pF102 to generate the plasmid pMF13. As with pMF5, this plasmid also had a deletion of 346 bp within the vector DNA [see Section 3A:1 (e); see also Fig. 19].

2. MUTANT PLASMIDS

In order to generate HSV-1 strain 17+ variants lacking various *Hind III* sites, cloned HSV-1 DNA fragments were either obtained (see Section 2A:6) or constructed (as described above) for their subsequent use in site-directed mutagenesis. There are 10 *Hind III* sites at 0.08, 0.1, two at 0.18, 0.26, 0.52, 0.58, 0.64, 0.88 and 0.91 map coordinates of the HSV-1 strain 17+ *wt* genome (see Fig. 21; refer to Table 4 for nucleotide number of the respective *Hind III* sites). The 0.88 and 0.91 m.c. *Hind III* sites are located in the short component (U_S) of the genome whereas the remaining 8 *Hind III* sites are contained within the long component (U_L).

Oligonucleotides containing conservative single-base mutations were synthesized (see Table 3) to destroy the *Hind III* sites without affecting the coding potential of the respective amino acid (aa). These oligonucleotides were then introduced into the *wt* recombinant plasmids to isolate mutant plasmids devoid of the *Hind III* sites. Site-directed mutagenesis was carried out using two approaches (see Section 2B:12). The isolation of mutant plasmids lacking the *Hind III* restriction endonuclease cleavage sites is described in the following sections.

(a) The *Hind III* restriction endonuclease site at 0.88 m.c.

The plasmid *Bam HI* ***n*** (see Section 2A:6) contains the *Hind III* site at 0.88 m.c. Using oligonucleotide 9 (see Table 3), the first approach for site-directed mutagenesis (Oostra *et al.*, 1983; Liang *et al.*, 1986; see Section 2B:12) was carried out. However, despite the analysis of over 500 bacterial colonies, a plasmid lacking the *Hind III* site within the *Bam HI* ***n*** fragment could not be isolated. As the vector plasmid pAT153 contains a *Hind III* site at position 29 (see Fig. 16), it was not possible to selection enrich the *in vitro* mutagenized and ligated DNA mixture with the *Hind III* restriction endonuclease. In order to have an extra selective pressure for the isolation of a mutant plasmid lacking a specific *Hind III* site, it was necessary to delete/remove the *Hind III* site present in pAT153. Therefore, pMF3 lacking the *Hind III* site of pAT153 was constructed (see above; see also Fig. 17). Site-directed mutagenesis was then carried out using pMF3 and

TABLE 4

	Hind III site at m.c.[1]	nucleotide no.[2]	Location	Status[3]
1.	0.08	12716	Within the gene UL5	essential
2.	0.1	15596	Within the gene UL6	essential
3.	0.18	27986	Within the UL13 gene	non-essential
4.	0.18	28038	Within the UL13 gene	non-essential
5.	0.26	39849	Within the UL19 gene	essential
6.	0.52	80707	Intergenic (between the UL36 genes)	not known and UL37
7.	0.58	90145	Within the gene UL40	essential
8.	0.64	98823	Within the UL46 gene	non-essential [4]
9.	0.88	133466	Within the US1 gene	non-essential
10.	0.91	138344	Promoter region of gD (US6)	essential

Table 4. **Location Of The *Hind III* Sites On The HSV-1 Strain 17± Genome**

1. Map coordinates (see Fig. 21)
2. The nucleotide numbers represent the numbering system for HSV-1 DNA sequence of McGeoch *et al.* (1988b). The numbers given represent the first base of the respective *Hind III* site.
3. See relevant text plus McGeoch *et al.* (1988b) for references.
4. Barker and Roizman (1990)

oligonucleotide 9. Following transformation of *in vitro* mutagenized plasmid DNA, bacterial colonies were screened and mutant plasmids lacking the *Hind III* site (0.88m.c.) were isolated. One of these plasmids was selected as a prototype (pMF21) and analysed further with various restriction endonucleases (data not shown). It was found that the plasmid pMF21 had a deletion of approximately 800 bases along with the *Hind III* site (data not shown). Therefore, it was decided that the plasmid pMF21 would not be used to marker rescue the RE site-deletion back into the virus.

One of the possible reasons for being unable to isolate a desired mutant plasmid could be an unknown mutation within the synthesized oligonucleotide. To avoid this possibility, another oligonucleotide (no. 10, Table 3) with a different conservative single-base change compared to that of oligonucleotide 9 (see Table 3) was synthesized and used with pMF3 in site-directed mutagenesis. As a result, mutant plasmids lacking the *Hind III* site were isolated and two of these (pMF22 and pMF22.1) were selected for further analysis. However, these were also found to contain a deletion of approximately 500 bases beside lacking the *Hind III* site (data not shown). It was therefore decided to reclone the HSV-1 *Bam HI* ***n*** fragment into the plasmid pF102 to generate pMF13 [see Section 3A:1 (g)]. A 24-mer oligonucleotide was synthesized from the complementary strand of the HSV-1 DNA sequence (oligonucleotide no. 22; compare oligonucleotides 9, 10 and 22 in Table 3) and used with the plasmid pMF13 in site-directed mutagenesis. Using the same procedure (as above) bacterial colonies were screened and several mutant plasmids isolated. Of these, pMF23 contained a deletion of approximately 250 bases, pMF24 had an approximate deletion of 150 bases and pMF25 contained an insertion of approximately 150 bases as well as lacking the desired *Hind III* site.

The *Hind III* site at 0.88 m.c. is located within the US1 gene which is dispensible in tissue culture (see Table 4; McGeoch *et al.*, 1985, 1988b; McGeoch, 1989). The HSV-1 *Bam HI* ***n*** fragment involves the internal short repeat (IR_S) region of the HSV-1 strain 17^+ *(wt)* genome (Murchie and McGeoch, 1982; McGeoch *et. al.*, 1985). Because of the presence of these reiterated sequences within the *Bam HI* ***n*** cloned fragment, it is possible that a mutant plasmid could not be isolated without having an insertion and/or deletion of DNA sequences in addition to the deletion of the *Hind III* site (see above) despite the analysis of a total of over 3000 bacterial colonies (data not shown).

(b) **The *Hind III* RE site at 0.91 m.c.**

For the removal of 0.91 m.c. *Hind III* site, two cloned HSV-1 fragments, *Kpn I* ***h*** and *Bam HI* ***j***, were obtained from Dr V G Preston [see Section 2A:6 (b)]. An 18-mer oligonucleotide containing a single-base conservative mutation was synthesized (no. 11, Table 3) and used in site-directed mutagenesis (method of Oostra *et al.*, 1983; Liang *et al.*, 1986; see Section 2B:12 (d) (i)]. Initial attempts using both *Kpn I* ***h*** and *Bam HI* ***j*** plasmids to delete the *Hind III* site have failed (data not shown) possibly because the vector (pAT153) contained a *Hind III* site. Therefore, pMF4 was constructed [see Section 3A:1 (d) and Fig. 18] and the mutagenized oligonucleotide was inserted into it. The resultant bacterial colonies were screened and a plasmid, pMF15, devoid of the *Hind III* site was finally isolated. The plasmid pMF15 was further analysed with various restriction endonucleases and the RE patterns were compared with that of the parental plasmid (pMF4). The RE patterns were found to be identical with the exception of that of *Hind III*, suggesting that the *Hind III* site deletion has not involved any other detectable (using RE analysis) alteration (data not shown).

(c) **The *Hind III* RE sites at 0.08 and 0.1 m.c.**

The plasmid *Kpn I* ***b*** (see Section 2A:6) contains the 0.08 and 0.1 m.c. *Hind III* sites. The HSV-1 *Kpn I* ***b*** fragment was cloned into the *Pst I* site of pAT153 (Dr V G Preston, personal communication). Using oligonucleotides 1 and 2 (see Table 3), site-directed mutagenesis was carried out (method of Oostra *et al.*, 1983; Liang *et al.*, 1986). However, attempts to remove these two *Hind III* sites (using the same procedure) have failed despite an analysis of over 1000 bacterial colonies (data not shown). Therefore, plasmids pMF6 and pMF7 containing these two *Hind III* sites were constructed [see above, Section 1 (f)] along with the synthesis of a 24-mer mutagenic oligonucleotide (for the 0.08 m.c. *Hind III* site; no. 12, Table 3) and a 27-mer mutagenic oligonucleotide (for the 0.1 m.c. *Hind III* site; no. 13, Table 3). Using the second approach for site-directed mutagenesis [see Section 2B:12 (d) (ii); Kunkel, 1985; Kunkel *et al.*, 1987; Figure 15], these oligonucleotides were introduced into the plasmid pMF6 and mutant plasmids lacking the *Hind III* sites at 0.08 (pMF16) and 0.1 m.c. (pMF17) were isolated. Further RE analysis of pMF16 and pMF17 has indicated that there is no other apparent deletion and/or insertion within the HSV-1 fragment containing the mutated *Hind III* sites (data not shown).

(d) **The *Hind III* RE sites at 0.18 m.c.**

The plasmid pMF2 [see above, 1 (b)] contains the two *Hind III* sites at 0.18 m.c. Using the method described by Oostra *et al.* (1983) and

Liang *et al.* (1986), the oligonucleotides 3 and 4 (see Table 3) were hybridized to the HSV-1 DNA contained within the plasmid pMF2 in an *in vitro* site-directed mutagenesis reaction. Following transformation in *E. coli* DH5 cells, the bacterial colonies were screened and mutant plasmids isolated. However, further RE analysis has revealed that these plasmids (pMF27 to pMF30) also contain deletions ranging from approximately 400 bp to 1 kb (data not shown). Further attempts to isolate a mutant plasmid without any additional deletion/insertion have also failed (data not shown).

The plasmid pMF8 [see Section 1 (f) above] was then constructed and employing the second approach for site-directed mutagenesis [see Section 2B:12 (d) (ii)], oligonucleotides 14 and 15 (see Table 3) were individually used in separate attempts to isolate a desired mutant plasmid. More than 500 bacterial colonies were screened without any success. In addition, it was later realized that the oligonucleotide 15 also contains a non-conservative mutation (indicated with a double underline in Table 3). Therefore, the oligonucleotide 16 (see Table 3) was synthesized and used in site-directed mutagenesis to isolate a mutant plasmid (pMF19) devoid of the two *Hind III* sites at 0.18 m.c. No apparent deletion/insertion except those of *Hind III* sites was found in pMF19 using RE analysis (data not shown).

(e) **The *Hind III* site at 0.26 m.c.**

The HSV-1 *Bgl II n* fragment containing the 0.26 m.c. *Hind III* site was cloned in plasmid vector pF101 to generate pMF1 [see above; Section 1 (a)]. This plasmid was subjected to site-directed mutagenesis (method of Oostra *et. al.*, 1983; Liang *et. al.*, 1986) using the oligonucleotide 5 (see Table 3). A number of mutant plasmids were isolated as a result of screening around 1500 bacterial colonies. However, every mutant plasmid also contained an additional deletion of approximately 500 to 850 bp. It was then realized that the oligonucleotide 5 contains another mutation (indicated with a double underline in Table 3) in addition to the conservative mutation to delete the *Hind III* site. Therefore, a second oligonucleotide (no. 17, Table 3) was synthesized and analysed using the Southern blot technique (see Section 2B:10). The Southern blot has failed to hybridize the oligonucleotide probe with the HSV-1 DNA fragment contained within the plasmid pMF1 (data not shown). Comparison of the DNA sequence with that of HSV-1 DNA has revealed that a single nucleotide is missing from the sequence of the oligonucleotide 17 (see Table 3).

During that period, the second approach for site-directed mutagenesis (Kunkel, 1985; Kunkel *et. al.*, 1987) was used successfully to isolate the plasmids pMF16 and pMF17 [see above (c)] devoid of the 0.08 and 0.1 m.c. *Hind III* sites respectively. Therefore, plasmid pMF9 was constructed [see Section 1 (f) above] and a 27-mer oligonucloetide was synthesized (no. 18, Table 3). These were used in site-directed mutagenesis to isolate a plasmid (pMF18) devoid of the 0.26 m.c. *Hind III* site. Further RE analysis of pMF18 DNA has confirmed that there is no other detectable deletion or insertion (data not shown).

(f) **The *Hind III* sites at 0.52 and 0.58 m.c.**

For the *Hind III* site at 0.52 m.c., the HSV-1 *Xho I* ***a'*** (n. 80175 to 82261) fragment was selected to carry out site-directed mutagenesis. This DNA fragment was cloned into the *Xho I* site in vector plasmid pKC7 (Rao and Rogers, 1979) and was kindly provided by Dr V G Preston. The oligonucleotides 6 and 19 (see Table 3) were synthesized to mutate the 0.52 m.c. *Hind III* site. Using the first approach for site-directed mutagenesis (see Section 2B:12), attempts to remove this *Hind III* site were not successful. Moreover, only 600 bacterial colonies were screened and the second approach for site-directed mutagenesis was not used. Therefore, a plasmid lacking the 0.52 m.c. *Hind III* site could not be isolated.

For the *Hind III* site at 0.58 m.c., the plasmids pMF5, pMF11 and pMF12 [see Section 1 (e) and (f) above] were generated. The plasmid pMF5 and the oligonucleotide 7 (see Table 3) were used following the first approach for site-directed mutagenesis (Oostra *et. al.*, 1983; Liang *et. al.*, 1986). However, despite the analysis of over 1500 colonies, a plasmid lacking this *Hind III* site could not be isolated. The second approach for site-directed mutagenesis (Kunkel, 1985; Kunkel *et. al.*, 1987) has involved the plasmids pMF11 and pMF12 and the oligonucleotide 20 (see Table 3). These attempts were also unsuccessful (data not shown).

(g) **The *Hind III* site at 0.64 m.c.**

The plasmid pMF10 [see Section 1 (f) above] containing the *Hind III* site at 0.64 m.c. was used to insert the oligonucleotide 21 (see Table 3), using the second approach for site-directed mutagenesis (as above). As a result, a plasmid (pMF20) containing the desired mutation was isolated and its DNA subjected to RE analysis (data not shown). The plasmid pMF20 was found satisfactory in that it contained no other detectable deletion and/or insertion (data not shown).

3. PLASMIDS CONTAINING THE *ESCHERICHIA COLI* β-GALACTOSIDASE GENE (*LAC Z*)

In order to use the *Escherichia coli* β-galactosidase gene (*lac Z*) as a marker to facilitate identification of HSV-1 genomes lacking *Hind III* sites, it was decided to insert the *lac Z* sequences into the unique *Hind III* sites of cloned HSV-1 DNA fragments. Once this is achieved, the strategy was to marker rescue the HSV-1 DNA fragments containing *lac Z* sequences back into the virus and to isolate recombinant viruses carrying the β-galactosidase gene at various *Hind III* sites. The plasmid lacking a specific *Hind III* site could then be marker rescued into an appropriate recombinant virus (identified by a blue colour virus plaque created because of the expression of β-galactosidase gene in the presence of a chromogenic substrate known as *X-gal*) to isolate a variant virus devoid of the *Hind III* site but not expressing the β-galactosidase gene (identified with a non-blue virus plaque). In this way, a selective procedure can be established to identify any particular kind of recombinant viruses.

The plasmid pFJ5ΔHind (Rixon and McLauchlan, 1990; see Fig. 13) was the parental plasmid carrying the β-galactosidase gene. Using the *Bam HI* and *Xba I* restriction endonucleases, the *Bam HI-Xba I* fragment of pFJΔHind containing the *lac Z* sequences was eluted out and purified (see Section 2B:9 (a)]. This fragment was then inserted into the corresponding RE sites of pGEM-1 (see Section 2A:6; and Fig. 20) to generate the plasmid pF106 (see Fig. 20). The *Sma I* site within the multiple cloning sites (MCS) of pF106 was then converted into a *Hind III* site by inserting a *Hind III* linker to generate a plasmid pF107. The MCS comprises *Hind III, Pst I, Sal I, Acc I, Hinc II, Xba I, Bam HI, Ava I, Sma I* (converted to *Hind III* in pF107), *Sac I* and *Eco RI* RE sites. As the *lac Z* sequences were inserted in between the *Xba I* and *Bam HI* sites in pF106, a *Hind III* fragment containing these sequences was created in pF107. This is illustrated diagramatically in Figure 20.

The plasmid pF107 was cleaved with *Hind III* and the *Hind III* fragment carrying the β-galactosidase coding sequences was eluted out and purified. This was then inserted into the unique *Hind III* sites of pMF1, pMF2, pMF4 and pMF13 to generate the plasmids pF108, pF109, pF110 and pF111 respectively (data not shown).

SECTION 3B : HSV-1 STRAIN 17± VARIANT VIRUSES

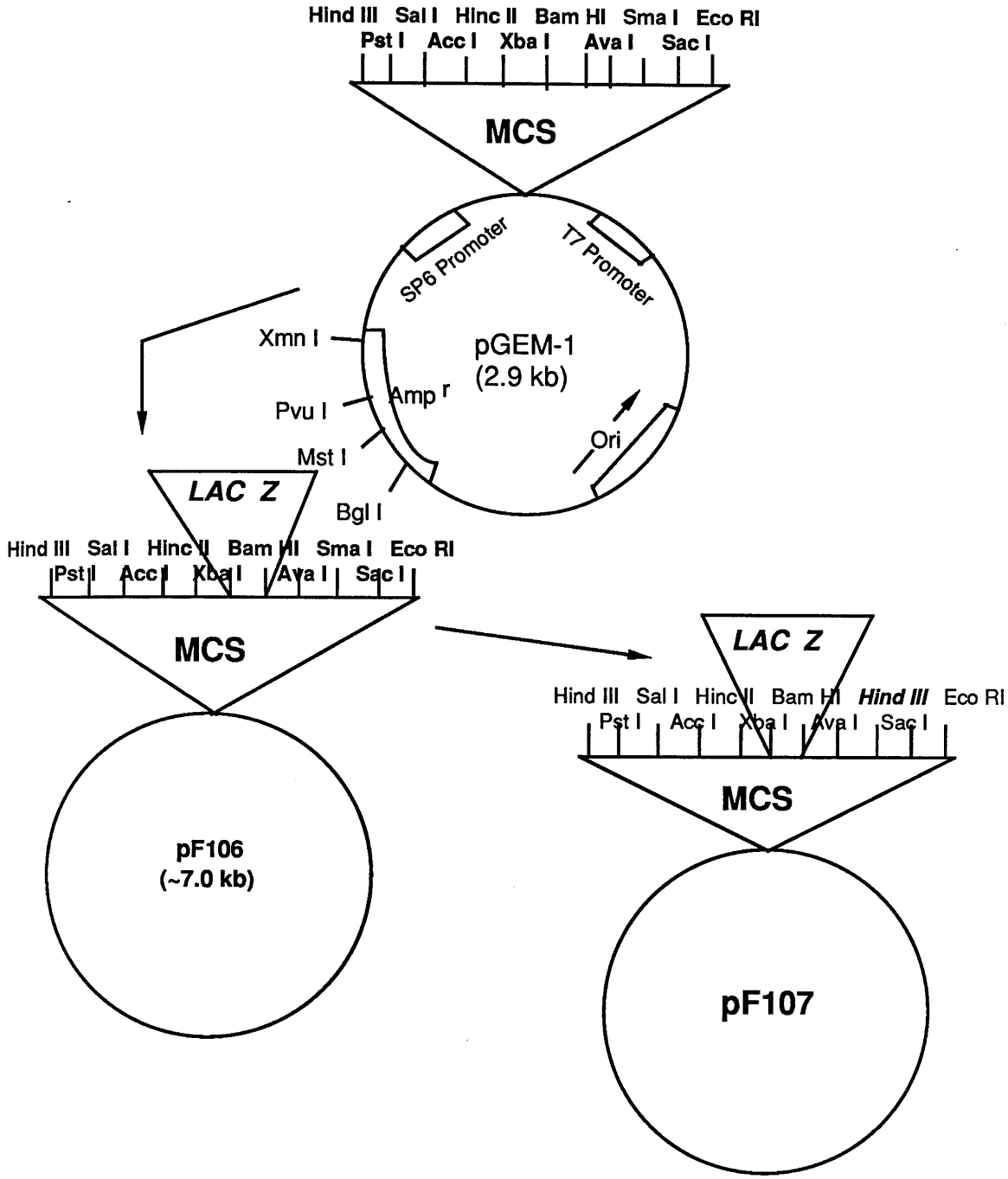

Figure 20. **Construction of plasmids pF106 and pF107**

The β-galactosidase coding sequences (*lac Z*) were contained in an *Xba I-Bam HI* fragment excised from plasmid pFJΔHind (Rixon and McLauchlan, 1990). This *Xba I-Bam HI* fragment was ligated to the corresponding RE sites of pGEM-1 to generate plasmid pF106. The *Sma I* site was then converted to the *Hind III* site to generate a plasmid pF107. Thus a *Hind III* fragment was created carrying the *lac Z* sequences.

1. RESTRICTION ENDONUCLEASE CLEAVAGE SITE-DELETION VARIANTS

The herpes simplex virus type 1 (HSV-1) Glasgow strain 17+ (*wt*) genome contains ten (10) *Hind III* and four (4) *Xba I* restriction endonuclease cleavage sites (Figure 21; Wilkie, 1976). The four normally occuring *Xba I* sites are located at 0.07, 0.29, 0.45 and 0.63 m.c. Using a modification of the selection enrichment technique described by Jones and Shenk (1978), an HSV-1 variant, X2, lacking the *Xba I* sites at 0.07 and 0.63 m.c. has been isolated and characterized (Brown *et al.*, 1984). The remaining *Xba I* sites in X2 have been removed by selection enrichment technique (the *Xba I* site at 0.45 m.c.) and site-directed mutagenesis (the *Xba I* site at 0.29 m.c.; using the method of Oostra *et. al.*, 1983; Liang *et. al.*, 1986) to generate a variant 1702, devoid of the all four *Xba I* sites (MacLean and Brown, 1987a).

Selection enrichment technique has been used to isolate the *Xba I* restriction endonuclease site-deletion variants because of non-availability of the complete DNA sequence of HSV-1 strain 17+ at that time. Consequently, 1702 is TK (thymidine kinase) negative (*tk*$^-$) and produces a truncated gC (MacLean and Brown, 1987a; Dr A R MacLean, personal communication). However, the restriction endonuclease profiles and growth characteristics of X2 and 1702 were found very similar to those of *wt* (strain 17+) virus, suggesting no detectable deletion and/or insertion (less than 150 bp) within the regions containing the deleted *Xba I* sites. The *Xba I* site at 0.63 m.c. was an exception in that it has been removed with a deletion of approximately 150 bp (Brown *et. al.*, 1984).

(a) Isolation of HSV-1 genomes devoid of the *Hind III* restriction endonuclease cleavage sites

With an objective to use RE sites as unselected markers in intrastrain recombination studies, it was decided to construct the HSV-1 genomes lacking the *Hind III* restriction endonuclease sites, using 1702 as the parental virus. As mentioned above, the *wt* virus contains 10 *Hind III* sites which are located at 0.08, 0.1, two at 0.18, 0.26, 0.52, 0.58, 0.64, 0.88 and 0.91 m.c (see Figure 21). Site-directed mutagenesis was used to mutate (delete) 7 *Hind III* sites during the course of the work described in this thesis (see Section 3A:2).

(i) Isolation of the variant 1721

In order to marker rescue the plasmid pMF15 (lacking the *Hind III* site at 0.91 m.c.; see Section 3A:2) back into the 1702 genome and to use the *Lac Z* sequences for the identification of the viral genomes lacking the *Hind III*

Figure 21. **Restriction Endonuclease Maps Of The HSV-1 Strain 17± Genome And The Variants 1721 To 1733.**

(a) The *Hind III* (above the line) and *Xba I* (below the line) maps for the DNA of HSV-1 strain 17^+ (Wilkie, 1976). Although the two *Hind III* sites at 0.18 map coordinates (m.c.) are located very close to each other (52 bp apart), they are shown separately to indicate them.

(b) The *Hind III* (above the line) and *Bgl II* (below the line) maps for the DNA of HSV-1 strain 17^+ (Wilkie, 1976; Wilkie *et al.*, 1977). There are two *Bgl II* sites each between the *Bgl II* fragments **k**/**o** (231 bp apart) and **o**/**p** (426 bp apart) (not shown).

(c) The *Hind III* (above the line) and *Bam HI* (below the line) maps for the DNA of HSV-1 strain 17^+.

The *Hind III* + *Xba I* maps for the DNA of HSV-1 strain 17^+ variants : (d) 1721, (e) 1722, (f) 1723, (g) 1724, (h) 1725, (i) 1726, (j) 1727, (k) 1728, (l) 1729, (m) 1730, (n) 1731 (o) 1732 and (p) 1733. As these variants are derived from the HSV-1 strain 17^+ variant 1702 (MacLean and Brown, 1987a), they are devoid of the normally occuring (four) *Xba I* sites.

The locations (as shown in this figure) of the various restriction endonuclease cleavage sites on the genome of HSV-1 strain 17^+ (*wt*) and its variants are approximate.

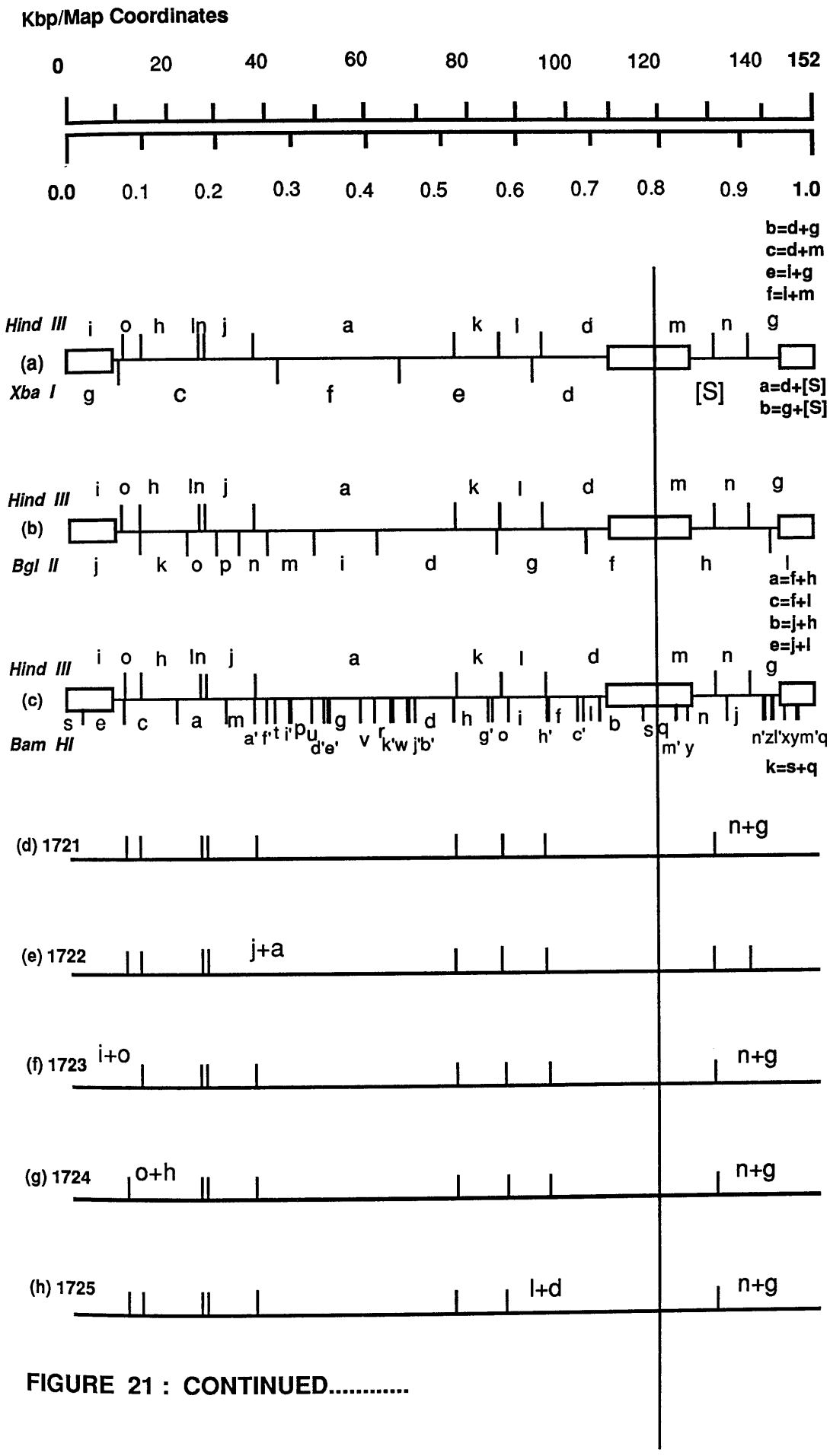

FIGURE 21 : CONTINUED.....

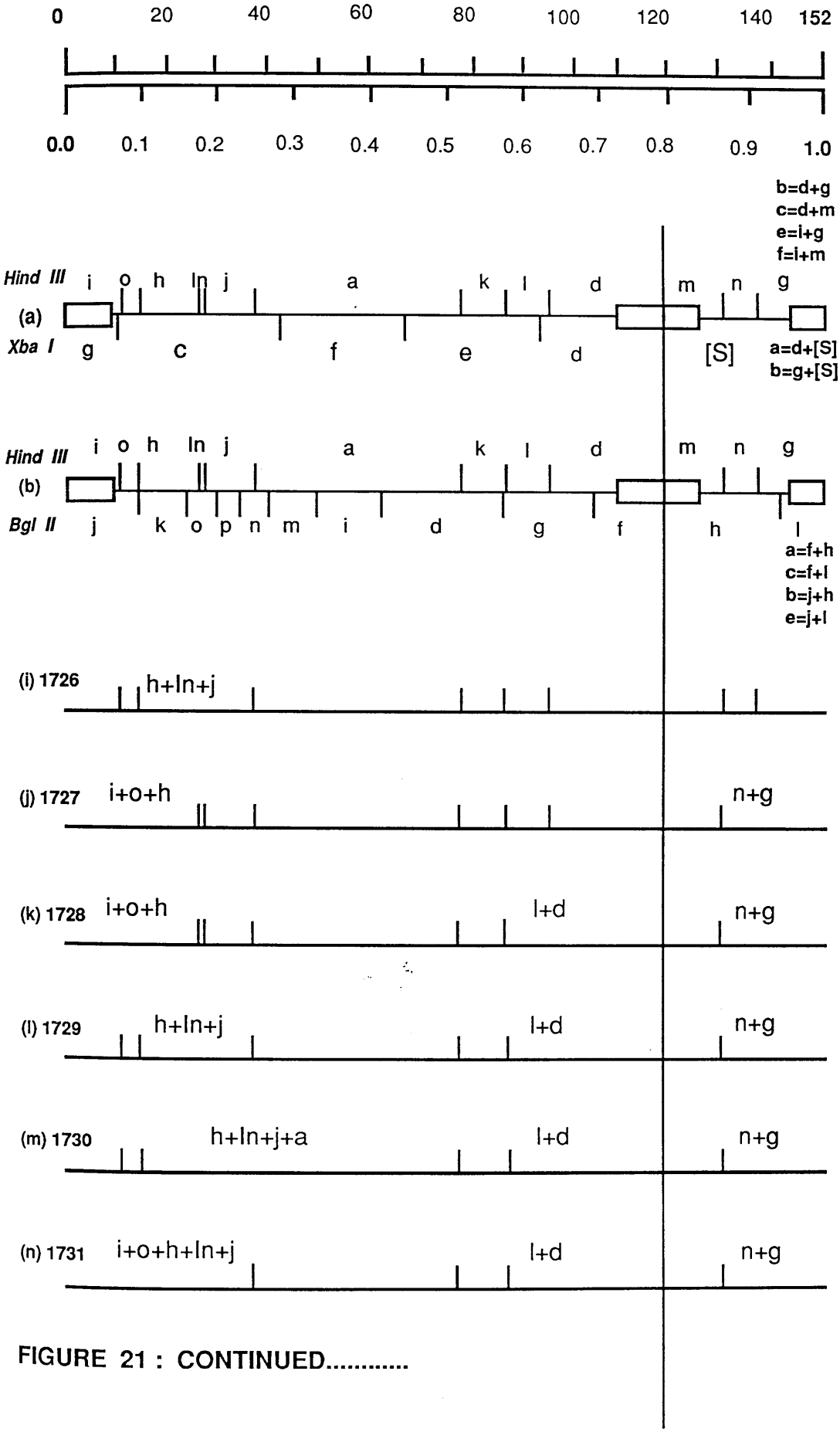

FIGURE 21 : CONTINUED.....

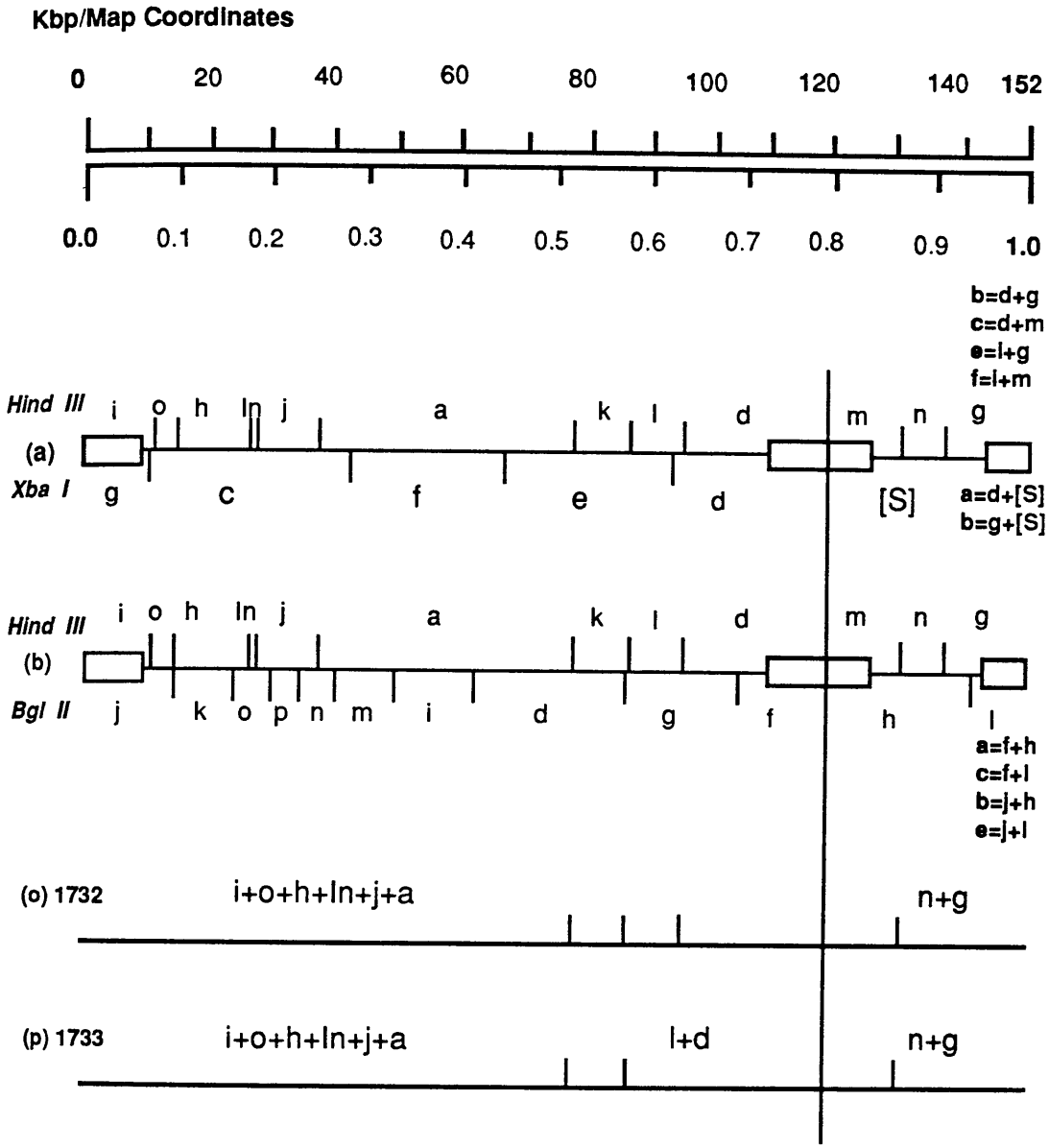

Figure 21. **The *Hind III, Xba I, Bgl II* And *Bam HI* Restriction Endonuclease Maps Of The HSV-1 Strain 17+ Genome And The *Hind III + Xba I* Maps Of The Variants 1721 To 1733.**

site, the plasmid pF110 (containing the *Lac Z* sequences at the unique *Hind III* site of pMF4; see Section 3A:3) was constructed. The plasmid (pF110) DNA was linearized with the restriction endonuclease *Sca I* and cotransfected with intact 1702 DNA using the calcium phosphate infectivity procedure of Stow and Wilkie (1976) [see Section 2B:4 (d)]. The resultant viral progeny was harvested and titrated out (see Section 2B: Methods). The recombinant plaques (identified by their blue colour in the presence of *X-gal*) were picked, grown into virus stocks and titrated out. In subsequent rounds of plaque purification, twelve blue plaques were picked and carried forward. However, after the fourth round, the blue plaques disappeared completely (see below).

The 0.91 m.c. *Hind III* site is located in between the US5 and US6 genes (McGeoch *et al.*, 1985), near the 5' terminus of the US6 ORF (see Table 4). The US6 gene encodes the glycoprotein D and is indispensible in cultured cells which do not express gD (Ligas and Johnson, 1988; McGeoch *et al.*, 1988b). Since the location of the 0.91 m.c. *Hind III* site is intergenic and the essential nature of the US6 gene was not known at that time, it was considered that the insertion of the *Lac Z* into the *Hind III* site would not interrupt any of the neighbouring genes. However, the disappearance of blue plaques (see above) has demonstrated that the US6 gene was indeed interrupted and the blue plaques were only detectable while there was enough 1702 virus for complementation (data not shown). Therefore, the blue plaque isolates could not be purified from the contaminating 1702 virus. As half of the *Hind III* sites are located within the known essential regions of the HSV-1 (strain 17^+) genome (see Table 4), it was decided that the *Lac Z* gene would not be used in further experiments.

The linearized pMF15 DNA (using the *Pst I* restriction endonuclease) was then cotransfected with intact 1702 DNA using the method mentioned above. Following transfection and titration of the progeny virus, ninety-six single, well-separated plaques were picked and grown in 35mm petri dishes of BHK21/C13 cells to generate virus stocks. These plaque isolates were examined by restriction endonuclease analysis of their ^{32}-P-labelled DNA [see Section 2B:4 (f)]. The *Hind III* restriction endonuclease (RE) profile of one of these differed from that of 1702 and *wt* (strain 17^+). This was designated as the HSV-1 (strain 17^+) variant 1721 [previously designated as H1; MacLean, A. R. *et al.*, 1991a; Figure 21 (d)]. The *Hind III* profile of 1721 is shown in Figure 22, lane 3 and the molecular weights of the fragments generated by the *Hind III* restriction endonuclease digestion of its DNA are given in Table 5. Comparison of the 1721 *Hind III* profile with that of the *wt*

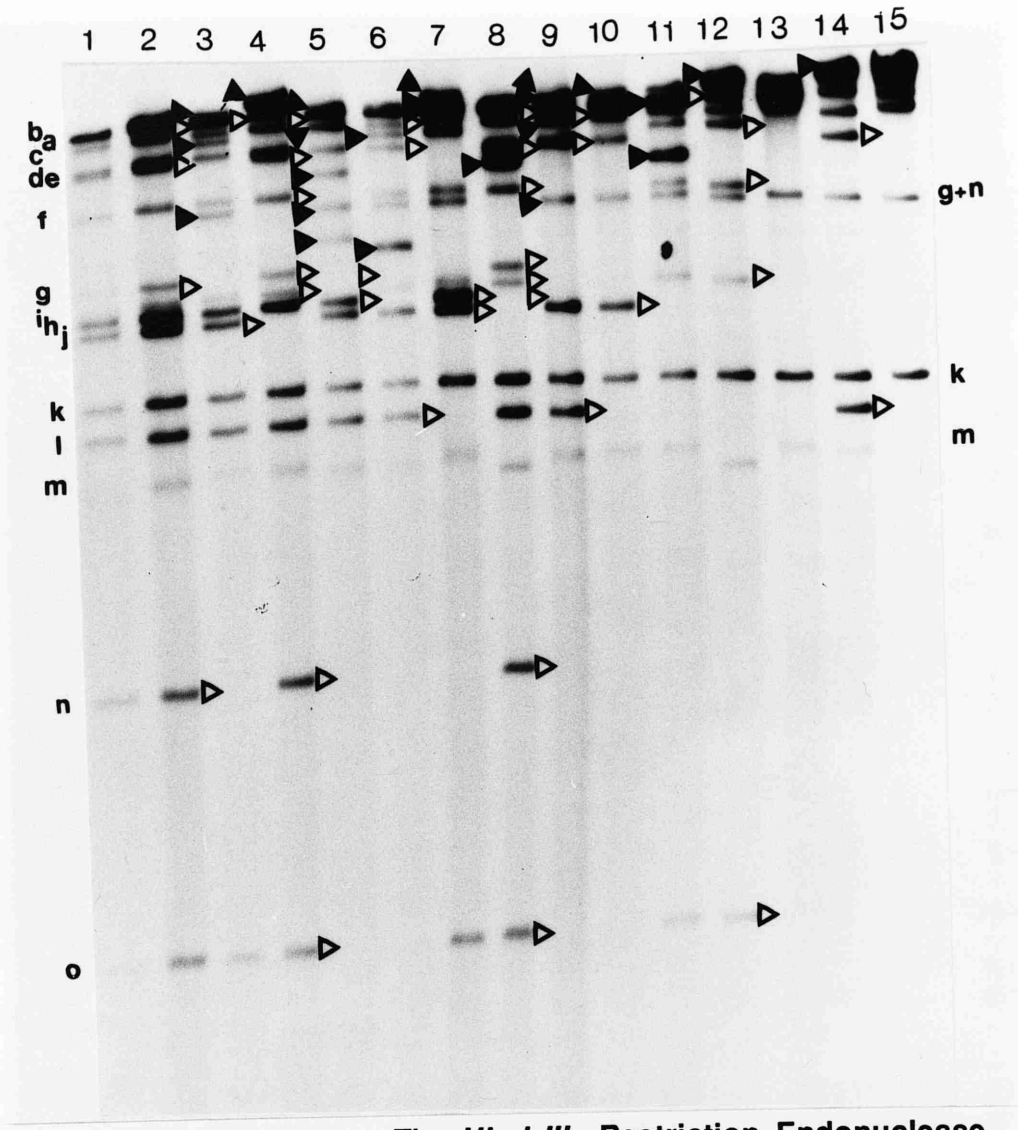

Figure 22. **The *Hind III* Restriction Endonuclease Profiles Of HSV-1 DNA And Its RE Site Deletion Variants**

The *Hind III* restriction endonuclease profiles of HSV-1 strain 17+ (lane 1), the *Xba I*- parental virus 1702 (lane 2; MacLean and Brown, 1987a) and the *Hind III* site deletion variants 1721 (lane 3), 1722 (lane 4), 1723 (lane 5), 1724 (lane 6), 1725 (lane 7), 1726 (lane 8), 1727 (lane 9), 1728 (lane 10), 1729 (lane 11), 1730 (lane 12), 1731 (lane 13), 1732 (lane 14) and 1733 (lane 15) DNAs ^{32}-P-labelled *in vivo* (0.5% agarose gel). The *wt* *Hind III* bands are labelled on left hand side. Few of the low molecular weight *Hind III* bands are also labelled on right hand side. The missing bands are indicated by open triangles (▷) whereas the novel bands by filled triangles (▶). If the missing/novel bands are absent/present in consecutive gel tracks, they are only indicated in the first track. Because of comigration of high molecular weight bands in the last few tracks (For example, lanes 10 to 15), it is difficult to distinguish the various *Hind III* bands and, therefore, they are not indicated (see Table 5).

TABLE 5

HIND III PROFILES

(Molecular Weights of ***Hind III*** fragments x 10^6)

	17 syn±	1721	1722	1723	1724	1725
			a+j 34.59	--	--	--
		b' 30.51	--	30.51	30.51	36.21
						c' 28.45
a	26.83	26.83	--	26.83	26.83	26.83
b	27.31	--	27.31	--	--	--
						d+l 23.87
c	22.75	22.75	22.75	22.75	22.75	--
		e' 20.69		22.58	20.69	20.69
d	18.17	18.17	18.17	18.17	18.17	--
e	17.49	--	17.49	--	--	--
				f' 14.82	--	--
f	12.93	12.93	12.93	--	12.93	12.93
		g+n 12.34	--	12.34	12.34	12.34
				i+o 10.24	h+o 10.03	--
g	9.14	--	9.14	--	--	--
h	8.14	8.14	8.14	8.14	--	8.14
i	8.35	8.35	8.35	--	8.35	8.35
j	7.76	7.76	--	7.76	7.76	7.76
k	6.20	6.20	6.20	6.20	6.20	6.20
l	5.70	5.70	5.70	5.70	5.70	--
m	4.58	4.58	4.58	4.58	4.58	4.58
n	3.20	--	3.20	--	--	--
o	1.89	1.89	1.89	--	--	1.89
ln	0.03	0.03	0.03	0.03	0.03	0.03

(ln = Innominate)

	17 syn±	1726	1727	1728	1729	1730
					a+h+j+ln	42.76
				b' 36.21	36.21	36.21
			e' 30.72	30.72	--	--
			b' 30.51	--	--	--
				c' 28.45	28.45	28.45
a	26.83	26.83	26.83	26.83	26.83	--
b	27.31	27.31	--	--	--	--
				d+l 23.87	23.87	23.87
			f' 22.96	22.96	--	--
c	22.75	22.75	22.75	--	--	--
			i+o+h 18.38	18.38	e' 20.69	20.69
d	18.17	18.17	18.17	--	--	--
e	17.49	17.49	--	--	--	--
		h+j+ln 15.93	--	--	15.93	--
f	12.93	12.93	--	--	12.93	12.93
			g+n 12.34	12.34	12.34	12.34
g	9.14	9.14	--	--	--	--
h	8.14	--	--	--	--	--
i	8.35	8.35	--	--	8.35	8.35
j	7.76	--	7.76	7.76	--	--
k	6.20	6.20	6.20	6.20	6.20	6.20
l	5.70	5.70	5.70	--	--	--
m	4.58	4.58	4.58	4.58	4.58	4.58
n	3.20	3.20	--	--	--	--
o	1.89	1.89	--	--	1.89	1.89
ln	0.03	--	0.03	0.03	--	--

(ln = Innominate)

TABLE 5 (continued)

HIND III PROFILES

(Molecular Weights of ***Hind III*** fragments x 10^6)

	17 syn±		1731		1732	1733
				e'	65.34	65.34
				f'	57.58	57.58
				a+h+i+j+o+ln	53.00	53.00
		e'	38.51		- -	- -
		b'	36.21		30.51	36.21
		f'	30.75		- -	- -
		c'	28.45		- -	28.45
a	26.83		26.83		- -	- -
b	27.31		- -		- -	- -
		h+i+j+o+ln	26.17		- -	- -
		d+l	23.87		- -	23.87
c	22.75		- -		22.75	- -
d	18.17		- -		18.17	- -
e	17.49		- -		- -	- -
f	12.93		- -		- -	- -
		g+n	12.34		12.34	12.34
g	9.14		- -		- -	- -
h	8.14		- -		- -	- -
i	8.35		- -		- -	- -
j	7.76		- -		- -	- -
k	6.20		6.20		6.20	6.20
l	5.70		- -		5.70	- -
m	4.58		4.58		4.58	4.58
n	3.20		- -		- -	- -
o	1.89		- -		- -	- -
ln	0.03		- -		- -	- -

(ln = Innominate)

Table 5. **The *Hind III* Restriction Endonuclease Profiles For The DNA Of HSV-1 Strain 17± And The Variants 1721 To 1733**

(Figure 22, lane 1; see also Table 5) and 1702 (Figure 22, lane 2) has revealed that the *Hind III* ***b***, ***e***, ***g*** and ***n*** bands are missing. Three novel bands were also detectable: one migrating below the *Hind III* ***f*** band, one running between the ***c*** and ***d*** bands and one running above the normal positions of the ***a*** and ***b*** bands. As the *Hind III* site at 0.91 m.c. is located in between the *Hind III* ***n*** and ***g*** fragments [see Figure 21 (a)], loss of this site should generate a fused ***g+n*** fragment, a larger ***e*** {(***l+g***)+***n***=***e***'} and a larger ***b*** {(***d+g***)+***n***=***b***'} fragments. These fused fragments would have molecular weights of 12.34 x 10^6, 20.69 x 10^6 and 30.51 x 10^6 respectively and should run at the positions of the three novel bands (see Figure 22, lane 3 and Table 5). Therefore, the *Hind III* profile of 1721 has indicated the absence of the 0.91 m.c. *Hind III* site. To confirm the 0.91 m.c. *Hind III* site loss, a *Bgl II-Hind III* digestion of ^{32}-P-labelled DNAs of the HSV-1 strain 17$^+$ (Figure 23, lane 1), 1702 (Figure 23, lane 2) and 1721 (Figure 23, lane 3) was carried out and the resulting DNA fragments were resolved on an agarose gel. The *Hind III* and *Bgl II* maps of the HSV-1 strain 17$^+$ DNA are shown in Figure 21 (b). It can be seen that the *Bgl II* ***h*** fragment contains the 0.88 and 0.91 m.c. *Hind III* sites. Thus, the *Bgl II* ***h***, a 11.31 x 10^6 molecular weight band, is cleaved by the 0.88 m.c. *Hind III* site to two smaller ***h'*** bands of approximate molecular weights of 6.20 x 10^6 and 5.11 x 10^6. The former is further cleaved with the 0.91 m.c. *Hind III* site to generate two smaller ***h''*** bands of 3.25 x 10^6 and 2.95 x 10^6 molecular weights respectively. The two *Bgl II* ***h''*** bands would now migrate above and below the uncleaved *Bgl II* ***p*** (M_r 3.04 x 10^6) band respectively (see Figure 23, lanes 1 and 2; see Table 6). The joints containing the *Bgl II* ***h*** fragment, ***a*** and ***b***, would be reduced by 6.20 x 10^6 and will migrate below ***c*** (***a'*** = 23.65-6.20 = 17.45 x 10^6) and ***e*** (***b'*** = 20.89-6.20 = 14.69 x 10^6) respectively. The joint ***b'*** containing the *Bgl II* ***j*** fragment would be further reduced by 1.23 (***b''*** = 14.69-1.23 = 13.46 x 10^6) because of the 0.08 m.c. *Hind III* site contained within the *Bgl II* ***j*** fragment and would migrate with the *Bgl II* ***f*** band (see Table 6 and Figure 23, lane 1). The *Bgl II-Hind III* restriction endonuclease profile of 1721 DNA is shown in Figure 23, lane 3. It can be seen that the two *Bgl II* ***h''*** bands are missing and a fused *Bgl II* ***h'*** novel band is running below the comigrating *Bgl II* ***k*** and ***l*** bands. The novel *Bgl II* ***h'*** band represents the expected molecular weight band that would be generated as a result of the fusion of the two *Bgl II* ***h''*** bands.

Although the *Hind III* site at 0.91 m.c. has been deleted through a conservative alteration in the third base of the respective amino acid within the DNA sequence (AAGCTT) recognized by the *Hind III* enzyme (see Table 3), it

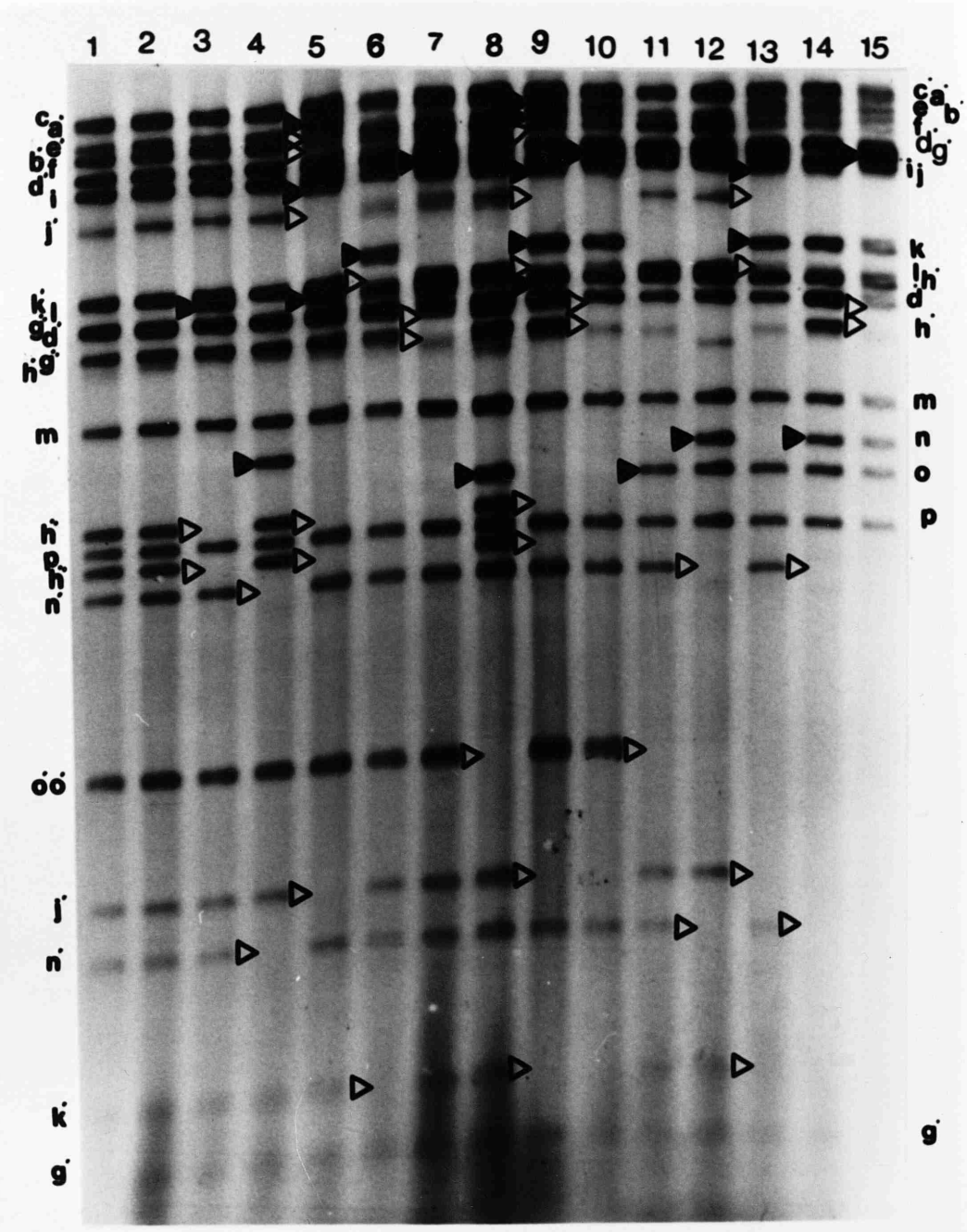

Figure 23. **The *Bgl II-Hind III* Profiles Of HSV-1 DNA**

The *Bgl II-Hind III* restriction endonuclease profiles of HSV-1 strain 17+ (lane 1), 1702 (lane 2), 1721 (lane 3), 1722 (lane 4), 1723 (lane 5), 1724 (lane 6), 1725 (lane 7), 1726 (lane 8), 1727 (lane 9), 1728 (lane 10), 1729 (lane 11), 1730 (lane 12), 1731 (lane 13), 1732 (lane 14) and 1733 (lane 15) DNAs ^{32}-P-labelled *in vivo* (0.5% agarose gel). The *wt Bgl II* bands are labelled on both sides. The missing bands are shown by open traingles (▷) whereas the novel *Bgl II* bands are indicated with filled triangles (▶).

TABLE 6

BGL II AND BGL II-HIND III PROFILES

(Molecular Weights of ***Bgl II*** fragments x 106)

	17 syn± (*Bgl II*)		17syn± (*Bgl II-Hind III*)	1721	1722	1723	1724	1725
a	23.65							
b	20.89							
c	18.58	c	18.58	18.58	18.58	18.58	18.58	18.58
d	17.00	a'	17.45	17.45	17.45	17.45	17.45	17.45
e	15.82					e 15.82	- -	- -
						b' 14.69	- -	- -
		e'	14.59	14.59	14.59	- -	14.59	14.59
		b'	13.46	13.46	13.46	- -	13.46	13.46
f	12.34	f	12.34	12.34	12.34	12.34	12.34	12.34
g	11.42							
h	11.31	d'	11.32	11.32	11.32	11.32	11.32	11.32
								g' 10.82
i	10.26	i	10.26	10.26	10.26	10.26	10.26	10.26
j	9.58					j 9.58	- -	- -
		j'	8.35	8.35	8.35	- -	8.35	8.35
k	6.94						k 6.94	- -
		k'	6.28	6.28	6.28	6.28	- -	6.28
l	6.24	l	6.24	6.24	6.24	6.24	6.24	6.24
				h' 6.20	- -	6.20	6.20	6.20
		g"	5.71	5.71	5.71	5.71	5.71	- -
		d'	5.68	5.68	5.68	5.68	5.68	5.68
		g"	5.21	5.21	5.21	5.21	5.21	- -
		h'	5.11	5.11	5.11	5.11	5.11	5.11
m	4.21	m	4.21	4.21	4.21	4.21	4.21	4.21
n	3.76				n 3.76	- -	- -	- -
o	3.48							
		h"	3.25	- -	3.25	- -	- -	- -
p	3.04	p	3.04	3.04	3.04	3.04	3.04	3.04
		h"	2.95	- -	2.95	- -	- -	- -
		n'	2.70	2.70	- -	2.70	2.70	2.70
		o'	1.73	1.73	1.73	1.73	1.73	1.73
		o'	1.72	1.72	1.72	1.72	1.72	1.71
		j'	1.23	1.23	1.23	- -	1.23	1.23
		n'	1.06	1.06	- -	1.06	1.06	1.06
		k'	0.66	0.66	0.66	0.66	- -	0.66
		g'	0.50	0.50	0.50	0.50	0.50	0.50
In	0.28	In	0.28	0.28	0.28	0.28	0.28	0.28
In	0.15	In	0.15	0.15	0.15	0.15	0.15	0.15
		In	0.03	0.03	0.03	0.03	0.03	0.03

(In = Innominate)

TABLE 6 (Continued)

BGL II AND BGL II-HIND III PROFILES

(Molecular Weights of ***Bgl II*** fragments x 106)

	17 syn± (*Bgl II*)		17syn± (*Bgl II-Hind III*)		1731	1732	1733
a	23.65						
b	20.89						
c	18.58	c	18.58		18.58	18.58	18.58
d	17.00	a'	17.45		17.45	17.45	17.45
e	15.82			e	15.82	15.82	15.82
				b'	14.69	14.69	14.69
		e'	14.59		- -	- -	- -
		b'	13.46		- -	- -	- -
f	12.34	f	12.34		12.34	12.34	12.34
g	11.42						
h	11.31	d'	11.32		11.32	11.32	11.32
				g'	10.82	- -	10.82
i	10.26	i	10.26		10.26	10.26	10.26
j	9.58			j	9.58	9.58	9.58
		j'	8.35		- -	- -	- -
k	6.94			k	6.94	6.94	6.94
		k'	6.28		- -	- -	- -
l	6.24	l	6.24		6.24	6.24	6.24
				h'	6.20	6.20	6.20
		g"	5.71		- -	5.71	- -
		d'	5.68		5.68	5.68	5.68
		g"	5.21		- -	5.21	- -
		h'	5.11		5.11	5.11	5.11
m	4.21	m	4.21		4.21	4.21	4.21
n	3.76			n	- -	3.76	3.76
o	3.48			o	3.48	3.48	3.48
		h"	3.25		- -	- -	- -
p	3.04	p	3.04		3.04	3.04	3.04
		h"	2.95		- -	- -	- -
		n'	2.70		2.70	- -	- -
		o'	1.73		- -	- -	- -
		o'	1.72		- -	- -	- -
		j'	1.23		- -	- -	- -
		n'	1.06		1.06	- -	- -
		k'	0.66		- -	- -	- -
		g'	0.50		0.50	0.50	0.50
In	0.28	In	0.28		0.28	0.28	0.28
In	0.15	In	0.15		0.15	0.15	0.15
		In	0.03		- -	- -	- -

(In = Innominate)

Table 6. ***Bgl II* And *Bgl II-Hind III* Restriction Endonuclease Profiles of The HSV-1 Strain 17± And The *Hind III* Site-Deletion Variants 1721 To 1733**

was further confirmed by carrying out a *Bam HI* and a *Bgl II* digestion of the ^{32}-P-labelled DNAs of strain 17^{+}, 1702 and 1721 that no other detectable deletion and/or insertion had arisen within the *Bam HI* and *Bgl II* fragments spanning the 0.91 m.c. *Hind III* site. The *Bam HI* profile of 1721 DNA (Figure 24, lane 3) was found identical to that of the *wt* (Figure 24, lane 1) and 1702 (Figure 24, lane 2) DNAs. The 0.91 m.c. *Hind III* site is located within the *Bam HI j* fragment [see Figure 21 (c)] and there was no detetectable change in the mobility of the *Bam HI j* fragment among the viral genomes compared (see Figure 24). Identical results were obtained by comparing the *Bgl II* DNA profiles of the *wt*, 1702 and 1721 viruses [see Figure 21 (b) and Figure 25, lanes 1, 2 and 3]. In addition, the 0.91 m.c. *Hind III* site is located in the promoter region of gD, disruption of which produces a non-viable virus in tissue culture (see above). Thus, the *Hind III* site loss has indeed taken place through a conservative change. An *Xba I* digestion of 1721 DNA has shown uncleaved mass at the top of the agarose gel track as that of the parental 1702 DNA, indicating the absence of the *Xba I* sites from these RE site-deletion variants (see Figure 26; MacLean and Brown, 1987a).

(ii) **Isolation of the variants 1723 and 1724**

The mutant plasmids pMF16 and pMF17 [see Section 3A: 2 (d)] were linearized with the *Bam HI* restriction endonuclease and individually transfected with intact 1721 DNA as described. The transfected petri dishes were harvested when complete *cpe* was observed and the harvested virus was sonicated and titrated out. From each transfection, ninety-six single plaques were isolated and virus stocks were generated in 35 mm petri dishes. Generally, ninety-six to two hundred plaque isolates were analysed from one transfection experiment and whenever the transfection experiment did not work, different batches of BHK21/C13 cells were used in further attempts to marker rescue the desired HSV-1 DNA fragment. The virus stocks were then analysed by the *Hind III* restriction endonuclease analysis of the ^{32}-P-labelled DNA (see Methods). Ten of these plaque isolates gave different restriction endonuclease profiles compared to that of 1721. Of these, six had an identical profile and one was designated as the prototype (1723). One of the remaining four isolates was designated as 1724, the RE profile of which differed from that of 1723 but identical to that of the other three isolates.

The *Hind III* maps of 1723 and 1724 are shown in Figure 21 (f) and (g) respectively. The *Hind III* profile of 1723 has shown that the *Hind III* ***e***, ***f***, ***i*** and ***o*** bands are missing compared to that of 1721 (see Figure 22, lanes 3 and 5). Three novel bands were also seen: one migrating in between the ***g+n***

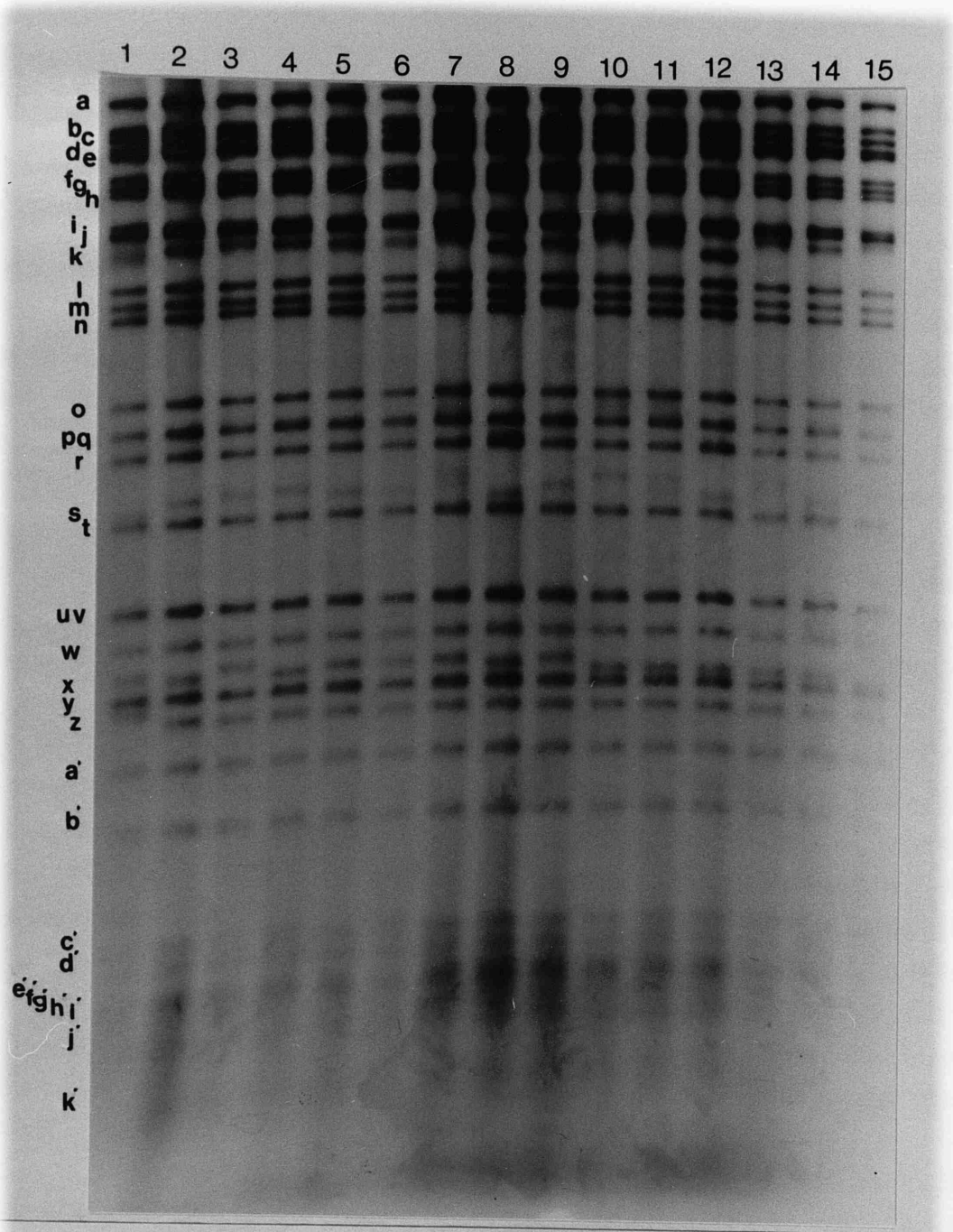

Figure 24. **The *Bam HI* Restriction Endonuclease Profiles Of HSV-1 DNA**

The *Bam HI* restriction endonuclease digests of HSV-1 strain 17+ (lane 1), 1702 (lane 2), 1721 (lane 3), 1722 (lane 4), 1723 (lane 5), 1724 (lane 6), 1725 (lane 7), 1726 (lane 8), 1727 (lane 9), 1728 (lane 10), 1729 (lane 11), 1730 (lane 12), 1731 (lane 13), 1732 (lane 14) and 1733 (lane 15) DNAs ^{32}-P-labelled *in vivo* (1.0% agarose gel). The *wt Bam HI* bands are labelled on left hand side.

(fused fragment in 1721) and ***h*** bands (approximate molecular wieght of 10.24 x 10^6 equivalent to the expected ***i+o*** fused fragment), one running in between the ***d*** and ***g+n*** bands [molecular weight of 14.82 x 10^6 equivalent to the expected ***f'*** band = (***l+m***)+***o*** fused fragment] and one comigrating with the ***c*** band [molecular weight of 22.58 x 10^6; the expected ***e'*** band = (***l+g+n***)+***o*** fused fragment] (see Figures 21 and 22; see also Table 5). Thus, 1723 had lost the *Hind III* site at 0.08 m.c. beside lacking the 0.91 m.c. *Hind III* site. A *Bgl II-Hind III* digestion of 1723 DNA provided further evidence regarding the absence of the 0.08 and 0.91 m.c. *Hind III* sites. The 0.08 m.c. *Hind III* site cleaves the *Bgl II* ***j*** fragment into two smaller ***j'*** fragments of approximate molecular weights of 8.35 x 10^6 and 1.23 x 10^6 respectively [see Figure 21 (b)]. The two *Bgl II* ***j'*** bands were missing and a normal *Bgl II* ***j*** band (molecular weight of 9.58 x 10^6) was migrating below the ***i*** band (compare lane 1 in Figure 25 with lane 5 in Figure 23). The ***j*** containing joints, ***b*** and ***e*** are now running above the *Bgl II* ***f*** (b' = 13.46+1.23 = 14.69 x 10^6) and below the *Bgl II* ***a'*** (e = 14.59+1.23 = 15.82 x 10^6) bands respectively (see Table 6 and Figure 23, lane 5).

The *Hind III* profile of the variant 1724 has indicated that the 0.1 m.c. *Hind III* site had been lost beside the loss of the 0.91 m.c. *Hind III* site. It can be seen (Figure 22, lane 6) that the *Hind III* ***h*** and ***o*** bands are missing and a novel band of 10.03 x 10^6 molecular weight is running between the ***g+n*** (fused fragment of 1721; see Figure 21) and ***i*** bands. A *Bgl II-Hind III* digestion of 1724 DNA gave a profile in which the two *Bgl II* ***k'*** bands were missing (the 0.1 m.c. *Hind III* site cuts the *Bgl II* ***k*** fragment into two smaller ***k'*** fragments; see Figure 21; see also Table 6) and a normal uncut *Bgl II* ***k*** band was migrating (see Figure 23, lane 6). Thus, confirming the loss of the *Hind III* site at 0.1 m.c.

The *Bam HI* (Figure 24, lanes 5 and 6), *Bgl II* (Figure 25, lanes 5 & 6) and *Xba I* (Figure 26, lanes 5 & 6) digestion of 1723 and 1724 DNAs gave identical profiles to the *Bam HI*, *Bgl II* and *Xba I* profiles of 1702 and 1721 DNAs. The 0.08 m.c. *Hind III* site is located within the coding sequence of the UL5 gene whereas the 0.1 m.c. *Hind III* site is located within the UL6 ORF (see Table 4). Both the UL5 and UL6 genes are essential for production of a viable virus in tissue culture (McGeoch, 1989) and the UL5 gene is one of the 7 genes required for HSV-1 DNA replication (Wu *et al.*, 1988; McGeoch *et al.*, 1988a, b). In addition, the oligonucleotides used to delete these *Hind III* sites contained conservative mutations and therefore should not produce any effect on the polypeptide coding potential of these viruses. Thus, the *Hind III*

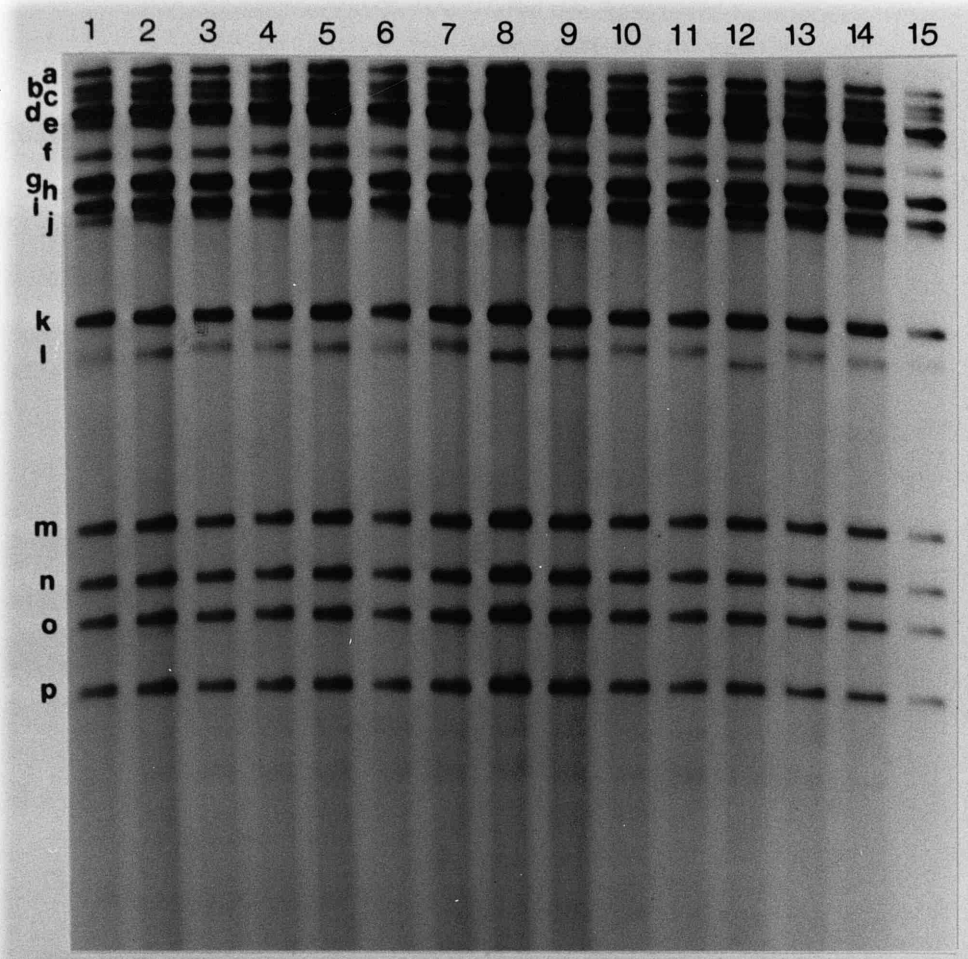

Figure 25. **The *Bgl II* Restriction Endonuclease Profiles Of HSV-1 DNA**

The *Bgl II* restriction endonuclease profiles of HSV-1 strain 17+ (*wt*; lane 1), 1702 (lane 2), 1721 (lane 3), 1722 (lane 4), 1723 (lane 5), 1724 (lane 6), 1725 (lane 7), 1726 (lane 8), 1727 (lane 9), 1728 (lane 10), 1729 (lane 11), 1730 (lane 12), 1731 (lane 13), 1732 (lane 14) and 1733 (lane 15) DNAs ^{32}-P-labelled *in vivo* (0.5% agarose gel). The *wt Bgl II* bands are labelled on left hand side.

sites at 0.08 and 0.1 m.c. were also lost without any additional deletion and/or insertion.

(iii) **Isolation of the variant 1727**

In order to isolate an HSV-1 variant lacking the *Hind III* sites at 0.08, 0.1 and 0.91 m.c., intact 1723 and 1724 DNAs were cotransfected and the resulting single plaque isolates were examined as described above. Of these, four gave an identical *Hind III* profile which was different from that of the 1723 or 1724 DNA. One of these was selected as the prototype and designated as the variant 1727. A *Hind III* profile of 1727 DNA is shown in Figure 22, lane 9. It can be seen that the *Hind III* ***e***, ***f***, ***g***, ***h***, ***i***, ***n*** and ***o*** bands are missing and the fused ***g+n*** band is running at an identical position to that of 1721 (compare lanes 3 and 9 in Figure 22). The fused ***i+o*** (of 1723) and ***h+o*** (of 1724) bands are also missing. The fused ***h+i+o*** (combined molecular weight of 18.38 x 10^6) band is comigrating with the *Hind III* ***d*** (mol. wt. 18.17 x 10^6) band and the novel ***f'*** band [mol. wt. of 22. 96 x 10^6 equivalent to the combined molecular weights of (***h+i+o***) + ***m*** bands] is comigrating with the *Hind III* ***c*** (mol. wt. 22.75 x 10^6) band. The novel ***e'*** [(***h+i+o***) + (***g+n***) = approx. mol. wt. of 30.72 x 10^6] and ***b'*** [***d*** + (***g+n***) = approx. mol. wt. of 30.51 x 10^6] bands are running at the top of the gel track and cannot be differentiated because of comigration. The *Bgl II-Hind III* profile of 1727 DNA (see Figure 23, lane 9) has confirmed that the variant was devoid of the 0.08, 0.1 and 0.91 m.c. *Hind III* sites [see above for the respective *Bgl II* fragments; see also Figure 21 (b) and Table 6]. The *Bam HI* and *Bgl II* profiles of 1727 DNA were identical to those of 17^+, 1702, 1721, 1723 and 1724 (see Figures 24, lane 9 and Figure 25, lane 9 and compare these with lanes 1, 2, 3, 5 and 6). An *Xba I* profile of 1727 DNA was also found identical to that of the parental viruses (see Figure 26, lane 9).

(iv) **Isolation of the variant 1722**

The mutant plasmid pMF18 [lacking the 0.26 m.c. *Hind III* site; see Section 3A:2 (e)] was linearized with the *Eco RI* digestion of its DNA and used in transfection experiments to marker rescue the *Hind III* site-deletion back into the virus. In order to isolate an HSV-1 variant lacking the *Hind III* sites at 0.08, 0.1, 0.26 and 0.91 m.c., initial transfection experiments involved the variant 1727 DNA (see above). The resultant plaque isolates were grown in 24-well Linbro trays to generate virus stocks as well as DNA [see Section 2B:4 (b)]. The DNA was then analysed by the *Hind III*, the *Hind III-Eco RI* and the *Hind III-Bgl II* restriction endonucleases using the Southern blot techniques and the plasmid pMF1 as a probe [see Sections 2B:10 (a), (b) &

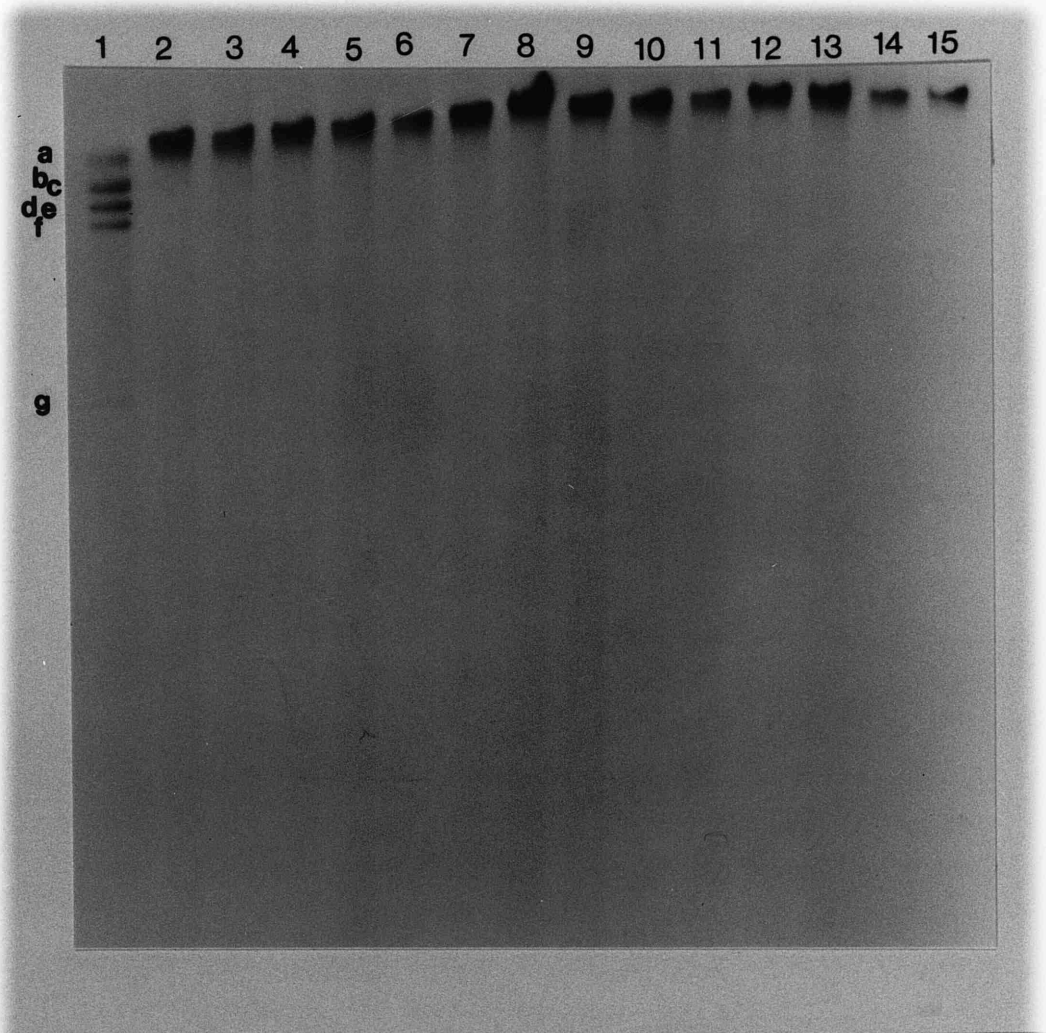

Figure 26. **The *Xba I* Restriction Endonuclease Profiles Of HSV-1 Strain 17± (*wt*) And The *Hind III + Xba I* Restriction Endonuclease Site-Deletion Variants DNAs**

Autoradiographs of the *Xba I* restriction endonuclease digests of HSV-1 strain 17+ (lane 1), 1702 (lane 2), 1721 (lane 3), 1722 (lane 4), 1723 (lane 5), 1724 (lane 6), 1725 (lane 7), 1726 (lane 8), 1727 (lane 9), 1728 (lane 10), 1729 (lane 11), 1730 (lane 12), 1731 (lane 13), 1732 (lane 14) and 1733 (lane 15) DNAs ^{32}-P-labelled *in vivo* and run on a 0.5% agarose gel. The *wt Xba I* bands are labelled on left hand side.

(d) and 3A:1 (a)]. However, despite the analysis of over 600 plaques, a desired variant virus could not be isolated (data not shown). Further plaque isolates were then examined as described in the above sections. The virus stocks were grown in 35mm petri dishes and their ^{32}-P-labelled DNA were subjected to the *Hind III* as well as *Hind III-Bgl II* digestion. In this way, more than 800 plaques were analysed without the isolation of a desired variant (data not shown).

The 0.26 m.c. *Hind III* site is located in between the *Hind III* fragments ***a*** and ***j*** (see Figure 21). The molecular weights of the *Hind III* ***a*** and ***j*** bands are 26.83 x 10^6 and 7.76 x 10^6 respectively (see Table 5). Loss of the 0.26 m.c. *Hind III* site would generate a fused ***a+j*** fragment with a mol. wt. of 34.59 x 10^6, which would migrate with the *Hind III* ***e'*** and ***b'*** bands of 1727 (see 1727 profile in Table 5) on successful marker rescue of this RE site-deletion into the genome of 1727. With the use of the *Hind III* restriction endonuclease alone in the analysis of the plaque isolates (see above), a variant virus could only be identified by the missing *Hind III* ***j*** band. However, if the variant is impure, i.e., a mixture of 1727 and the variant lacking the 0.08, 0.1, 0.26 and 0.91 m.c. *Hind III* sites (as was the case with most of the marker rescue experiments described here), it cannot be identified because of the comigration of the ***a+j*** band with those of the *Hind III* ***e'*** and ***b'*** bands. Therefore, two restriction endonucleases (in combination; either the *Hind III-Eco RI* or *Hind III-Bgl II*) were also used in the analysis of the >1400 plaque isolates described above. However, because of the different restriction endonuclease buffer conditions, partial digestion of the DNA by one of the combined restriction endonucleases has also been observed at least in some samples out of a batch of 48 analysed at one time. Therefore, it was not possible to differentiate a partially digested sample from a desired variant using the Southern blot techniques unless it was repeated and a complete digestion of DNA by both of the restriction endonucleases used was obtained. In addition, it was also necessary to analyse such samples with some other suitable combination of restriction endonucleases to see whether a particular sample is the desired variant. As it is necessary to analyse a large number of plaque isolates in marker rescue experiments, it was decided to use the restriction endonulease analysis of ^{32}-P-labelled DNA [see Section 2B:4 (f)] in further analysis to avoid such repetition. Moreover, this technique was found to be a better one in our hands.

As described above, the marker rescue experiments remained unsuccessful despite the analysis of over 1400 plaques. Because of the

accumulation of several high molecular weight bands at the top of the gel track (see Table 5; see also Figure 22, lane 9) and difficulties encountered in the analysis of these plaques (as discussed above), it was decided to use the variant 1721 instead of 1727. Therefore, the linearized plasmid DNA (pMF18; see above) was then cotransfected with 1721 DNA as described. The progeny virus (single plaque isolates) was analysed as before. Once again, a desired variant could not be isolated despite the analysis of over 500 plaque isolates.

The *Hind III* site at 0.26 m.c. is located within the open reading frame of the UL19 gene (MCP; VP5; Vmw 155; McGeoch *et al.*, 1988b; see also Table 4). The protein product of the UL19 gene, the major capsid protein, is essential for the assembly of the virions (see Section 1D:4 & 6). It is possible, therefore, that because of the presence of unknown lethal mutations within the population of the plasmid molecules lacking the 0.26 m.c. *Hind III* site, a variant virus could not be isolated. To explore this possibility, it was necessary to sequence the HSV-1 DNA fragment contained within the plasmid pMF18 along with carrying out further transfection experiments. However, the DNA sequence was not determined because a variant lacking the 0.26 m.c. *Hind III* site was finally isolated (see below).

The plasmid pMF18 was cotransfected with intact 1702 DNA and the resulting plaque isolates examined as before. An HSV-1 (strain 17^+) variant virus [designated as 1722; see Figure 21 (e)] lacking the *Hind III* site at 0.26 m.c. was isolated within the 192 plaque isolates analysed. The *Hind III* profile of 1722 is shown in Figure 22, lane 4 (see also Table 5). The *Hind III* bands ***a*** and ***j*** are missing and a novel band representing the fused ***a+j*** fragments is running at the top of the gel track. The loss of the 0.26 m.c. *Hind III* site was further confirmed by a *Bgl II-Hind III* digestion of 1722 DNA (see Figure 23, lane 4). The *Bgl II* ***n*** fragment is cleaved by the 0.26 m.c. *Hind III* site [see Figure 21 (b)] into two smaller ***n'*** fragments of mol. wts. 2.70×10^6 and 1.06×10^6 respectively (see Table 6). It can be seen in Figure 23, lane 4 that the *Bgl II* ***n*** band is uncut and running at its normal position (compare with the *Bgl II* profile of HSV-1 strain 17^+ DNA in Figure 25, lane 1) suggesting that the 0.26 m.c. *Hind III* site has indeed been deleted out. As this site is located within an essential HSV-1 gene (see above) and the site-deletion has been carried out by site-directed mutagenesis using a conservative single base alteration within the respective amino acid (see Section 3A:2), the presence of other mutations within the region spanning it are not possible. A high titre 1722 virus stock has also been obtained (see later). No other detectable

change has been found by analysing the 1722 DNA with the *Bam HI* (Figure 24, lane 4), the *Bgl II* (Figure 25, lane 4) or the *Xba I* (Figure 26, lane 4) restriction endonucleases and comparing these profiles with those of strain 17^+ and 1702 DNAs (see Figures 24, 25 and 26, lanes 1 and 2).

(v) **Isolation of the variant 1725**

The plasmid pMF20 [see Section 3A:2 (g)] lacking the 0.64 m.c. *Hind III* site was linearized with the *Eco RI* restriction enzyme and cotransfected with 1721 DNA as described. The infected (transfected) cells were harvested and titrated out (see Methods). Single, well-separated plaques were isolated, grown into virus stocks and analysed as before. A variant virus lacking the 0.64 and 0.91 m.c. *Hind III* sites was isolated and designated as 1725. The *Hind III* sites present on the genome of 1725 are shown in Figure 21 (h) whereas the *Hind III* profile of 1725 DNA is shown in Figure 22, lane 7. The *Hind III* ***b'*** (of 1721), ***c***, ***d*** and ***l*** bands are missing (compare lanes 3 and 7 in Figure 22; see also Table 5) and three novel bands, one migrating at the top of the gel track [***b'***= (***d+g+n***)+***l***; molecular weight of 36.21 x 10^6], one migrating between the *Hind III* ***b'*** and ***a*** bands [***c'***= (***d+m***)+***l***; mol. wt. of 28.45 x 10^6] and the third migrating between the *Hind III* ***a*** and ***e'*** bands (***d+l***; mol. wt. of 23.87 x 10^6) are present in the group of bands running at the top of the gel track. The loss of the 0.64 m.c. *Hind III* site was further confirmed by a *Bgl II-Hind III* digestion of 1725 DNA (see Figure 23, lane 7). The *Bgl II* ***g*** fragment is cleaved by the 0.58 m.c. *Hind III* site into two smaller ***g'*** fragments with molecular weights of 10.91 and 0.50 x 10^6 respectively. The larger fragment is further cleaved by the 0.64 m.c. *Hind III* site into two ***g"*** fragments of 5.70 and 5.21 x 10^6 M_r respectively [see Figure 21 (b); see also Table 6]. The two *Bgl II* ***g"*** bands comigrate with the *Bgl II* ***d'*** and ***h'*** bands respectively (see Figure 23, lanes 1 and 2). The deletion of the 0.64 m.c. *Hind III* site would generate the larger *Bgl II* ***g'*** (M_r 10.91 x 10^6) band. It can be seen in Figure 23, lane 7 that the two ***g"*** bands are missing and a novel ***g'*** band is running in between the ***d'*** and ***i*** bands, thus confirming the loss of the *Hind III* site at 0.64 m.c. The *Bam HI*, *Bgl II* and *Xba I* profiles of 1725 DNA were found identical to those of the parental viruses, indicating no other detectable deletion and/or insertion within the region spanning the 0.64 m.c. *Hind III* site (see lanes 1, 2 and 7 in Figures 24, 25 and 26).

(vi) **Isolation of the variant 1726**

For the isolation of an HSV-1 strain 17^+ variant lacking the 0.18 m.c. *Hind III* sites, a mutant plasmid pMF19 devoid of the two *Hind III* sites at

0.18 m.c. was constructed [see Section 3A:2 (d)] and used in transfection experiments. The plasmid DNA was linearized with either of the *Sca I*, *Sph I* or *Xba I* restriction endonuclease and cotransfected with intact 1721 DNA as described. Using the same procedures (see above), over 1500 plaque isolates were analysed but a variant lacking the 0.18 and 0.91 m.c. *Hind III* sites could not be isolated.

The 0.18 m.c. *Hind III* sites are located within the UL13 gene (McGeoch *et al.*, 1988b), whose product, a protein kinase (Smith and Smith, 1989; see Table 4) is dispensible in tissue culture (Miss L Coulter, personal communication). Therefore, the possible presence of lethal mutations beside the conservative changes responsible for the destruction of the *Hind III* sites within the coding DNA sequence of the UL13 gene would have had no effect on the viablity of the variant virus. Different batches of BHK21/C13 cells were used in separate transfection experiments. In addition, HSV-1 strain 17^+ (*wt*) DNA has always been used as a positive control in these experiments and produced a satisfactory cytopathic effect on the transfected cell monolayers. No other reason could be found for being unable to isolate a desired variant virus.

The linearized plasmid (pMF19) DNA was then cotransfected with intact 1702 DNA and the resulting plaque isolates were examined as before. The variant 1726 [see Figure 21 (i)] lacking the two *Hind III* sites at 0.18 m.c. has finally been isolated and analysed. The *Hind III* profile of 1726 DNA is shown in Figure 22, lane 8 (see also Table 5). The 0.18 m.c. *Hind III* sites are located in between the *Hind III* fragments ***h*** and ***j*** (see Figure 21). It can be seen that the *Hind III* ***h*** and ***j*** bands are missing (Figure 22, lane 8) and a novel band is migrating in between the ***d*** and ***f*** bands. The combined molecular weights of the *Hind III* ***h***, ***j*** and the innominate fragment (**In**) (8.14×10^6, 7.76×10^6 and 0.03×10^6 respectively; see Table 5) was found equivalent to the apparent molecular weight of the novel band. Thus, indicating the loss of the 0.18 m.c. *Hind III* sites. On *Bgl II-Hind III* digestion of 1726 DNA, the two *Bgl II* ***o'*** bands (M_r 1.73 and 1.72×10^6 respectively; the *Bgl II* ***o*** fragment is cleaved by the 0.18 m.c. *Hind III* sites; see Figure 21; see also Table 6) were found missing along with the formation of a normal *Bgl II* ***o*** band (see Figure 23, lane 8). Moreover, no other detectable deletion and/or insertion was found on comparison of the *Bam HI*, *Bgl II* or *Xba I* profiles of 1726 DNA with that of the 17^+ or 1702 DNA profiles (see Figures 24, 25 and 26).

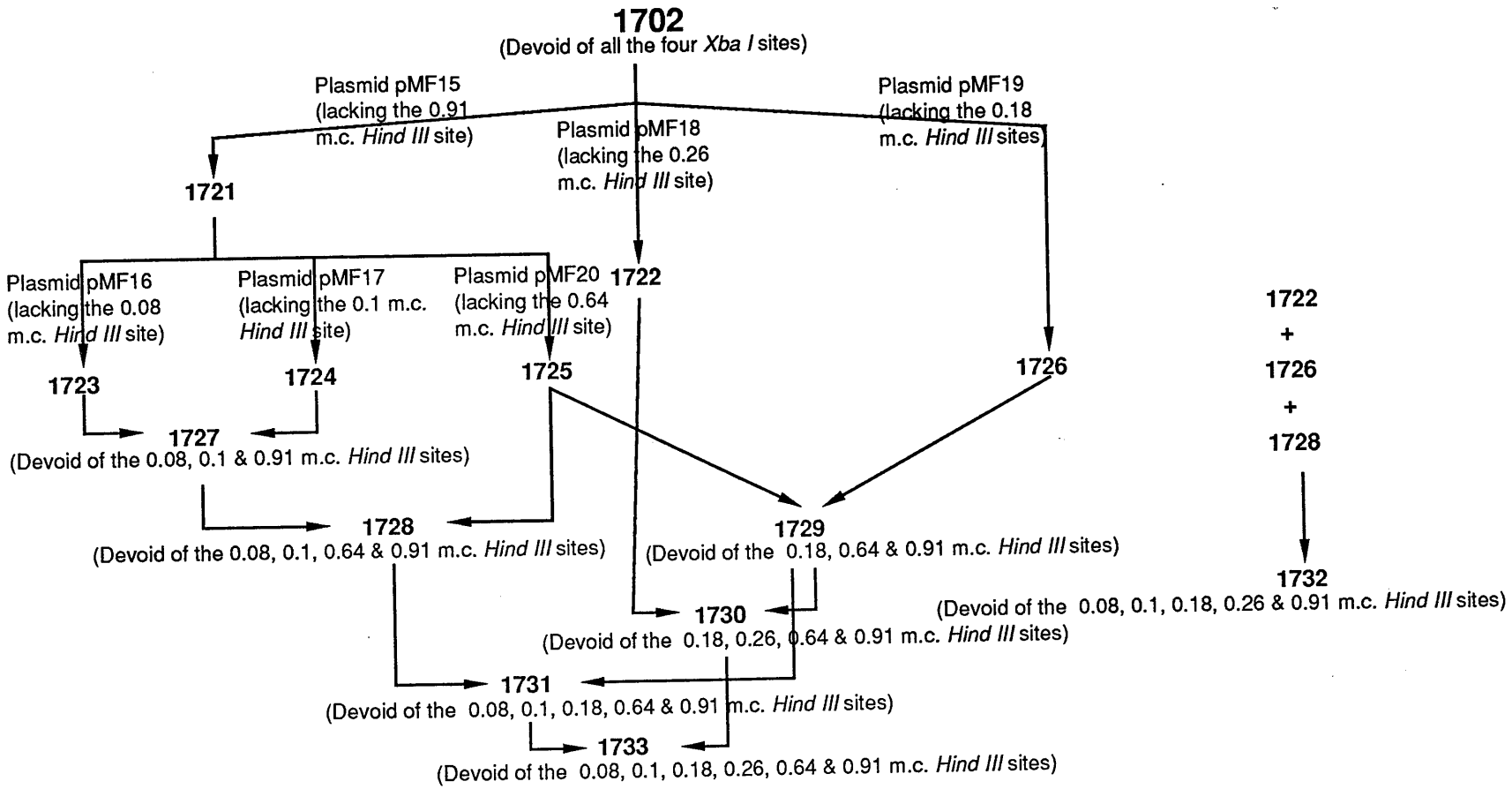

FIGURE 26.5 **CONSTRUCTION OF THE VARIANTS 1721 TO 1733**

Summary of HSV-1 strain 17^+ *Hind III* site-deletion variant's generation.

Since all the *Hind III* sites were deleted by making conservative single-base mutations (see Table 3) and as most of these *Hind III* sites (four out of seven) are located within the essential regions of the HSV-1 genome (see Table 4), it was assumed that no other changes such as deletions or insertions have occurred within the regions spanning the mutated *Hind III* sites. In addition, no changes in either the plaque morphology (data not shown) or growth characteristics (see later) of the variants described above was observed when compared with the parental viruses.

(vii) **Isolation of the variants 1728, 1729, 1730, 1731, 1732 and 1733**

In order to isolate an HSV-1 genome lacking the 7 deleted *Hind III* sites (as above), the variants 1725 and 1727 were recombined by infecting confluent BHK21/C13 cell monolayers at an moi of 5 pfu/cell of each virus. The infected cells were harvested at 24 h *pi* and titrated out at 37°C (see Section 2B:16). The resulting plaques were isolated and their DNA analysed with the *Hind III* as well as the *Bgl II-Hind III* restriction endonuclease digestion. Several plaque isolates showed different RE profiles compared to that of the 1725 and 1727 viruses. Of these, one was designated as the variant 1728 lacking the *Hind III* sites at 0.08, 0.1, 0.64 and 0.91 m.c {see Figure 21 (k)}. The *Hind III* profile of 1728 is shown in Figure 22, lane 10 with missing ***b***, ***c***, ***d***, ***e***, ***f***, ***g***, ***h***, ***i***, ***l***, ***n*** and ***o*** bands [compare with the ***wt*** (lane 1) and 1702 (lane 2) DNA profiles; see also Table 5]. The fused bands, such as, ***g+n*** (M_r 12.34 x 10^6) and ***h+i+o*** (M_r 18.38 x 10^6) can also be seen. However, other fused bands, such as, ***b'***, ***e'***, ***c'***, ***d+i*** and ***f'*** are difficult to differentiate from each other because of smaller differences between their respective moleuclar weights and comigration at the top of the gel track. A *Bgl II-Hind III* digestion of 1728 DNA has confirmed the absence of 0.08, 0.1, 0.64 and 0.91 m.c. *Hind III* sites (Figure 23, lane 10; see above sections for description regarding the respective *Hind III* sites and the *Bgl II* fragments containing these *Hind III* sites).

Similarly, the variants 1725 and 1726 were recombined to obtain a variant 1729 lacking the *Hind III* sites at 0.18, 0.64 and 0.91 m.c. [see Figure 21 (l)]. The *Hind III* profile of 1729 DNA is given in Table 5 and shown in Figure 22, lane 11. The *Hind III* ***b***, ***c***, ***d***, ***e***, ***g***, ***h***, ***j***, ***l***, ***n*** and ***In*** [the Innominate (***In***) *Hind III* band is too small (M_r 0.03 x 10^6) to be observed on a 0.5% agarose gel and runs off from the bottom of the gel during electrophoresis. The oligonucleotide used to delete the 0.18 m.c. *Hind III* sites contained the *Hind III* ***In*** fragment to ensure its presence within the fused ***h+In+j*** *Hind III*

* was isolated during a previous attempt to isolate the variant 1733 directly by infecting cell monolayers at an moi of 3.33 pfu/cell of each of the three variants 1722, 1726 and 1728.

** The construction of the *Hind III* site-deletion variants 1721 to 1733 is illustrated in Figure 26.5.

fragment spanning these cleavage sites (see Table 3)] fragments are missing and fused fragments ***b'***, ***c'*** ,***d+l***, ***e'*** and ***g+n*** are migrating at an identical position to those of 1725 (compare lanes 7 and 11 in Figure 22) whereas the fused *Hind III* fragment ***h+j+ln*** is running identically as in 1726 (compare lanes 8 and 11 in Figure 22). The *Bgl II-Hind III* digestion of 1729 DNA has confirmed the absence of the 0.18, 0.64 and 0.91 m.c. *Hind III* sites (see Figure 23, lane 11 and compare with lanes 7 and 8; see also Table 6).

The variants 1730 lacking the 0.18, 0.26, 0.64 and 0.91 m.c. *Hind III* sites [see Figure 21 (m)] and 1731 lacking the *Hind III* sites at 0.08, 0.1, 0.18, 0.64 and 0.91 m.c. [Figure 21 (n)] were subsequently isolated by recombining the variants 1722 with 1729 (to obtain 1730) and 1728 with 1729 (to obtain 1731). The *Hind III* profiles of 1730 and 1731 DNAs are shown in Figure 22, lanes 12 and 13 whereas their *Bgl II-Hind III* profiles are shown in Figure 23, lanes 12 and 13 respectively (see Tables 5 & 6). Comparison of the *Hind III* as well as *Bgl II-Hind III* profiles of 1730 and 1731 with that of the parental viruses has indicated the absence of the specific *Hind III* sites as mentioned (see above).

Finally, the variants 1730 and 1731 were recombined as described and the resulting plaque isolates examined as before. The desired variant (1733) lacking the 7 *Hind III* sites at 0.08, 0.1, two at 0.18, 0.26, 0.64 and 0.91 m.c. was isolated [see Figure 21 (p)] . The variant (1732) devoid of the *Hind III* sites at 0.08, 0.1, 0.18, 0.26 and 0.91 m.c. [see Figure 21 (o)]*. The *Hind III* profiles of 1732 and 1733 DNAs are shown in Figure 22, lanes 14 and 15 whereas their *Bgl II-Hind III* profiles are shown in Figure 23, lanes 14 & 15 respectively. These profiles were compared with those of the parental viruses and were found satisfactory (see Figures 22 and 23; see also Tables 5 & 6)**.

The *Bam HI* (Figure 24), *Bgl II* (Figure 25) and *Xba I* (Figure 26) DNA profiles of the variants 1728, 1729, 1730, 1731, 1732 and 1733 were found identical to that of the parental viruses. Thus, no other detectable deletion/insertion was observed within the genomes of these variants using RE analysis.

(viii) **Growth properties of the variants 1721 to 1733**

The growth properties of the variants 1721 to 1733 were found very similar to those of HSV-1 strain 17+ and 1702 (MacLean and Brown, 1987a). Single-cycle growth experiments were carried out in BHK21/C13 cells over a 36 h period [see Section 2B:3 (e)]. Both 1721 and 1733 showed the *wt* growth characteristics of strain 17+ (Figure 27). Multiple-cycle growth experiments involving 1721 and 1733 produced similar results (data not

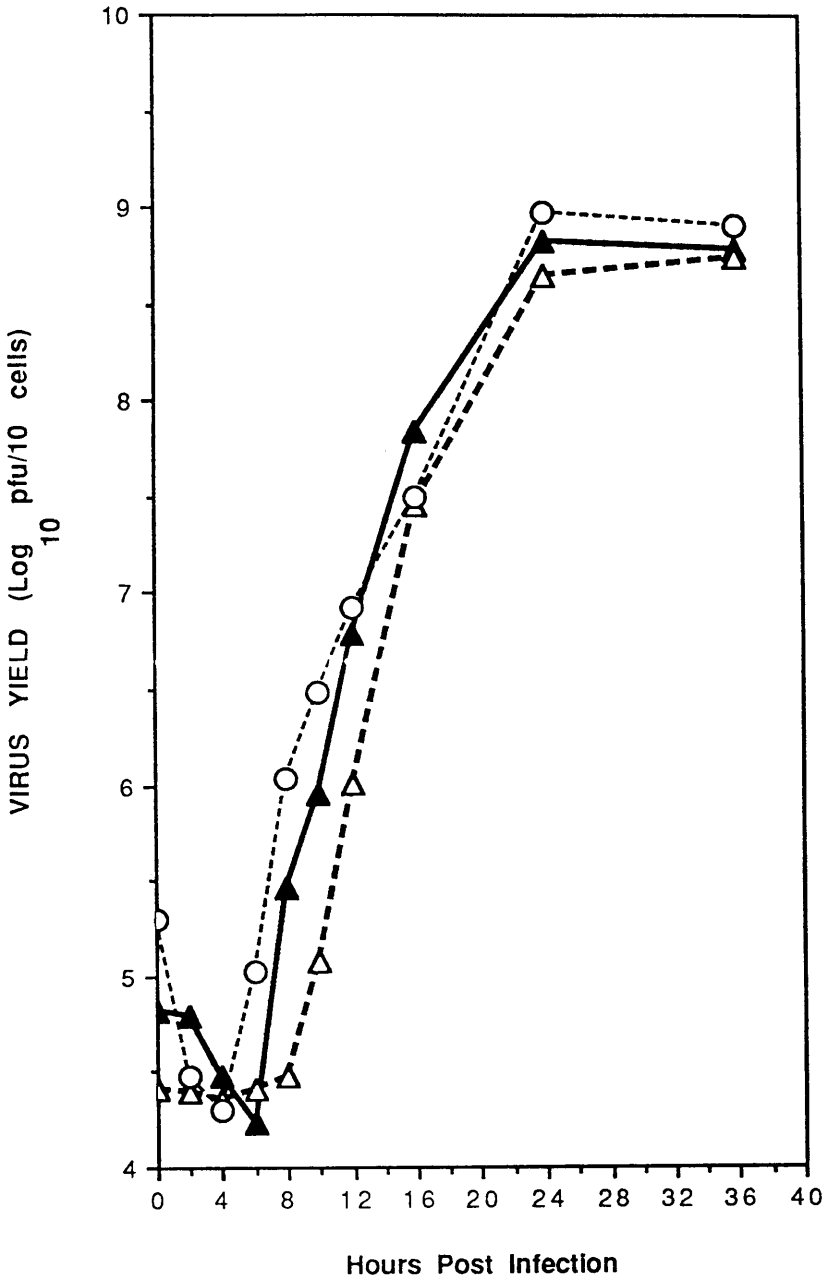

Figure 27. **Single-Cycle Growth Curves Of HSV-1 Strain 17+, 1721 And 1733**

Single-cycle growth curves of HSV-1 strain 17+ (—▲—), 1721 (--△--) and 1733 (---○---). BHK21/C13 cells were infected at an *moi* of 5 pfu/cell, the cell monolayers were washed twice with PBS-calf, overlaid with ETC5 or ETC10 and incubated at 37°C. Cells were harvested at 0, 2, 4, 6, 8, 10, 12, 16, 24 and 36 h *pi* (see Section 2B : Methods).

shown). High titre stocks of 1721, 1722, 1723, 1724, 1725, 1726, 1727, 1728, 1729, 1730, 1731, 1732 and 1733 have been obtained (see Table 7).

As the variant 1733 was derived from the variants 1722 to 1732 and virus titres obtained for these variants were very similar (Table 7), single-cycle growth experiments were not carried out for these viruses (1722 to 1732).

(b) **Isolation of an HSV-1 (strain 17±) genome lacking a *Bgl II* restriction endonuclease cleavage site**

During the process of marker rescuing the HSV-1 DNA fragments contained within the plasmids pMF18 and pMF19 [see Sections 3A:2 (d) & (e) and 3B:1 (a) (iv) & (vi)] back into the virus, nearly 4000 plaque isolates were examined using the restriction endonuclease analysis. In addition to the isolation of the desired variants (1722 and 1726; see above), a number of other variants with aberrant restriction endonuclease profiles were also isolated. One of these spontaneously isolated variants was designated as 1734 (see Figure 28). The plaque isolates were usually examined by the *Hind III* as well as *Bgl II-Hind III* restriction endonucleases. The *Hind III* profile of 1734 [see Figure 29 (b), lane 3] was identical to that of the parental virus 1721 [Figure 29 (b), lane 2]. Like 1721, 1734 was also devoid of the 0.91 m.c. *Hind III* site plus the four *Xba I* sites [see above; Section 3B:1 (a) (i)]. However, the *Bgl II-Hind III* digestion of 1734 DNA has shown that the *Bgl II* ***m*** (M_r 4.21 x 10^6) and one of the ***n'*** (M_r 1.06 x 10^6) bands were missing and a novel band (M_r 5.27 x 10^6) was comigrating with the *Bgl II* ***g"*** (M_r 5.21 x 10^6) band [see Figure 29 (a), lane 4; compare the *Bgl II-Hind III* profile with that of 1721 (lanes 5 & 6 as well as Figure 23, lane 3)]. The remaining *Bgl II* bands were found identical to that of 1721(see Tables 6 & 8). Thus, indicating that the 0.272 m.c. *Bgl II* site [located in between the *Bgl II* ***m*** and ***n*** fragments; see Figure 28 (a)] is lost because of some change within the *Bgl II* recognition sequence (AGATCT) and/or a small deletion within the region spanning it. A *Bgl II* digestion of 1734 DNA has further confirmed the loss of the *Bgl II* site at 0.272 m.c. [see Figure 29 (a), lanes 1 to 3]. It can be seen in Figure 29 (a), lane 2 that the *Bgl II* ***m*** and ***n*** (M_r 3.76 x 10^6) bands are missing and a novel band (approx. M_r 7.97 x 10^6) representing the fused *Bgl II* ***m+n*** band is running in between the *Bgl II* ***j*** (M_r 9.58 x 10^6) and ***k*** (M_r 6.94 x 10^6) bands (see Table 8).

In order to find out whether the loss of the *Bgl II* site at 0.272 m.c. has involved any other deletion/insertion, 1734 DNA was analysed with various other restriction endonucleases. A *Bam HI* digest of 1734 DNA gave an identical profile [see Figure 29 (c), lane 3] to that of 1702 [Figure 29 (c),

TABLE 7

	VIRUS	STOCK TITRE
1.	HSV-1 strain 17+	1.80×10^{10} pfu/ml
2.	1702	1.70×10^{9} pfu/ml
3.	1721	7.50×10^{9} pfu/ml
4.	1722	2.50×10^{10} pfu/ml
5.	1723	2.20×10^{9} pfu/ml
6.	1724	1.88×10^{9} pfu/ml
7.	1725	1.16×10^{11} pfu/ml
8.	1726	2.50×10^{11} pfu/ml
9.	1727	1.05×10^{10} pfu/ml
10.	1728	1.15×10^{11} pfu/ml
11.	1729	6.50×10^{10} pfu/ml
12.	1730	1.00×10^{10} pfu/ml
13.	1731	2.50×10^{10} pfu/ml
14.	1732	3.50×10^{9} pfu/ml
15.	1733	2.25×10^{10} pfu/ml

Table 7. **Virus Stock Titres Of HSV-1 Strain 17+ And Restriction Endonuclease Site-Deletion Variants 1702, And 1721 To 1733**

See Section 2B : 'Methods' for the preparation of high titre virus stocks.

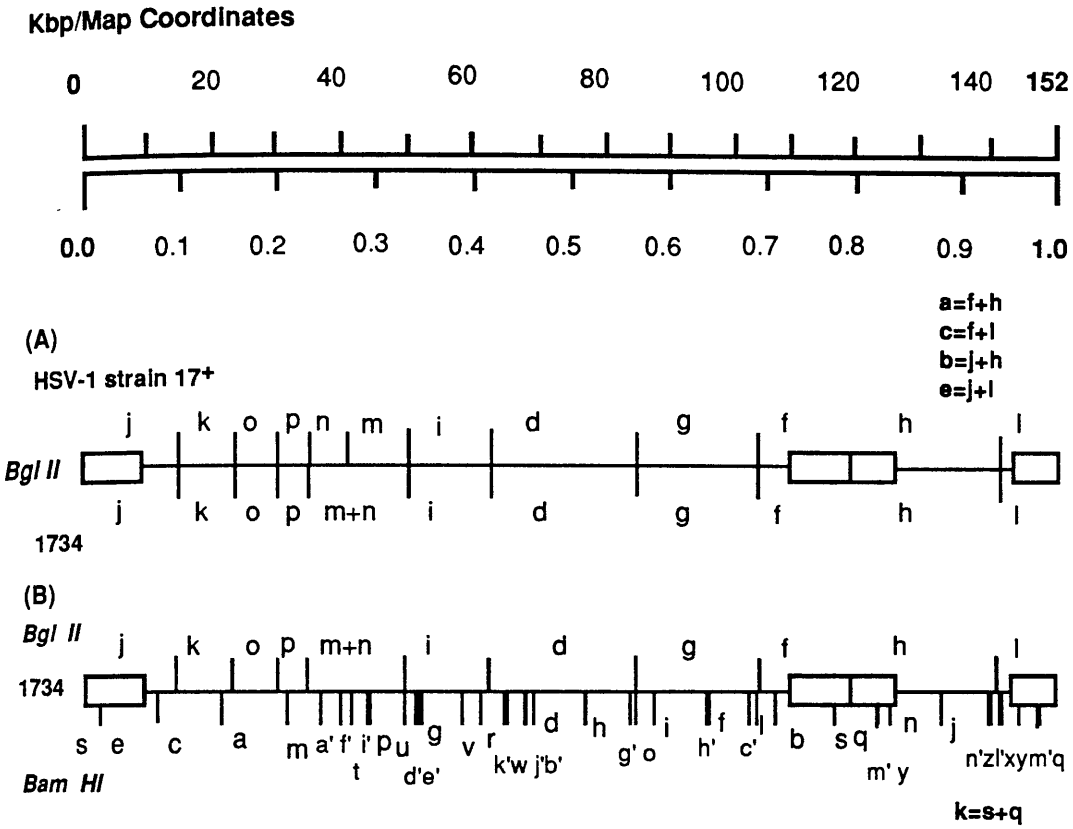

Figure 28. **The *Bgl II* And *Bam HI* Restriction Endonuclease Maps Of The HSV-1 Strain 17± And The Variant 1734 Genomes**

(A) The *Bgl II* maps for the DNA of HSV-1 strain 17+ (above the line) and 1734 (Below the line). There are two *Bgl II* sites each between the *Bgl II* ***k/o*** fragments (231 bp apart) and ***o/p*** fragments (426 bp apart) (not shown in the figure).

(B) The Bgl II map of 1734 (above the line) and the *Bam HI* map (below the line) for the DNA of HSV-1 strain 17+.

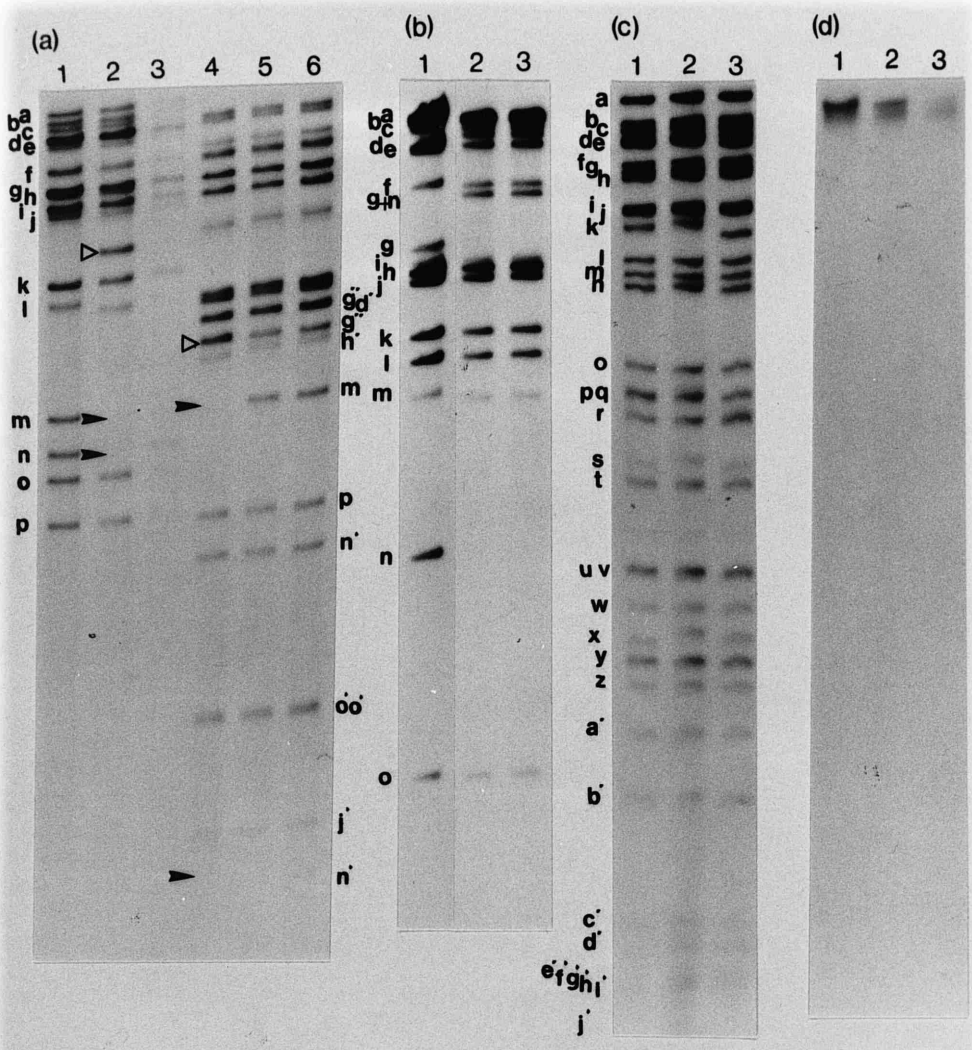

Figure 29. The Restriction Endonuclease Profiles Of HSV-1 (Strain 17±) Variant 1734 DNA

(a) The *Bgl II* restriction endonuclease profiles of HSV-1 strain 17+ variants 1702 (lane 1; identical to that of *wt*; see Figure 25, lanes 1 & 2), 1721 (lane 3) and 1734 (lane 2) DNAs ^{32}-P-labelled *in vivo* (0.5% agarose gel). The *wt Bgl II* bands are labelled on left hand side. The missing bands are indicated with a filled arrowhead (►) and the novel band with an open triangle (▷).

The *Bgl II-Hind III* profiles of 1734 (lane 4) and 1721 (lanes 5 & 6). The HSV-1 variant 1734 was derived from the variant 1721 whose profile is identical to that of *wt* except that the 0.91 m.c. *Hind III* site is missing (see Figure 22, lane 3). The lower *wt Bgl II* bands are labelled on right hand side (see Figure 23 for labelling of the remaining *Bgl II* bands). The missing *Bgl II m* and one of the *n'* bands are indicated (►) and the fused *m+n'* band is comigrating with the Bgl II g" h' bands (▷).

(b) The *Hind III* profiles of 1702 (lane 1), 1721 (lane 2) and 1734 (lane 3) DNAs. The *wt Hind III* bands are labelled on left hand side. The fused *g+n* band in lanes 2 and 3 is also labelled.

(c) The *Bam HI* profiles of 1702 (lane 1), 1721 (lane 2) and 1734 (lane 3) DNAs. The *wt Bam HI* bands are labelled on left hand side.

(d) The *Xba I* profiles of 1702 (lane 1), 1721 (lane 2) and 1734 (lane 3) DNAs. As the parental virus (1702) contain no *Xba I* site, the DNA remained undigested when subjected to the *Xba I* restriction endonuclease digestion. See Figure 26 for the *wt Xba I* bands.

TABLE 8

BGL II AND BGL II-HIND III PROFILES

(Molecular Weights of ***Bgl II*** fragments x 106)

	17 syn± (*Bgl II*)	1734		17syn± (*Bgl II-Hind III*)	1734	1735		1736
a	23.65	23.65						
b	20.89	20.89						
c	18.58	18.58	c	18.58	18.58	18.58		18.58
d	17.00	17.00	a'	17.45	17.45	17.45		17.45
e	15.82	15.82						
			e'	14.59	14.59	14.59		14.59
			b'	13.46	13.46	13.46		13.46
f	12.34	12.34	f	12.34	12.34	12.34		12.34
g	11.42	11.42						
h	11.31	11.31	d'	11.32	11.32	11.32		11.32
						g' 10.82		10.82
i	10.26	10.26	i	10.26	10.26	10.26		10.26
j	9.58	9.58						
	m+n	7.97						
			j'	8.35	8.35	8.35		8.35
k	6.94	6.94						
			k'	6.28	6.28	6.28		6.28
l	6.24	6.24	l	6.24	6.24	6.24		6.24
					h' 6.20	6.20		6.20
			g"	5.71	5.71	- -		- -
			d'	5.68	5.68	5.68		5.68
					m+n' 5.27	5.27		5.27
			g"	5.21	5.21	- -		- -
			h'	5.11	5.11	5.11		5.11
m	4.21	- -	m	4.21	- -	- -		- -
n	3.76	- -						
o	3.48	3.48					o	3.48
			h"	3.25	- -	- -		- -
p	3.04	3.04	p	3.04	3.04	3.04		3.04
			h"	2.95	- -	- -		- -
			n'	2.70	2.70	2.70		2.70
			o'	1.73	1.73	1.73		- -
			o'	1.72	1.72	1.72		- -
			j'	1.23	1.23	1.23		1.23
			n'	1.06	- -	- -		- -
			k'	0.66	0.66	0.66		0.66
			g'	0.50	0.50	0.50		0.50
In	0.28	0.28	In	0.28	0.28	0.28		0.28
In	0.15	0.15	In	0.15	0.15	0.15		0.15
			In	0.03	0.03	0.03		- -

(In = Innominate)

TABLE 8 (Continued)

BGL II AND BGL II-HIND III PROFILES

(Molecular Weights of ***Bgl II*** fragments x 106)

	17 syn± (*Bgl II*)	1734		17syn± (*Bgl II-Hind III*)	1737		1738
a	23.65	23.65					
b	20.89	20.89					
c	18.58	18.58	c	18.58	18.58		18.58
d	17.00	17.00	a'	17.45	17.45		17.45
e	15.82	15.82				e	15.82
						b'	14.69
			e'	14.59	14.59		- -
			b'	13.46	13.46		- -
f	12.34	12.34	f	12.34	12.34		12.34
g	11.42	11.42					
h	11.31	11.31	d'	11.32	11.32		11.32
					g' 10.82		10.82
i	10.26	10.26	i	10.26	10.26		10.26
j	9.58	9.58				j	9.58
	m+n	7.97				m+n	7.97
			j'	8.35	8.35		- -
k	6.94	6.94			k 6.94		6.94
			k'	6.28	- -		- -
l	6.24	6.24	l	6.24	6.24		6.24
					h' 6.20		6.20
			g"	5.71	- -		- -
			d'	5.68	5.68		5.68
					m+n' 5.27		- -
			g"	5.21	- -		- -
			h'	5.11	5.11		5.11
m	4.21	- -	m	4.21	- -		- -
n	3.76	- -					- -
o	3.48	3.48			o 3.48		3.48
			h"	3.25	- -		- -
p	3.04	3.04	p	3.04	3.04		3.04
			h"	2.95	- -		- -
			n'	2.70	2.70		- -
			o'	1.73	- -		- -
			o'	1.72	- -		- -
			j'	1.23	1.23		- -
			n'	1.06	- -		- -
			k'	0.66	- -		- -
			g'	0.50	0.50		0.50
In	0.28	0.28	In	0.28	0.28		0.28
In	0.15	0.15	In	0.15	0.15		0.15
			In	0.03	- -		- -

(In = Innominate)

Table 8. **The *Bgl II* And *Bgl II-Hind III* Restriction Endonuclease (RE) Profiles Of HSV-1 Strain 17± And RE Site-Deletion Variants 1734 To 1738**

lane 1] and 1721 [Figure 29 (c), lane 2] DNAs. Similarly, the *Hind III* [Figure 29 (b)], *Xba I* [Figure 29 (d)], *Eco RI*, *Hpa I* and *Kpn I* (data not shown) profiles of 1734 DNA were found identical to those of the parental viruses.

The 0.272 m.c. (n. 41448) *Bgl II* site is located within the *Bam HI* ***f'*** (n. 40778 to 41545 = 767), *Eco RI* ***g*** (n. 29338 to 45570 = 16232), *Hind III* ***a*** (n. 39849 to 80707 = 40858), *Hpa I* ***i*** (n. 41132 to 49833 = 8701) and *Kpn I* ***i*** (n. 34188 to 43620 = 9432) fragments. Therefore, the best restriction endonuclease DNA fragment was the *Bam HI* ***f'*** to see the extent of deletion and/or insertion leading to the destruction of the 0.272 m.c. *Bgl II* site. However, as the *Bam HI* ***e'***, ***f'***, ***g'***, ***h'*** and ***i'*** bands comigrate with each other because of smaller differences between their respective molecular weights [see Figure 29 (c)], it was decided to use the Southern blot technique to see any change in the mobility of the *Bam HI* ***f'*** band of 1734 DNA compared to that of the strain 17+, 1702 and 1721 DNAs.

The *Bam HI* digested DNAs of HSV-1 strain 17+, 1702, 1721 and three different plaque isolates of 1734 were resolved on a 1.2% agarose gel along with a 123 bp DNA ladder (BRL). The DNA was then transferred to Hybond nylon membranes [see Section 2B:10 (a)]. These were hybridized to the nick-translated DNA [see Section 2B:10 (b)] prepared from either the plasmid pMF1 [see Section 3A:1 (a)] or the plasmid containing the HSV-1 *Kpn I* ***i*** fragment [see Section 2A:6 (b)] following the method described in Section 2B:10 (d). Autoradiographs of these DNA-DNA hybridization experiments are shown in Figure 30 (a) and (b). It can be seen that the mobility or size of the *Bam HI* ***f'*** fragment of 1734 DNA (lanes 5, 6 & 7) is identical to that of HSV-1 strain 17+ (lane 2), 1702 (lane 3) and 1721 (lane 4). Thus indicating that a semiconservative change has led to the removal of the *Bgl II* site at 0.272 m.c.

The *Bgl II* site at 0.272 m.c. is located within the UL20 gene (n. 40822 to 41488; McGeoch *et al.*, 1988b). Because of non-isolation of a *Lac Z* insertion mutant for UL20 (MacLean, C.A. *et al.*, 1991), it was suggested that this gene may be essential for virus growth in tissue culture. Therefore, it was interesting to see whether 1734 virus produces normal levels of the UL20 gene product. For this purpose, immunoprecipitation experiments were kindly carried out by Dr C A MacLean (see Section 2B:14). The results of immunoprecipitations are shown in Figure 31. It can be seen that precipitation of the 22K protein product of the UL20 gene (MacLean, C.A. *et al.*, 1991) is specifically inhibited by the peptide against which the serum was raised from extracts of cells infected with HSV-1 strain 17+ (lane 5), 1721 (lane 9) and 1734 (lane 7) but not by an unrelated control peptide (lanes 4, 8 and 6

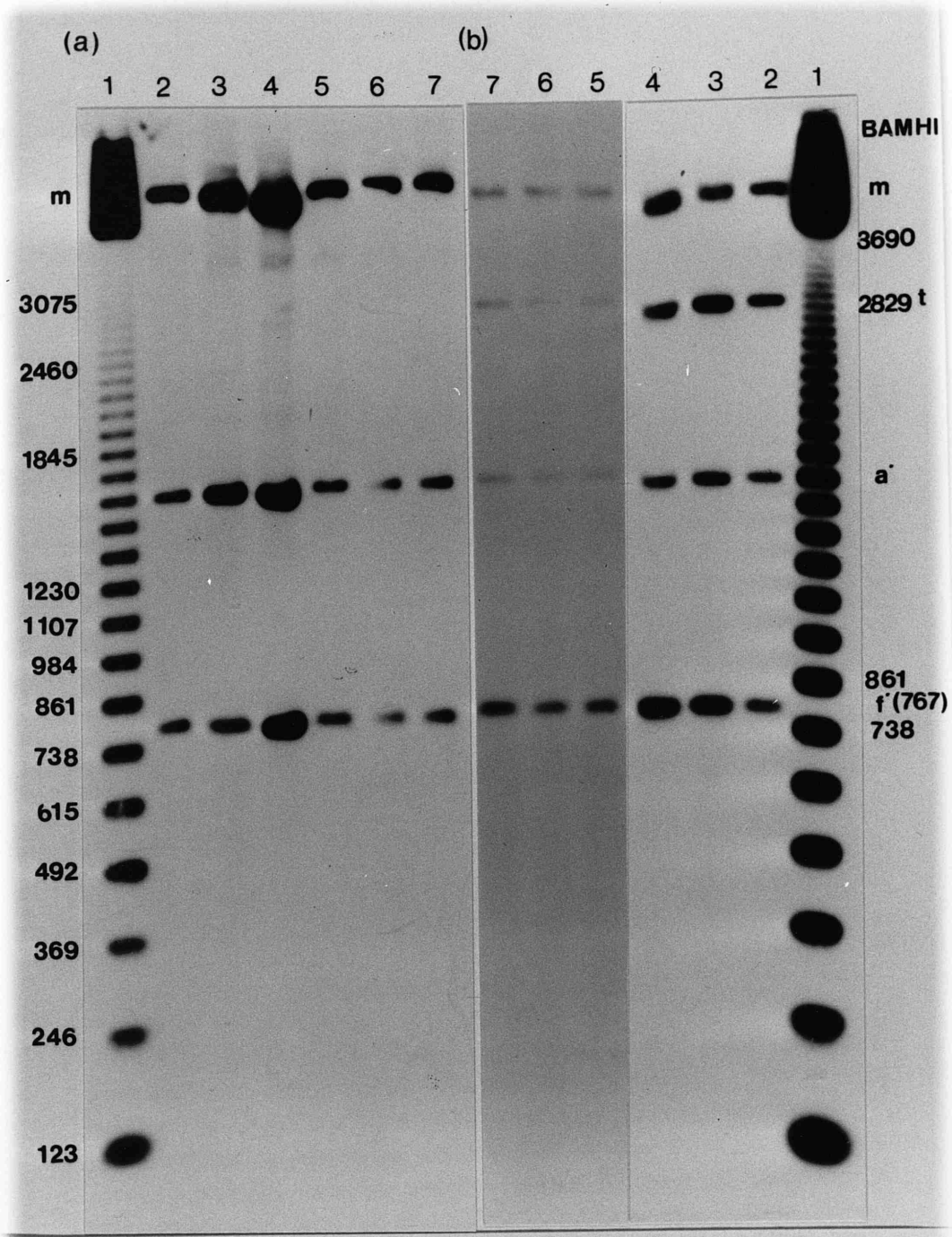

Figure 30. **Southern Blot of 1734 DNA Using (a) The Plasmid pMF1 Probe And (b) The Plasmid *Kpn I i* Probe**

Autoradiographs of Southern blot hybridization experiments. The nick translated DNA from pMF1 (a) or *Kpn I i* (b) was hybridized to *Bam HI* digested DNA of HSV-1 strain 17+ (*wt*; lane 2), 1702 (lane 3), 1721 (lane 4) and three different plaque isolates of 1734 (lanes 5 to 7). The digested DNA was resolved on a 1.2% agarose gel along with a 123 bp DNA ladder (BRL; lane 1). The 123 bp DNA ladder is labelled on the left hand side whereas the *wt Bam HI* bands and few of the 123 bp ladder bands are labelled on the right hand side. The *Bgl II n/m* site (0.272 m.c.) is located within the 767 bp *Bam HI f'* fragment [see Figure 27 (b)].

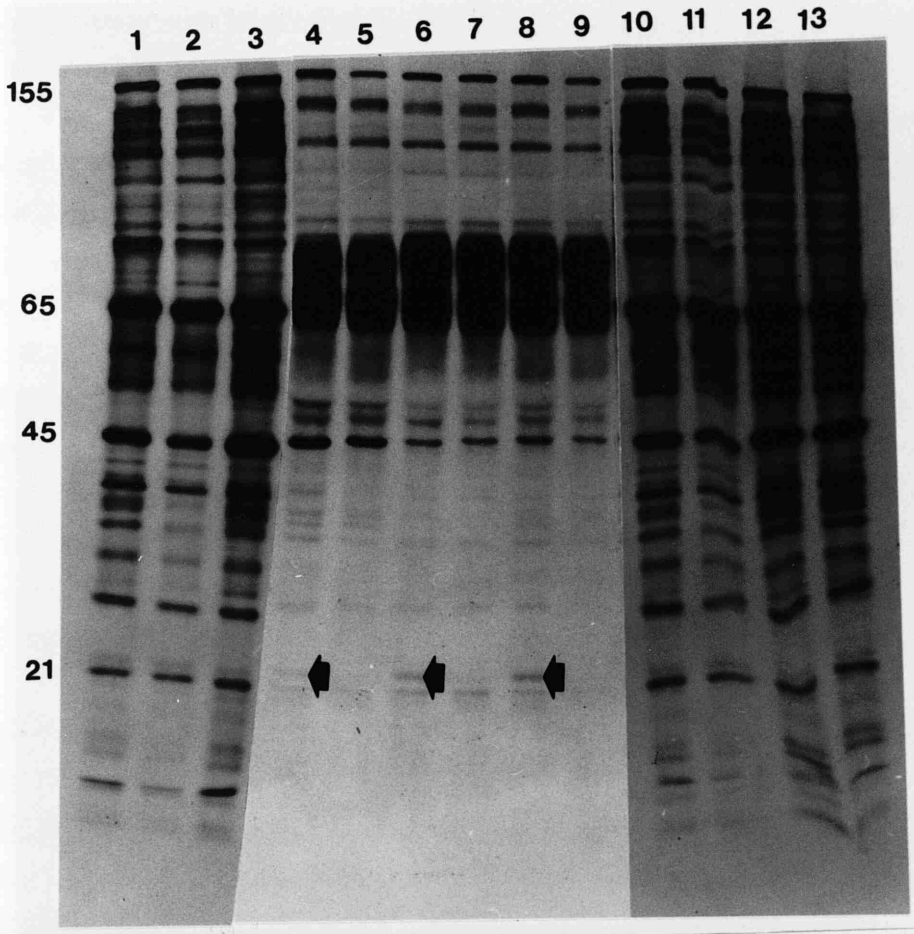

Figure 31. Immunoprecipitation For The Variant 1734

Immunoprecipitation of the UL20 gene product. Antiserum was raised against a synthetic oligopeptide, NH_2-Gln-Met-Leu-Pro-Pro-Thr-Asp-Pro-Leu-Arg-Thr-Arg-Tyr-COOH, representing amino acids 168 to 179 of the UL20 ORF with a carboxy-terminal tyrosine residue added to facilitate coupling to BSA (MacLean, C.A. *et al.*, 1991). Proteins from HSV-1 (strain 17$^+$)-infected (lanes 4 & 5), 1734-infected (lanes 6 & 7) or 1721-infected (lanes 8 & 9) cell extracts labelled with [^{35}S]methionine were precipitated using immune serum in the presence of 10 μg of the peptide against which the serum was raised (lanes 5, 7 & 9) or an unrelated control peptide (lanes 4, 6 & 8), and analysed on 5 to 12.5% SDS-PAGE (see Methods : Section 2B). A band which appeared to be specifically precipitated is shown with a filled arrowhead (◀). Molecular weights x 10^3 are shown on left hand side. HSV-1 (strain 17$^+$)-infected (lanes 3, 12 & 13), 1734-infected (lanes 2 & 11) and 1721-infected (lanes 1 & 10) cell extracts labelled with [^{35}S]methionine are also shown.

respectively). The approximate molecular weight (22K) of the UL20 gene product is comparable in all the three viruses (see Figure 31), indicating that the *Bgl II* site loss had no effect, whatsoever, on the production of the UL20 protein. Therefore, it appeared that the UL20 ORF was not interrupted because of the changes leading to the destruction of the 0.272 m.c. *Bgl II* site, suggesting a semiconservative alteration within the relevant amino acids. However, a recent report has shown the isolation of a viable UL20 DNA negative mutant of HSV-1 strain F in at least some cell lines in tissue culture (Baines *et al.*, 1991), suggesting that the UL20 gene product is not required for virus growth at least in cell lines which provide a substitute function for the UL20 gene. The report also indicates that the UL20 gene product is required for the efficient transport of virions to the extracellular space. Therefore, the presence of a small undetectable (using the analysis described above) deletion and/or insertion leading to the loss of the 0.272 m.c. *Bgl II* site cannot be ruled out.

The variant 1734 has shown similar growth characteristics to that of *wt* (strain 17^+) on a one-step growth experiment (Figure 32). High titre stocks of 1734 were obtained (see Table 9).

(c) **Generation of an HSV-1 (strain $17^\pm$) genome devoid of one *Bgl II*, seven *Hind III* and four *Xba I* restriction endonuclease cleavage sites**

In order to obtain an additional unselected marker (RE sites) for subsequent use in intrastrain recombination studies, it was decided to recombine the variants 1733 (lacking 7 *Hind III* and 4 *Xba I* sites) and 1734 (lacking 1 *Bgl II*, the 0.91 m.c. *Hind III* sites and 4 *Xba I* sites; see above). BHK21/C13 cell monolayers were infected at an *moi* of 5 pfu/cell of each virus and the resulting plaque isolates were analysed using the *Bgl II-Hind III* digestion of their ^{32}-P-labelled DNAs. As a result, a variant virus, designated as 1735, lacking the 0.64 and 0.91 m.c. *Hind III* sites plus the 0.272 m.c. *Bgl II* site plus the 4 *Xba I* sites was isolated [see Figures 33 (e) and 34, lane 5; see also Table 8]. BHK21/C13 cells were then coinfected with 1733 and 1735 viruses and the resulting plaques screened as before. In this way, several rounds of recombination experiments were carried out to isolate the variants 1736 [lacking the 0.18, 0.64 and 0.91 m.c. *Hind III* sites plus the 0.272 m.c. *Bgl II* site plus the 4 *Xba I* sites; see Figures 33 (f) and 34, lane 6], 1737 [devoid of the 0.1, 0.18, 0.64 and 0.91 m.c. *Hind III* sites plus the 0.272 m.c. *Bgl II* site plus the 4 *Xba I* sites; see Figures 33 (g) and 34, lane 7] and finally 1738 lacking the 0.272 m.c. *Bgl II* site, the 0.08, 0.1, two at 0.18, 0.26, 0.64 and 0.91

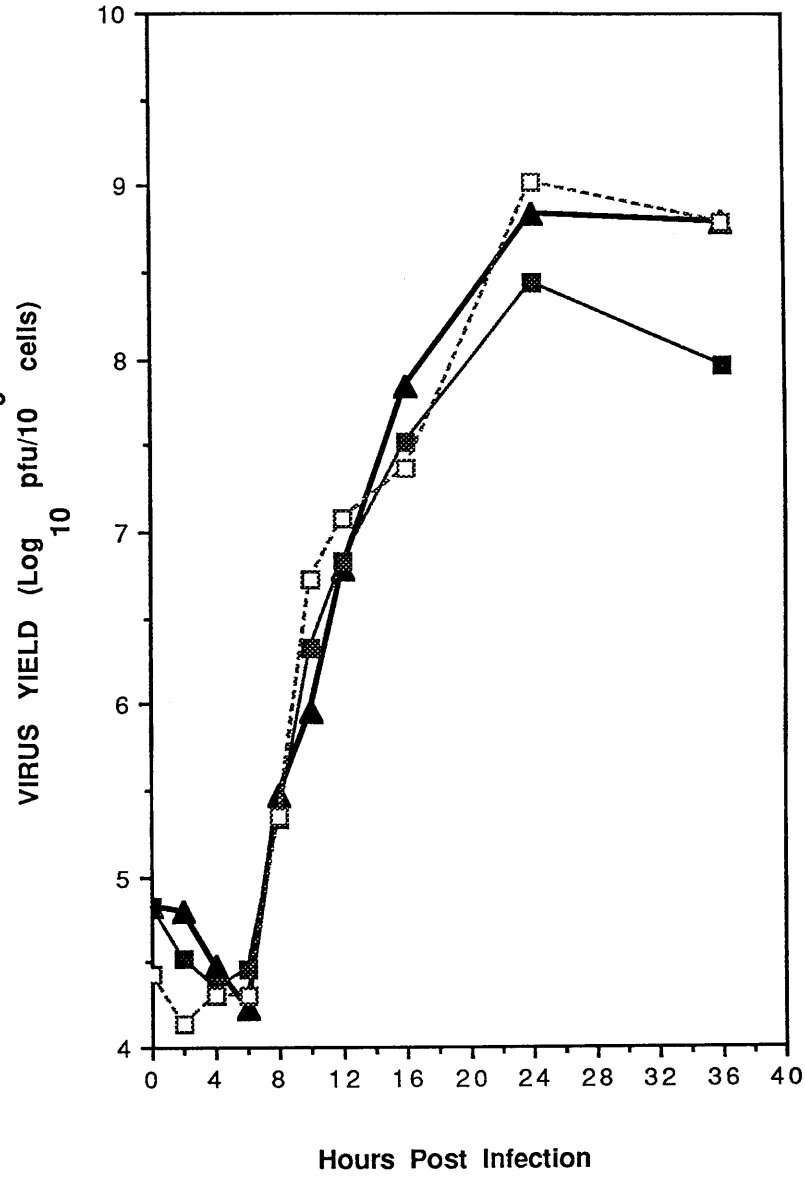

Figure 32. **One-Step Growth Curves Of HSV-1 Strain 17+, 1734 And 1738**

One-step growth curves of HSV-1 strain 17+ (—▲—), 1734 (—■—) and 1738 (--□--). The one-step growth experiments were carried out in BHK21/C13 cells (see Methods: Section 2B).

TABLE 9

	VIRUS	STOCK TITRE
1.	HSV-1 strain 17^{+}	1.80×10^{10} pfu/ml
2.	1734	1.00×10^{11} pfu/ml
3.	1735	4.00×10^{10} pfu/ml
4.	1736	2.50×10^{10} pfu/ml
5.	1737	5.00×10^{9} pfu/ml
6.	1738	9.00×10^{9} pfu/ml

Table 9. **Virus Stock Titres Of HSV-1 Strain 17^{+} And Restriction Endonuclease Site-Deletion Variants 1734 To 1738**

Figure 33. **Restriction Endonuclease Maps Of The HSV-1 Strain 17+ And The Variants 1734 To 1738 Genomes.**

(a) The *Hind III* (above the line) and *Xba I* (below the line) maps for the DNA of HSV-1 strain 17+ (Wilkie, 1976). The two *Hind III* sites at 0.18 map coordinates (m.c.) are shown separately although these are located very close to each other (52 bp apart) on the HSV-1 genome.

(b) The *Hind III* (above the line) and *Bgl II* (below the line) maps for the DNA of HSV-1 strain 17+ (Wilkie, 1976; Wilkie *et al.*, 1977). There are two *Bgl II* sites each between the *Bgl II* fragments **k**/**o** (231 bp apart) and **o**/**p** (426 bp apart). These *Bgl II* sites are not shown in the figure.

(c) The *Hind III* (above the line) and *Xba I* (below the line) restriction endonuclease maps for the DNA of HSV-1 (strain 17+) variant 1733.

(d) The *Hind III* (above the line) and *Bgl II* (below the line) restriction endonuclease maps for the DNA of HSV-1 (strain 17+) variant 1734.

The *Hind III* (above the line) and *Bgl II* (below the line) restriction endonuclease maps of HSV-1 strain 17+ variants : (e) 1735, (f) 1736, (g) 1737 and (h) 1738. As these variants are derived from the HSV-1 strain 17+ variant 1702 (MacLean and Brown, 1987a), they are devoid of the normally occuring (four) *Xba I* sites.

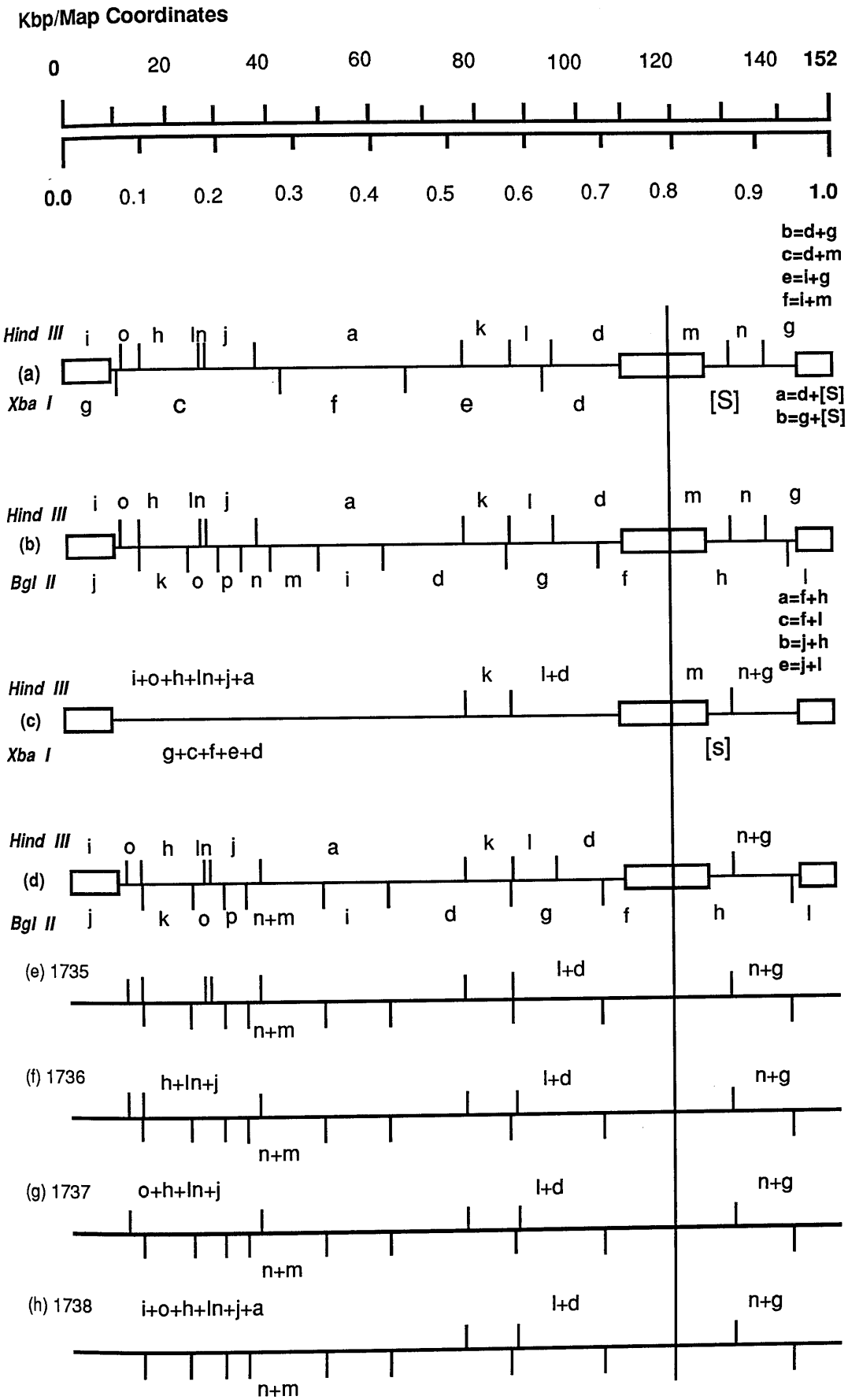
Kbp/Map Coordinates
0 20 40 60 80 100 120 140 152
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
b=d+g
c=d+m
e=i+g
f=i+m
Hind III
(a)
Xba I
a=d+[S]
b=g+[S]
Hind III
(b)
Bgl II
a=f+h
c=f+l
b=j+h
e=j+l
Hind III
(c)
Xba I
Hind III
(d)
Bgl II
(e) 1735
(f) 1736
(g) 1737
(h) 1738

Figure 33.

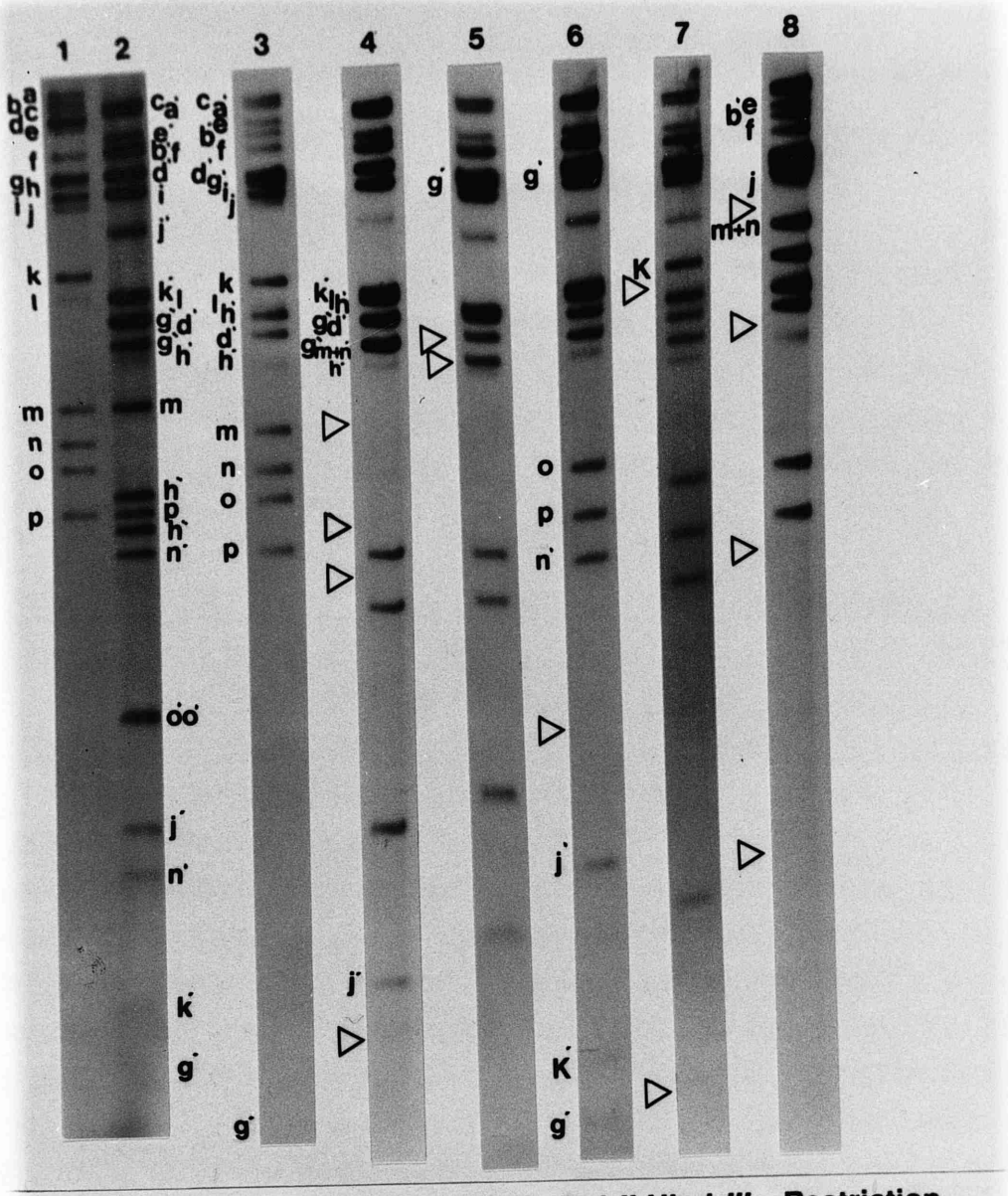

Figure 34. The *Bgl II* And *Bgl II-Hind III* Restriction Endonuclease Profiles Of HSV-1 DNA

The *Bgl II* profile of HSV-1 strain 17+ DNA (lane 1). The *wt* bands are labelled on left hand side.

The *Bgl II-Hind III* restriction endonuclease profiles of HSV-1 strain 17+ (lane 2), 1733 (lane 3), 1734 (lane 4), 1735 (lane 5), 1736 (lane 6), 1737 (lane 7) and 1738 (lane 8) DNAs ^{32}P-labelled *in vivo* (0.5% agarose gel). The *wt Bgl II* bands are labelled on the right hand side of lane 2 as well as on the left hand side of lane 3. The missing bands in lane 4 (1734 DNA profile) are indicated with open triangles (▷) whereas the novel bands are shown by labelling the respective *Bgl II* bands along with the surrounding bands. These missing and novel bands are shown in comparison with the *wt* profile (lane 2). In the subsequent gel tracks, the missing bands are indicated with open triangles and the novel bands with the respective *Bgl II* bands. However, once the missing/novel bands are indicated, they are not shown in subsequent lanes. M_r values of the various *Bgl II* bands are given in Table 8.

m.c. *Hind III* sites and the 0.07, 0.29, 0.45 and 0.63 m.c. *Xba I* sites [see Figures 33 (h) and 34, lane 8]. The *Bgl II-Hind III* profiles of 1735, 1736, 1737 and 1738 are given in Table 8.

The *Bam HI* and *Xba I* profiles of 1735, 1736, 1737 and 1738 were found identical to that of the parental viruses (data not shown).

A one-step growth curve of 1738 over a 36 h period in BHK21/C13 cells showed no marked difference in growth characteristics from those of 1734 and strain 17^+ (Figure 32). High titre stocks were obtained from the variants 1735, 1736, 1737 and 1738 (Table 9).

2. VARIANTS CONTAINING ADDITIONAL RESTRICTION ENDONUCLEASE CLEAVAGE SITES

(a) Isolation of an HSV-1 genome (1739) containing an additional *Hind III* restriction endonuclease cleavage site

As mentioned earlier, the *wt* HSV-1 genome contains 10 *Hind III* sites at 0.08, 0.1, two at 0.18, 0.26, 0.52, 0.58, 0.64, 0.88 and 0.91 m.c. During the process of screening a large number of HSV-1 genomes using restriction endonuclease analysis to obtain the variants 1722 and 1726 (see above sections), a variant showing aberrant *Hind III* as well as *Bgl II-Hind III* profiles was isolated. This variant was designated as 1739 (see Figure 35). The *Hind III* digest of 1739 DNA has shown that the *Hind III* ***a*** band (approx M_r 26.83 x 10^6; see Table 5) is missing and two novel bands with molecular weights of approximately 15.33 x 10^6 and 11.50 x 10^6 are running above and below the *Hind III* ***f*** band respectively (see Figure 36, lane 2 and compare with the *wt Hind III* profile, lane 1). A *Bgl II-Hind III* profile of 1739 DNA (Figure 36, lane 5) has shown that the *Bgl II* ***i*** (M_r 10.26 x 10^6) band is missing and two novel bands with approximate molecular weights of 6.66 x 10^6 and 3.60 x 10^6 are migrating slightly above the *Bgl II* ***k'*** and uncleaved ***o*** bands respectively. In the HSV-1 strain 17^+ *Bgl II-Hind III* profile (see Figure 36, lane 4; see Table 8), the *Bgl II* ***k'*** band comigrates with the ***l*** band whereas the *Bgl II* ***o*** band is cleaved with the 0.18 m.c. *Hind III* sites. Therefore, the smaller novel band (M_r 3.60 x 10^6) in 1739 DNA profile is migrating in between the *Bgl II* ***m*** and ***p*** bands (compare lanes 3, 4 &5 in Figure 36). Comparison of the *Hind III* and *Bgl II-Hind III* profiles of 1739 DNA has suggested the presence of an additional *Hind III* site generated within the *Hind III* ***a*** fragment. The apparent molecular weights of the novel *Hind III* (***a'***) and *Bgl II* (***l'***) bands have suggested the location of this site around 0.374 m.c. This conclusion was further supported by obtaining various other combinations of *Hind III* plus

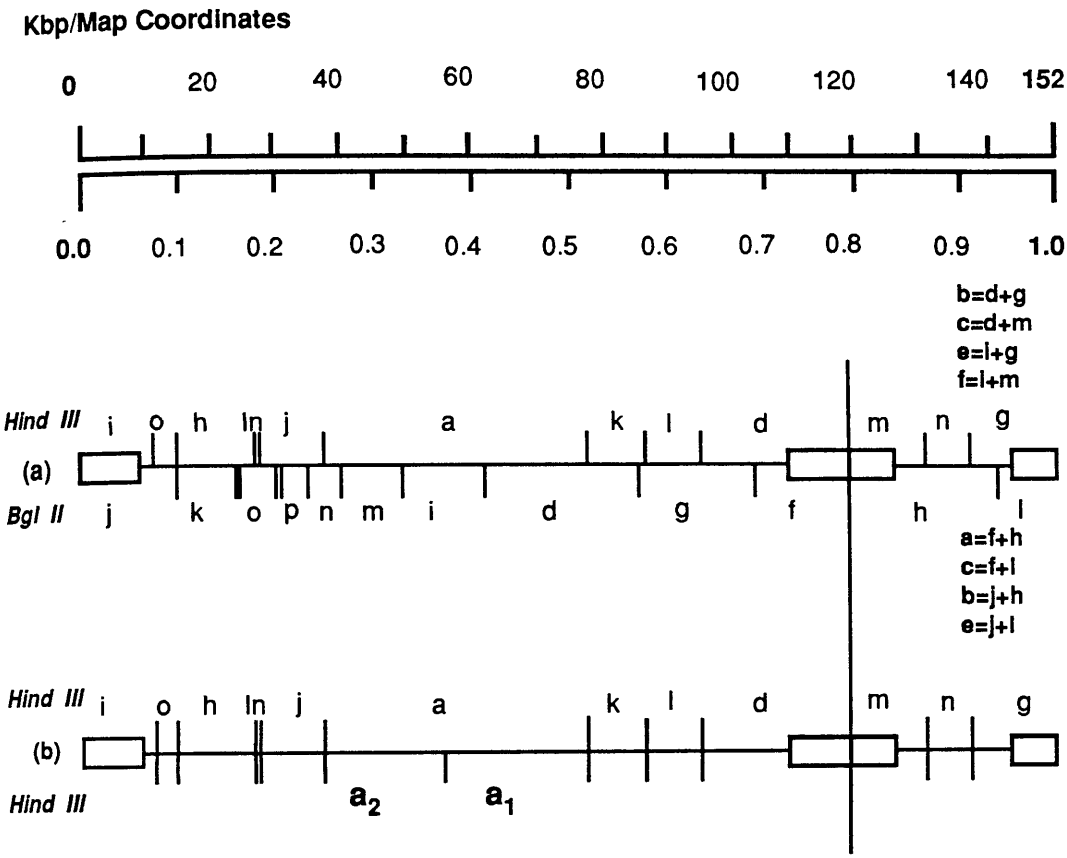

Figure 35. **The *Hind III* And *Bgl II* Restriction Endonuclease Maps Of The HSV-1 Strain 17+ And The Variant 1739 Genomes**

(a) The *Hind III* (above the line) and *Bgl II* (below the line) maps for the DNA of HSV-1 strain 17+.

(b) The *Hind III* maps for the DNA of HSV-1 strain 17+ (above the line) and the variant 1739 (below the line).

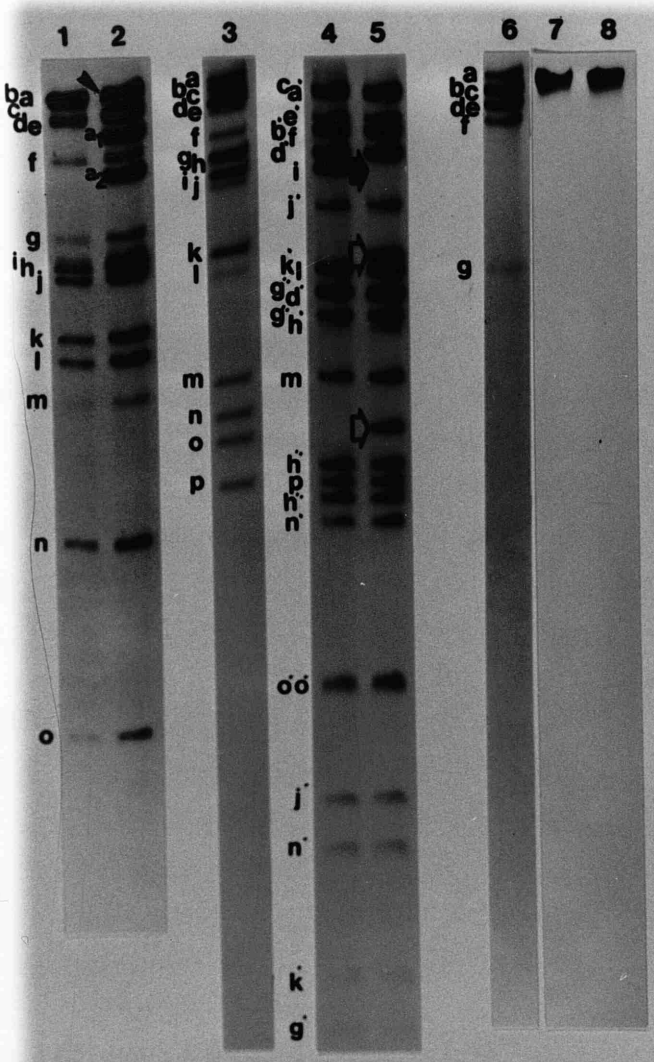

Figure 36. **Restriction Endonuclease Profiles Of HSV-1 Strain 17+, 1721 And 1739 DNAs**

Autoradiographs of *Hind III* restriction endonuclease digests of HSV-1 strain 17+ (lane 1) and 1739 (lane 2) DNA. The *wt Hind III* restriction fragments are labelled on the left side of the figure. The missing *Hind III a* band is indicated with a filled arrowhead and the novel bands are labelled as a_1 and a_2 respectively.

Autoradiograph of *Bgl II* restriction endonuclease digest of HSV-1 strain 17+ (lane 3) DNA. The *wt Bgl II* bands are labelled on the left. The *Bgl II-Hind III* restriction endonuclease profiles of HSV-1 strain 17+ (lane 4) and 1739 (lane 5) DNAs. The *wt Bgl II* bands are labelled on the left hand side. The missing band is shown by a solid arrowhead (➧) while the novel bands are indicated with open arrowheads (⇨).

Autoradiographs of *Xba I* restriction endonuclease digests of HSV-1 strain 17+ (lane 6), 1721 (lane 7) and 1739 (lane 8) DNAs. Since the variant 1721 and 1739 were derived from the *Xba I* negative genome of 1702, their DNA remained undigested. The *wt Xba I* bands are labelled on the left hand side.

restriction endonuclease DNA profiles of 1739 (data not shown). The *Bam HI* (Figure 37), *Bgl II* (Figure 37), *Eco RI* (data not shown), and *Kpn I* (data not shown) profiles of 1739 DNA were found identical to that of the strain 17+, 1702 and 1721 parental viruses, suggesting no other detectable (within a range of 150 bp) deletion or insertion within the restriction endonuclease fragments containing the additional *Hind III* site. It should, however, be noted that since the variant 1739 was derived from the variant 1721 [see above; Section 3B:1 (a) (i)], it contained no *Xba I* sites (see Figure 36).

The virus (1739) exhibits similar growth characteristics to that of strain 17+ on a single-cycle growth experiment (Figure 38). High titre virus stocks were obtained from 1739 (Table 10).

(b) **Generation of an HSV-1 variant containing 11 *Hind III* and 5 *Xba I* restriction endonuclease cleavage sites**

MacLean and Brown (1987c) have reported the isolation of an HSV-1 strain 17+ variant 1708 containing 5 *Xba I* sites at 0.07, 0.29, 0.45, 0.63 and 0.74 m.c., as opposed to the normal 4 *Xba I* sites in strain 17+ at 0.07, 0.29, 0.45 and 0.63 m.c. To increase the number of multiple unselected markers (restriction endonuclease sites) in intrastrain recombination crosses, it was decided to transfer the additional *Hind III* site from the variant 1739 (see above) to the variant 1708. Therefore, recombination experiments were carried out between the variants 1708 and 1739. BHK21/C13 cells were infected at a total moi of 10 *pfu*/cell, virus was harvested from the infected cells at 24 h *pi*, titrated out, single plaques isolated and stocks prepared from Linbro wells. ^{32}-P *in vivo* labelled DNAs were prepared from these isolates and subjected to *Bgl II-Xba I* and *Hind III* restriction endonuclease analysis to identify recombinant genomes. Several recombinant genomes were identified and isolated, some of which gave RE profiles indicative of 11 *Hind III* and 3 *Xba I* sites. Of these, one was selected as a prototype and designated 1740. The *Hind III-Xba I* map of the variant 1740 is shown in Figure 39 (c). The *Hind III* profile of 1740 DNA was found identical to that of 1739 (data not shown; see Figure 36, lane 2). A *Bgl II-Xba I* double digest of 1740 DNA has shown the presence of 0.45, 0.63 and 0.74 m.c. *Xba I* sites.

The five *Xba I* sites in 1708 are contained within the *Bgl II* ***j***, ***m***, ***d***, ***g*** and ***f*** fragments, respectively [see Figure 39 (b)]. Thus, the *Bgl II* ***j*** (M_r 9.58 x 10^6) is cleaved into two smaller ***j'*** bands of approximately 6.98 x 10^6 and 2.60 x 10^6 M_r by the 0.07 m.c. *Xba I* site. The larger ***j'*** band comigrates with the *Bgl II* ***k*** (M_r 6.94 x 10^6) band while the smaller ***j'*** band runs below the *Bgl II* ***p*** band. In addition, the ***j*** containing joints ***b*** and ***e*** comigrate with ***c*** (***b'*** = 18.30

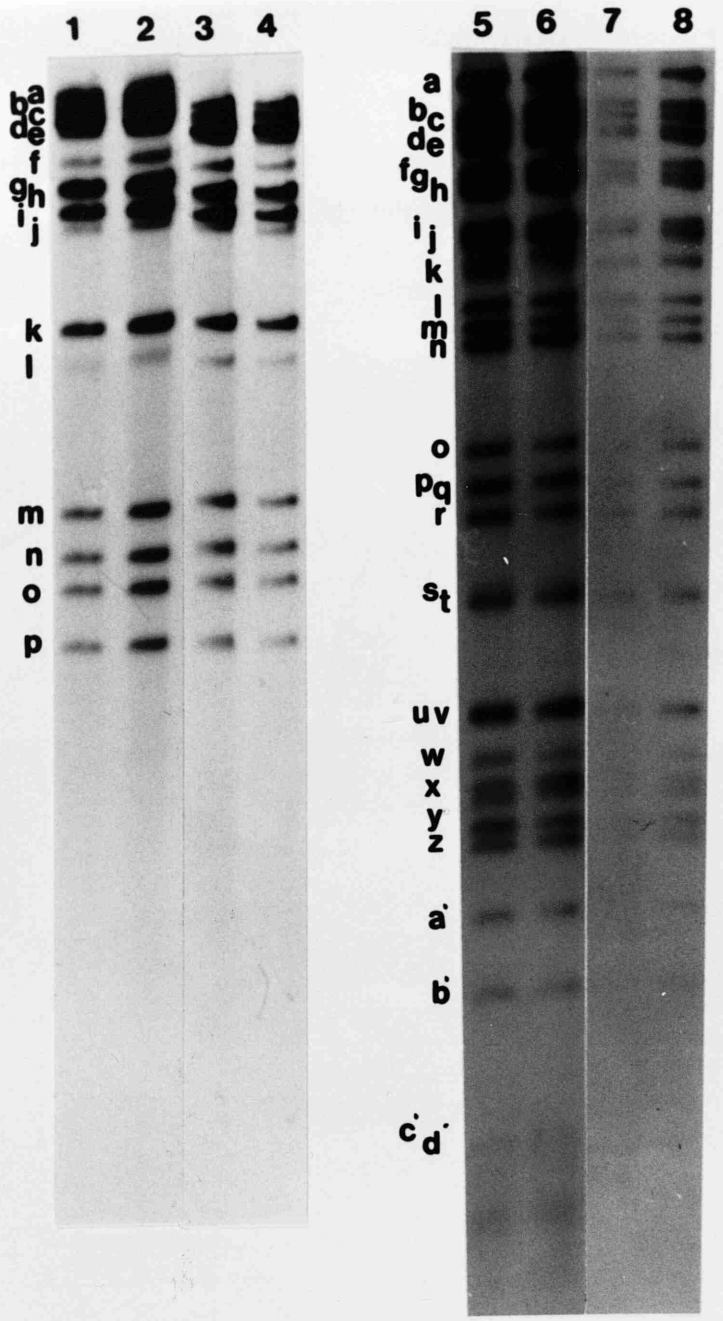

Figure 37. **<u>*Bgl II* And *Bam HI* Restriction Endonuclease Profiles Of HSV-1 DNA</u>**

The *Bgl II* (lanes 1 to 4) and *Bam HI* (lanes 5 to 8) restriction endonuclease profiles of HSV-1 strain 17^+ (lanes 1 & 5), 1702 (lanes 2 & 6), 1721 (lanes 3 & 7) and 1739 (lanes 4 & 8) DNAs ^{32}-P-labelled *in vivo* (0.5% and 1.2% agarose gels, respectively). The *wt Bgl II* and *Bam HI* bands are labelled on the left hand side of lanes 1 and 5 respectively.

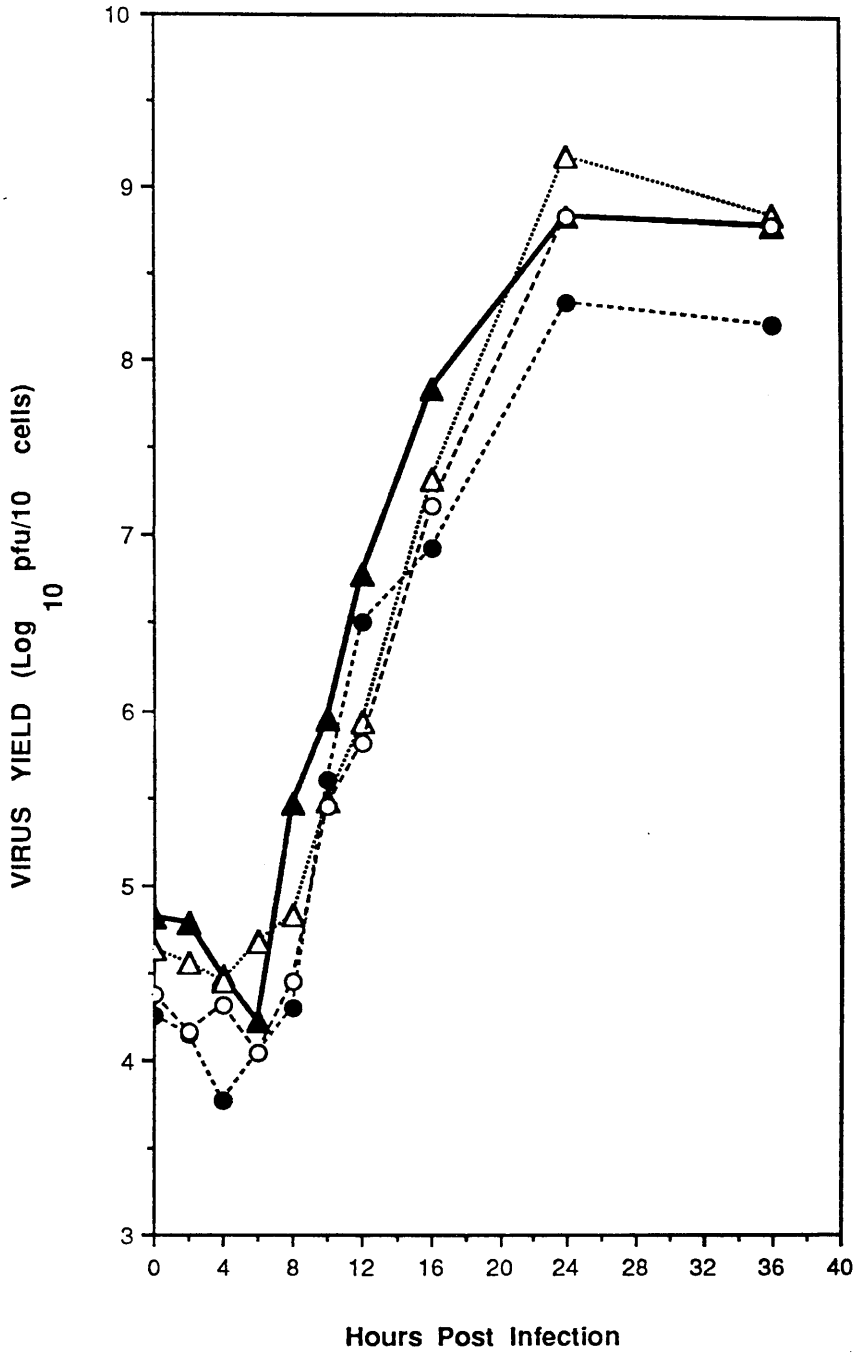

Figure 38. **One-Step Growth Curves Of HSV-1 Strain 17+, 1708, 1739 And 1743**

One-step growth curves of HSV-1 strain 17+ (—▲—), 1708 (·····△·····), 1739 (···●····) and 1743 (- - -○- - -). The one-step growth experiments were carried out in BHK21/C13 cells (see Methods: Section 2B).

TABLE 10

	VIRUS	STOCK TITRE
1.	HSV-1 strain 17+	1.80×10^{10} pfu/ml
2.	1708	1.00×10^{10} pfu/ml
3.	1739	1.00×10^{10} pfu/ml
4.	1740	3.20×10^{10} pfu/ml
5.	1741	1.70×10^{10} pfu/ml
6.	1742	1.25×10^{10} pfu/ml
7.	1743	4.00×10^{10} pfu/ml

Table 10. **Virus Stock Titres Of HSV-1 Strain 17+ And Its Variants 1708, And 1739 To 1743**

Figure 39. **The *Bgl II*, *Hind III* And *Xba I* Restriction Endonuclease Maps Of The HSV-1 Strain 17+ And The Variants 1708, 1739, 1740, 1741, 1742 And 1743 Genomes.**

(a) The *Hind III* (above the line) map for the DNA of HSV-1 (strain 17^+) variant 1739 and the *Xba I* (below the line) map for the DNA of HSV-1 strain 17^+ variant 1708 (MacLean and Brown, 1987c).

(b) The *Xba I* (above the line) map of 1708 and the *Bgl II* (below the line) map for the DNA of HSV-1 strain 17^+ (Wilkie, 1976; Wilkie *et al.*, 1977). There are two innominate (***In***) *Bgl II* sites each between the *Bgl II* fragments **k/o** (231 bp apart) and **o/p** (426 bp apart).

(c) The *Hind III* (above the line) and *Xba I* (below the line) restriction endonuclease maps for the DNA of HSV-1 (strain 17^+) variants 1740, (d) 1741, (e) 1742 and (f) 1743.

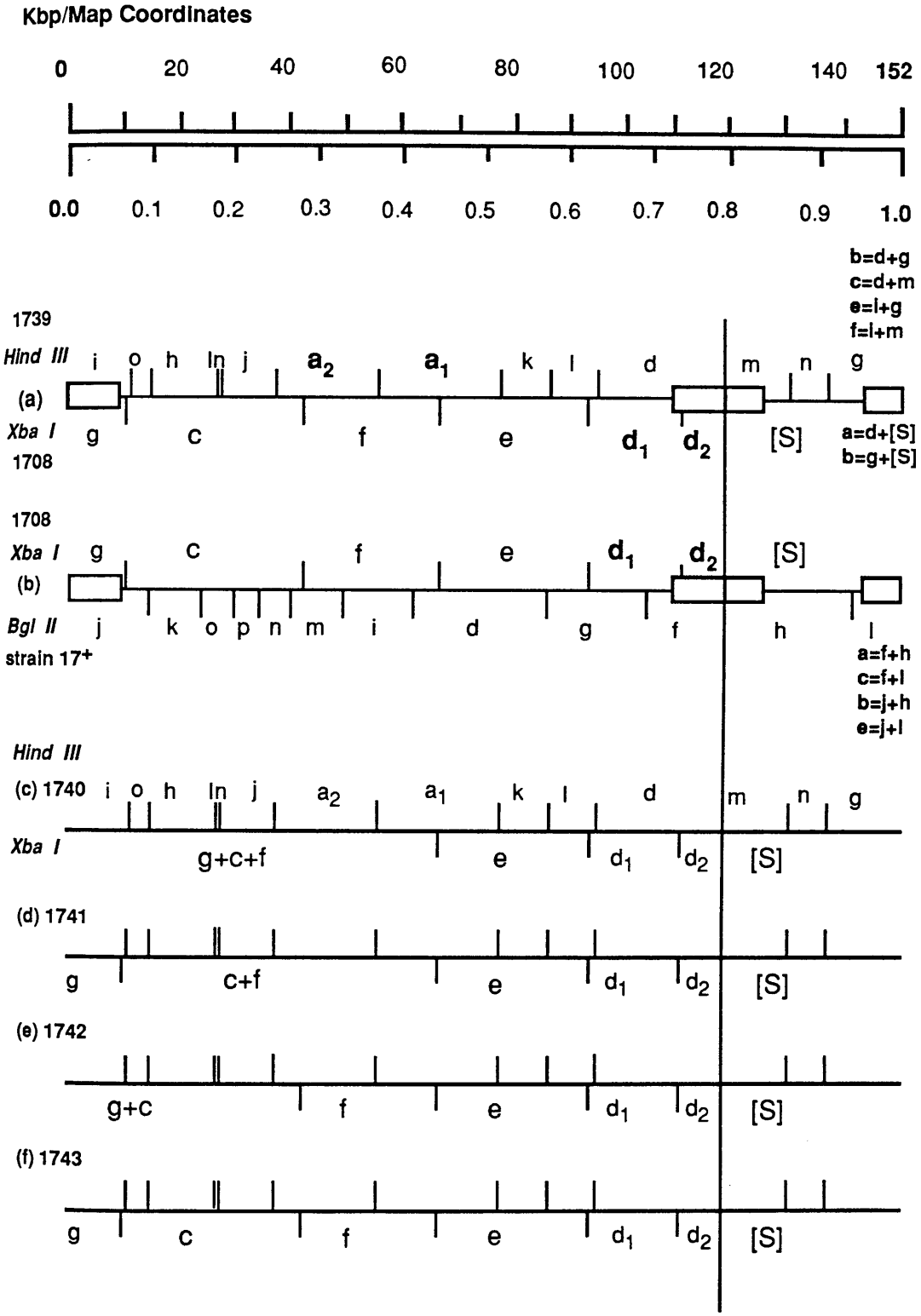

Figure 39. **Restriction Endonuclease Maps Of The HSV-1 Strain 17± And The Variants 1708, 1739, 1740, 1741, 1742 And 1743 Genomes**

x 10^6 mol. wt.) and ***d'*** (see below; ***e'*** = 13.22 x 10^6 mol. wt.) respectively (see Table 11). The *Bgl II* ***m*** (M_r 4.21 x 10^6) is cleaved by the 0.29 m.c. *Xba I* site into two smaller ***m'*** (M_r 2.15 x 10^6 and 2.06 x 10^6 respectively) bands which migrate below the ***j'*** (M_r 2.60 x 10^6) band. The *Xba I* site at 0.45 m.c. cuts the *Bgl II* ***d*** (M_r 17.00 x 10^6) into two smaller ***d'*** bands of approximate M_r of 13.21 x 10^6 and 3.79 x 10^6 respectively, which comigrate with ***e'*** and ***n*** respectively. The *Bgl II* ***g*** (M_r 11.42 x 10^6) is cleaved by the *Xba I* site at 0.63 m.c. to two ***g'*** bands of around 5.96 x 10^6 and 5.45 x 10^6 molecular weights, which run below the *Bgl II* ***l***. The additional *Xba I* site around 0.74 m.c. cleaves the *Bgl II* ***f*** (M_r 12.34 x 10^6) into ***f'*** bands of approximate M_r of 7.84 x 10^6 and 4.50 x 10^6, running below ***i*** and above ***m***, respectively. The ***f*** containing joints, ***a*** and ***c*** are now reduced and migrate above and below the *Bgl II* ***b'*** band, respectively (see Table 11). A *Bgl II-Xba I* DNA profile of 1740 is given in Table 11 and shown in Figure 40, lane 4. The *Bgl II* ***d***, ***g*** and ***f*** bands are cleaved and the resulting smaller bands are running at their expected positions (as discussed above), indicating the presence of 0.45, 0.63 and 0.74 m.c. *Xba I* sites.

The variant 1740 was then recombined with 1708 to transfer the remaining (0.07 and 0.29 m.c.) *Xba I* sites. The resulting plaque isolates were analysed with the *Hind III* and *Bgl II-Xba I* double digest of their ^{32}-P *in vivo* labelled DNAs. A number of recombinants were isolated, some of which giving a pattern indicating the presence of 0.07, 0.45, 0.63 and 0.74 m.c. *Xba I* sites while the DNA profile of several recombinants showed that the *Xba I* sites at 0.29, 0.45, 0.63 and 0.74 m.c. are present. A prototype from each group of recombinants was selected and designated as 1741 and 1742, respectively. The *Hind III* profiles of 1741 and 1742 were found identical to that of 1739 (data not shown). A *Bgl II-Xba I* profile of 1741 DNA is shown in Figure 40, lane 5 whereas that of 1742 is shown in Figure 40, lane 6. The 1741 *Bgl II-Xba I* DNA profile showed that the *Bgl II* ***j***, ***d***, ***g*** and ***f*** are cleaved, thus indicating the presence of 0.07, 0.45, 0.63 and 0.74 m.c. *Xba I* sites [see Table 11; Figure 39 (d) and Figure 40, lane 5] whereas the genome of 1742 contained the *Xba I* sites at 0.29, 0.45, 0.63 and 0.74 m.c. [Figures 39 (e) and 40, lane 6].

The final round of recombination experiments involved 1741 and 1742 viruses and a variant, designated as 1743, was isolated. The *Hind III* and *Xba I* maps of 1743 are illustrated in Figure 39 (f). A *Hind III* digest of 1743 DNA has shown the presence of all 11 *Hind III* sites (data not shown). The *Bgl II-Xba I* DNA profile of 1743 (Figure 40, lane 7), indicating the

TABLE 11

BGL II AND BGL II-XBA I PROFILES

(Molecular Weights of ***Bgl II*** fragments x 106)

	17 syn± (*Bgl II*)		17syn± (*Bgl II-Hind III*)		1708		1739	1740
a	23.65	a	23.65		- -	a	23.65	- -
b	20.89					b	20.89	20.89
				a'	19.15		- -	19.15
c	18.58	c	18.58			c	18.58	- -
		b'	18.30	b'	18.30		- -	- -
d	17.00					d	17.00	
e	15.82					e	15.82	15.82
				c'	14.08		- -	14.08
		e'	13.22	e'	13.22		- -	- -
		d'	13.21	d'	13.21		- -	13.21
f	12.34	f	12.34			f	12.34	- -
g	11.42					g	11.42	
h	11.31	h	11.31	h	11.31		11.31	11.31
i	10.26	i	10.26	i	10.26		10.26	10.26
j	9.58					j	9.58	9.58
				f'	7.84		- -	7.84
		j'	6.98	j'	6.98		- -	- -
k	6.94	k	6.94	k	6.94		6.94	6.94
l	6.24	l	6.24	l	6.24		6.24	6.24
		g'	5.96	g'	5.96		- -	5.96
		g'	5.46	g'	5.46		- -	5.46
				f'	4.50		- -	4.50
m	4.21					m	4.21	4.21
		d'	3.79	d'	3.79		- -	3.79
n	3.76	n	3.76	n	3.76		3.76	3.76
o	3.48	o	3.48	o	3.48		3.48	3.48
p	3.04	p	3.04	p	3.04		3.04	3.04
		j'	2.60	j'	2.60		- -	- -
		m'	2.15	m'	2.15		- -	- -
		m'	2.06	m'	2.06		- -	- -
In	0.28	In	0.28	In	0.28		0.28	0.28
In	0.15	In	0.15	In	0.15		0.15	0.15

(In = Innominate)

TABLE 11 (Continued)

BGL II AND BGL II-XBA I PROFILES

(Molecular Weights of ***Bgl II*** fragments x 106)

	17 syn± (*Bgl II*)		17syn± (*Bgl II-Hind III*)		1741		1742	1743
a	23.65	a	23.65		- -		- -	- -
b	20.89					b	20.89	- -
				a'	19.15		19.15	19.15
c	18.58	c	18.58					
		b'	18.30	b'	18.30		- -	18.30
d	17.00							
e	15.82					e	15.82	- -
				c'	14.08		14.08	14.08
		e'	13.22	e'	13.22		- -	13.22
		d'	13.21	d'	13.21		13.21	13.21
f	12.34	f	12.34					
g	11.42							
h	11.31	h	11.31	h	11.31		11.31	11.31
i	10.26	i	10.26	i	10.26		10.26	10.26
j	9.58					j	9.58	- -
				f'	7.84		7.84	7.84
		j'	6.98	j'	6.98		- -	6.98
k	6.94	k	6.94	k	6.94		6.94	6.94
l	6.24	l	6.24	l	6.24		6.24	6.24
		g'	5.96	g'	5.96		5.96	5.96
		g'	5.46	g'	5.46		5.46	5.46
				f'	4.50		4.50	4.50
m	4.21			m	4.21		- -	- -
		d'	3.79	d'	3.79		3.79	3.79
n	3.76	n	3.76	n	3.76		3.76	3.76
o	3.48	o	3.48	o	3.48		3.48	3.48
p	3.04	p	3.04	p	3.04		3.04	3.04
		j'	2.60	j'	2.60		- -	2.60
		m'	2.15		- -	m'	2.15	2.15
		m'	2.06		- -	m'	2.06	2.06
In	0.28	In	0.28	In	0.28		0.28	0.28
In	0.15	In	0.15	In	0.15		0.15	0.15

(In = Innominate)

Table 11. ***Bgl II* And *Bgl II-Xba I* Restriction Endonuclease Profiles of The HSV-1 Strain 17± And The Variants 1708, And 1739 To 1743 DNAs**

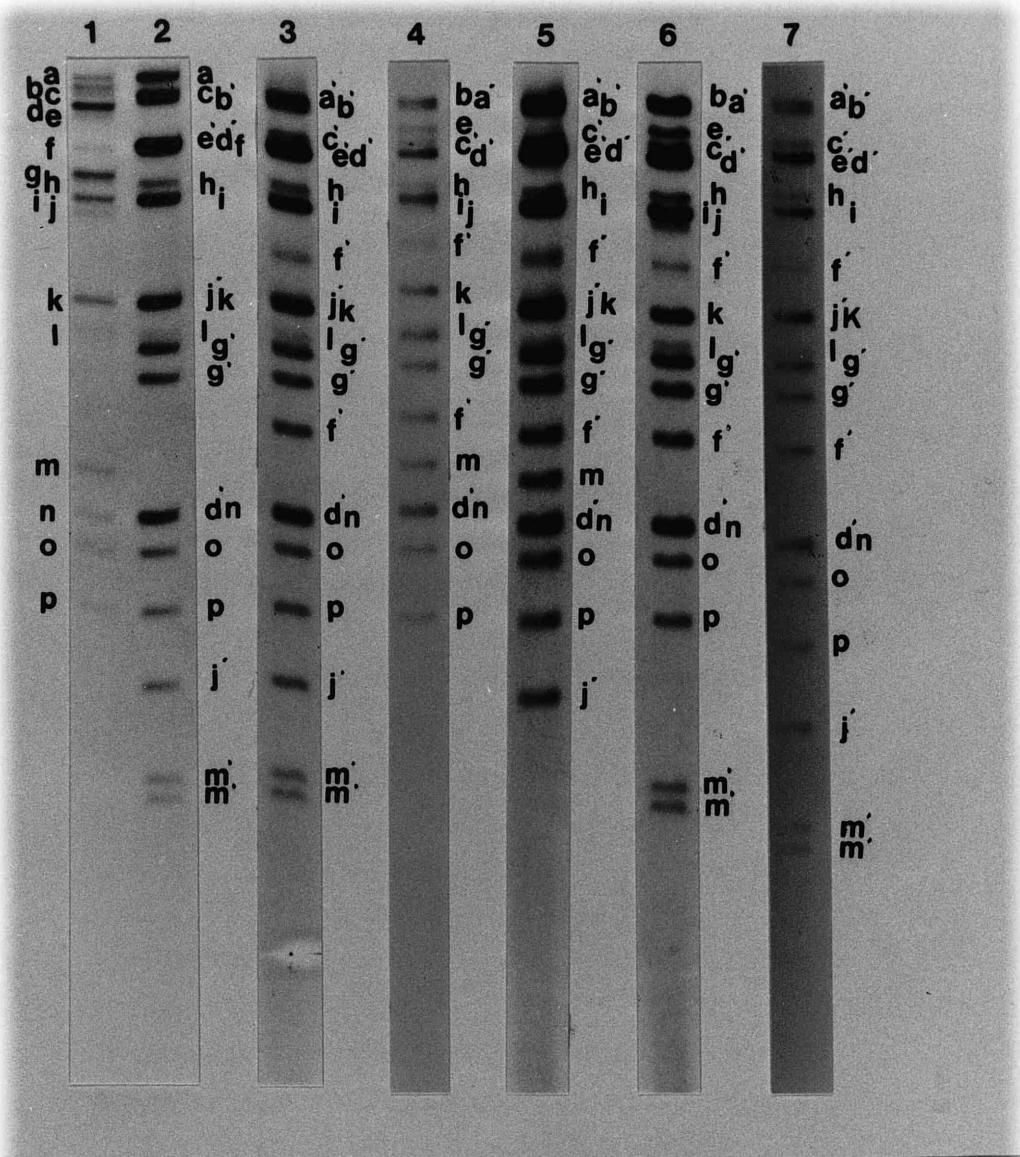

Figure 40. **<u>*Bgl II* And *Bgl II-Xba I* Restriction Endonuclease Profiles Of HSV-1 DNA</u>**

Autoradiograph of *Bgl II* restriction endonuclease digest of HSV-1 strain 17+ DNA (lanes 1) labelled with ^{32}-P *in vivo* (0.5% agarose gel). The *wild-type Bgl II* bands are labelled on the left hand side.

Autoradiographs of *Bgl II-Xba I* restriction endonuclease double digests of HSV-1 strain 17+ (lanes 2), the variant 1708 containing an additional Xba I site around 0.74 m.c. (lanes 3; MacLean and Brown, 1987c), 1740 (lanes 4), 1741 (lane 5), 1742 (lane 6) and 1743 (lanes 7) DNAs ^{32}-P-labelled *in vivo* (0.5% agarose gel). The *wt Bgl II* bands are labelled on the right hand side of every gel track (lanes 2 to 7).

presence of all 5 *Xba I* sites. A *Bgl II-Hind III* digest of 1740, 1741, 1742 and 1743 DNAs was found identical to that of 1739 (see Figure 36; data not shown). These viruses (1740 to 1743) were also analysed with the *Bam HI, Bgl II, Eco RI, Kpn I* and *Xba I* restriction endonucleases and gave identical DNA profiles to that of HSV-1 strain 17$^+$ (data not shown), suggesting no other alterations within the DNA fragments spanning the additional *Hind III* and the *Xba I* restriction endonuclease cleavage sites.

One-step growth experiments over a 36 h period at 37°C have shown that the variant 1743 replicated as well as *wild-type*. Results of these experiments are shown in Figure 38. It can be seen that strain 17$^+$, 1708, 1739 and 1743 have grown at a similar rate and produced a similar 36 h yield. One-step growth experiments were not carried out for the variants 1740, 1741 and 1742 because the variant 1743 was derived from these viruses and has shown *wild-type* growth characteristics. Comparable high titre stocks for all the viruses (1739 to 1743) have been obtained (see Table 10).

3. GENERAL POLYPEPTIDE PROFILES OF HSV-1 STRAIN 17± VARIANTS 1702, 1708, 1721, 1733, 1738, 1739 AND 1743

The general polypeptide profiles of 1721, 1733, 1738, 1739 and 1743 were compared with those of strain 17$^+$, 1702 and 1708 (Figure 41). These profiles were identical to that of 1702 (MacLean and Brown, 1987a), which is *tk* negative and produce a truncated form of gC (Dr A R MacLean, personal communication). Apart from these, the polypeptide profiles of 1721, 1733, 1738, 1739 and 1743 were essentially similar to those of strain 17$^+$ and 1708 (MacLean and Brown, 1987c) under the conditions used.

Since 1733, 1738 and 1743 had been derived from 1722 to 1732, 1734 to 1737 and 1740 to 1742 respectively, the polypeptide profiles for the variants 1722 to 1732, 1734 to 1737 and 1740 to 1742 were not analysed and assumed to be identical to those of the parental viruses, 1702, 1721 and 1739 (Figure 41).

4. HSV-1 STRAIN 17± DNA DELETION/INSERTION VARIANTS

During the course of transfection experiments to isolate HSV-1 (strain 17$^+$) variants lacking *Hind III* sites [see Section 3B:1 (a) (i) to (vi)], over 4,500 single plaque isolates were analysed using the *Hind III* restriction endonuclease alone and/or a combination of *Hind III-Bgl II* and *Hind III-Eco RI*

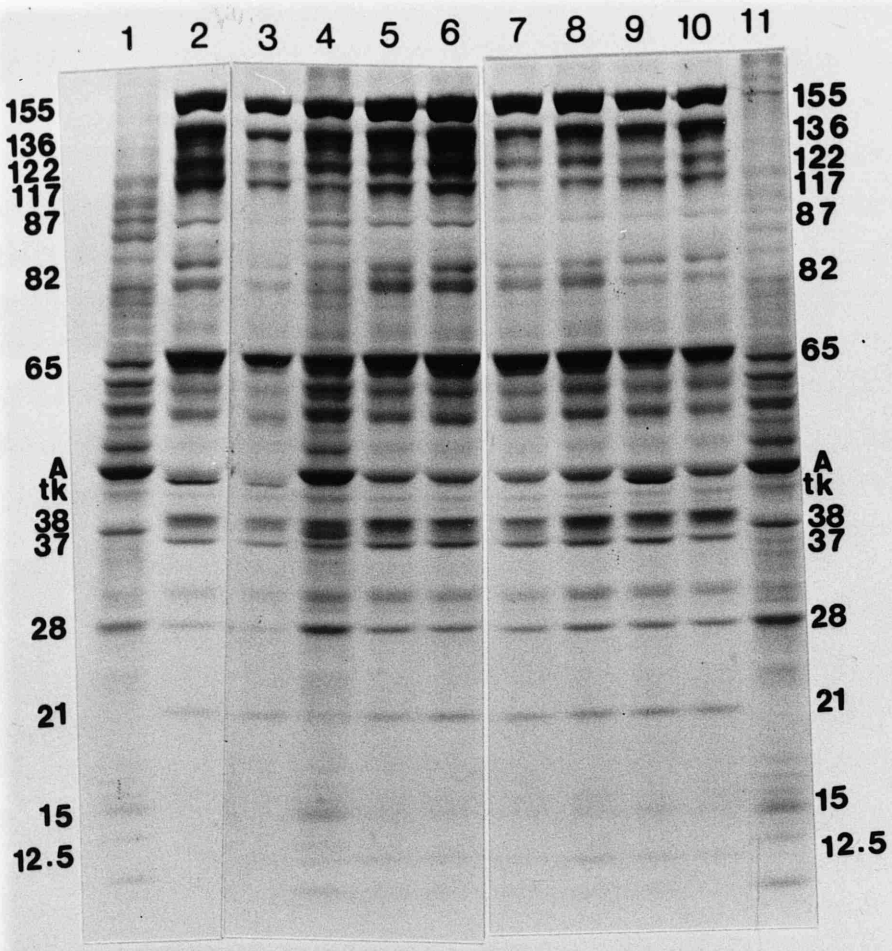

Figure 41. **The General Polypeptide Profiles Of HSV-1 Strain 17± And Its Variants**

The general polypeptide profiles of HSV-1 strain 17+ (lanes 2 & 3), 1702 (lane 4), 1721 (lane 5), 1733 (lane 6), 1738 (lane 7), 1739 (lane 8), 1708 (lane 9) and 1743 (lane 10). Lanes 1 and 11 are mock infected. The molecular weights (KD) of predominant HSV-1 polypeptides are marked on both sides of the figure. A is actin; *tk* is thymidine kinase. *tk* is absent in all the HSV-1 variants except 1708 (lane 9) because of their derivation from 1702 (MacLean and Brown, 1987a).

restriction endonucleases. In addition, approximately 1,000 single plaques were analysed to isolate the variants 1735 to 1738 [see Section 3B:1 (c)] and 1740 to 1743 [see Section 3B:2 (b)] using the above restriction endonucleases along with the *Bgl II-Xba I* double digestion of their DNAs. Furthermore, another 1,008 single plaque isolates were examined in the final intrastrain recombination experiments between the variants 1738 and 1743 (see Section 3C) using the *Bgl II-Hind III* as well as *Bgl II-Xba I* restriction endonucleases. As well as the desired variants, a large number of these gave aberrant restriction endonuclease profiles differing from that of the parental viruses. On further analysis using various restriction endonucleases, most of these single plaque isolates have shown DNA deletion and/or insertion. Only preliminary characterization of these spontaneously isolated variants have been carried out using restriction endonuclease analysis. Moreover, most of these have not been plaque purified three times and, therefore, may show contamination by the parental viruses (within a range of 5 to 25%). Some are briefly described in the following sections.

(a) **Isolation of the variant 1714**

This variant was spontaneously isolated during the marker rescue experiments to isolate the variant 1726 [see Section 3B:1 (a) (vi)] and had a deletion in both TR_L and IR_L. Like the parental virus 1721, 1714 was also devoid of the 4 *Xba I* sites and the 0.91 m.c. *Hind III* site [see Section 3B:1 (a) (i)]. However, the *Hind III* restriction endonuclease profile of 1714 DNA differed from that of 1721 [see Figure 42 (a)]. It can be seen in Figure 42 (a), lane 2 that the *Hind III* ***c***, ***e'***, ***d*** and ***f*** bands are reduced and migrating slightly lower than their normal positions compared to that of 1721 (lane 3). In addition, the *Hind III* ***i*** (Mr 8.35 x 10^6) band is missing and the novel ***i'*** band is possibly comigrating with the ***j*** (Mr 7.76 x 10^6) band. The remaining *Hind III* bands appear to be identical to those of 1721 [compare lanes 2 and 3 in Figure 42 (a)].

The *Hind III* ***i*** fragment contains TR_L whereas *Hind III* ***d*** contains IR_L [see Figure 45 (b)]. The *Hind III* ***i*** fragment forms the joint fragments ***e (i+g)*** and ***f(i+m)*** while the ***d*** fragment forms the joint fragments ***b (d+g)*** and ***c (d+m)***. Because of the 0.91 m.c. *Hind III* site-deletion from the genomes of 1721 and 1714, the joint fragments ***b*** and ***e*** contain the fused ***g+n*** fragment and are migrating at the top of the gel track (***b'***) and above the *Hind III* ***c*** band (***e'***) respectively [see Figure 42 (a), lanes 2 &3; see also the *Hind III* DNA profile of 1721 in Table 5]. In the 1714 *Hind III* profile [Figure 42 (a) lane 2], the ***e'*** band is migrating faster than the equivalent 1721 band [Figure 42 (a),

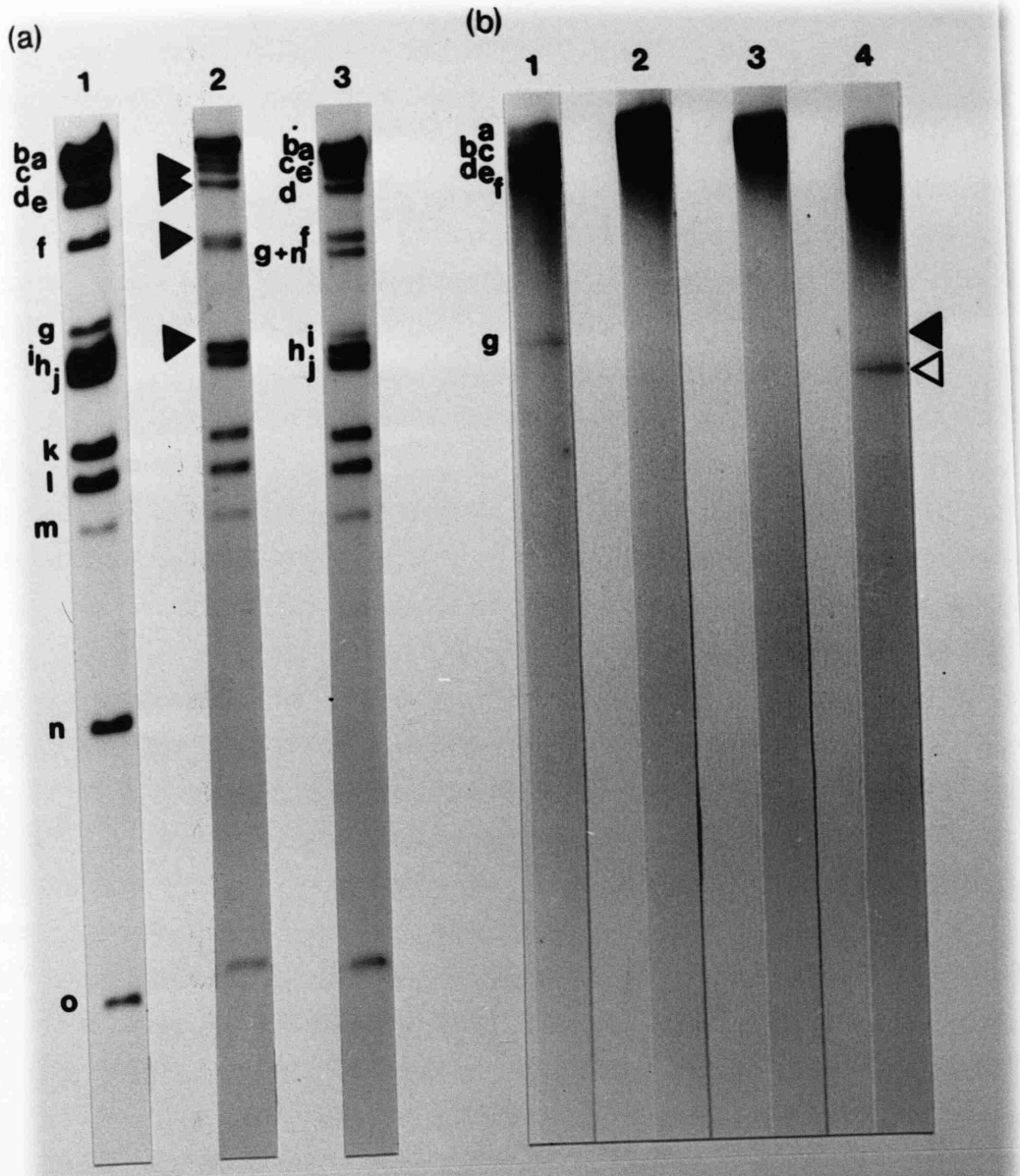

Figure 42. The *Hind III* And *Xba I* Profiles Of HSV-1 DNA

(a) The *Hind III* restriction endonuclease profiles of HSV-1 strain 17+ (lanes 1), 1714 (lane 2) and 1721 (lane 3) DNAs labelled with ^{32}-P *in vivo* (0.5% agarose gel). The *wt Hind III* bands are labelled on the left hand side of lanes 1 and 3. The missing *Hind III i* band and the reduced *e'*, *d* and *f* bands in 1714 DNA profile (lane 2) are indicated (▶).

(b) The *Xba I* profiles of HSV-1 strain 17+ (lane 1), 1702 (lane 2), 1714 (lane 3) and 1716 (lane 4) DNAs ^{32}-P-labelled *in vivo* (0.5% agarose gel) (MacLean A. R. *et al.*, 1991). The *wt Xba I* bands are labelled on left hand side. The missing *Xba I g* band is indicated with a filled triangle and the novel *g'* band with an open triangle.

lane 3] whereas the mobility of the ***b'*** band is not so obvious because of its comigration with the *Hind III* ***a*** band. Moreover, the ***d*** containing joint ***c*** is migrating faster than that of 1721 and the ***i*** containing joint ***f*** (Mr 12. 93 x 10^6) is running marginally above the ***g+n*** (Mr 12.34 x 10^6) band but below its equivalent 1721 band. Thus, it appeared that the variant 1714 was deleted in both the *Hind III* ***d*** and ***i*** fragments by approximately 0.5 x 10^6 Mr (approximately 700 to 800 bp).

The genome of the variant 1714 was further analysed with the *Bam HI*, *Kpn I* and *Hpa I* restriction endonucleases. A *Bam HI* digest of 1714 DNA is shown in Figure 43 (c) lane 3. It can be seen that the *Bam HI* ***s*** (Mr 1.91 x 10^6) band is missing and a novel band with an approximate Mr of 1.41 x 10^6 is running below the comigrating ***u***, ***v*** bands. In addition, the ***s*** containing joint ***k*** (***s+q***; Mr 3.90 x 10^6) is also missing and a novel band (approximate Mr 3.40 x 10^6) is migrating below the *Bam HI* ***l*** band. Similarly, the *Kpn I* restriction endonuclease profile of 1714 DNA [Figure 43 (a), lane 3] was compared to that of strain 17^+ [Figure 43 (a), lane 1] and 1702 [Figure 43 (a), lane 2] DNAs. The *Kpn I* ***r*** band (Mr 2.40 x 10^6) was missing and a novel band of about 1.9 x 10^6 Mr was migrating between the ***t*** and ***u*** bands. The joints ***a*** and ***e*** containing the *Kpn I* ***r*** fragment comigrate with the ***b***, ***c*** & ***d*** and ***f*** & ***g*** bands respectively and therefore, no obvious alteration in the mobility of these bands could be detected. On *Hpa I* digestion of 1714 DNA, *Hpa I* ***m*** (Mr 3.87 x 10^6) was found to be missing and a novel band with an approximate molecular weight of 3.37 x 10^6 was observed below the ***n*** band [Figure 43 (b), lane 3]. The *Hpa I* ***m*** containing joint fragments ***a (m+c)*** and ***d (m+g)*** were found to be migrating marginally faster compared to that of ***wt*** (lane 1) and 1702 (lane 2) bands, confirming that the 1714 genome was deleted in both copies of the terminal portion of R_L between 0 and 0.095 m.c. and 0.81 and 0.83 m.c. with an approximate deletion of 750-800 bp in each copy.

Southern blot experiments were carried out by Dr A R MacLean to confirm that the deletion in the 1714 genome was present in both copies of R_L. The HSV-1 strain 17^+ and 1714 DNAs were digested with the *Bam HI* restriction endonuclease and resolved on a 1% agarose gel. The DNA was transferred to a nitrocellulose membrane and hybridized with a randomly primed *Bam HI* ***k*** fragment of strain 17^+ DNA (see Figure 44). It can be seen that the *Bam HI* ***k*** probe hybridized to the fragments ***k***, ***q*** and ***s*** in the ***wt*** (strain 17^+) sample (lane 2) but in 1714 DNA sample, the probe hybridized to a novel ***k***, a normal ***q*** and a novel ***s*** fragment respectively (Figure 44, lane 3). The probe also hybridized to a novel ***k*** with additional *'a'* sequences. Comparison

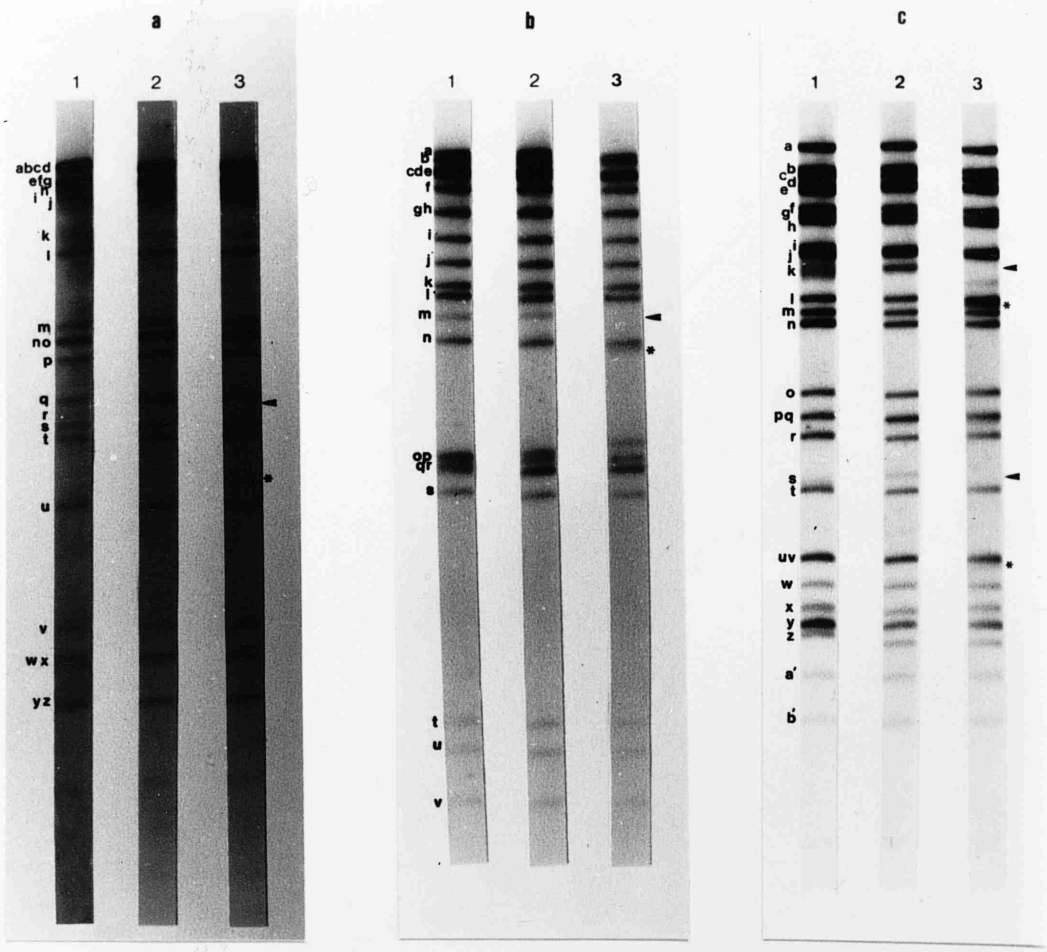

Figure 43. **The *Kpn I*, *Hpa I* And *Bam HI* Restriction Endonuclease Profiles Of HSV-1 DNA**

The *Kpn I* (a), *Hpa I* (b) and *Bam HI* (c) restriction endonuclease profiles of HSV-1 strain 17+ (lane 1), 1702 (lane 2) and 1714 (lane 3) DNAs ^{32}-P-labelled *in vivo* (0.8%, 0.7% and 1.0% agarose gels respectively). The *wt* restriction endonuclease bands are labelled on the left hand side of the strain 17+ DNA profiles (lane 1). The missing bands are indicated with a filled arrowhead and the novel bands with an asterisk (MacLean A.R. *et al.*, 1991).

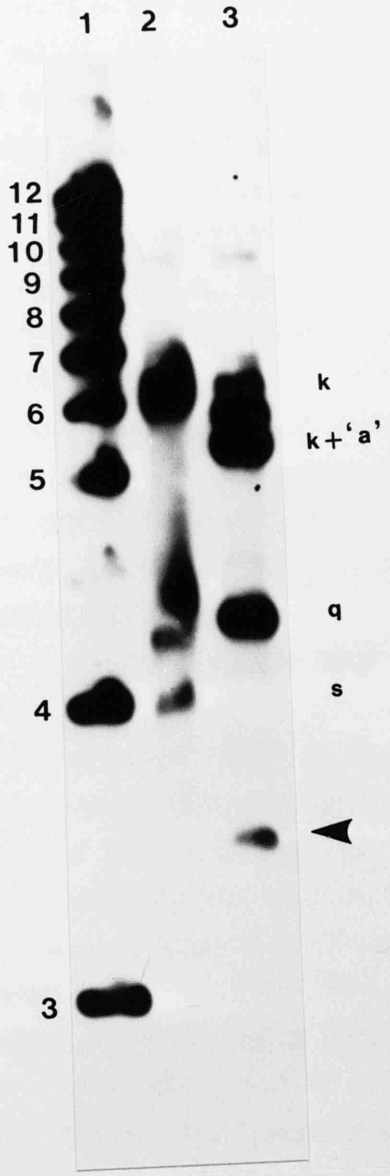

Figure 44. **Southern Blot of 1714 DNA Using The *Bam HI k* Probe**

The *Bam HI* restriction endonuclease digested HSV-1 strain 17+ (*wt*; lane 2), and the variant 1714 (lane 3) DNAs were resolved on a 1% agarose gel and transferred to a nitrocellulose membrane. These were then hybridized to a randomly primed *Bam HI k* fragment (MacLean A.R. *et al.*, 1991). The *wt Bam HI* bands are labelled on the right hand side. The novel k+'a' (sequences) band is also labelled. The novel *Bam HI s'* band is indicated (◀). Lane 1 shows a Kb DNA ladder (BRL).

of the *Bam HI* ***k*** and ***s*** fragments of the *wt* and 1714 DNA and incorporation of a size ladder (1 kb DNA ladder, BRL; lane 1) has confirmed the size and location of the deletion (see above) within the genome of 1714.

Sequence determination of the *Sma I* subfragments from the *Bam HI* ***k*** fragment of 1714 DNA has revealed that the deletion was 759 bp in length, located between nucleotides 125213 and 125972 [see Figure 45 (c); data not shown; see MacLean, A. R. *et al.*, 1991a]. The deletion has precisely removed an 18 bp DR_1 element of the *'a'* sequences located at the IR_L/IR_S joint region of the HSV-1 strain 17^+ genome. The other end (nucleotide 125213) of the deletion was found to be 1105 bp upstream of the 5' end of the IE-1 (IE110) gene. No gene had previously been predicted within the region spanning the deletion from sequence determination of the HSV-1 strain 17^+ genome (Perry and McGeoch, 1988; McGeoch *et al.*, 1988b). However, in HSV-1 strain F, a gene termed ICP34.5 was reported to be located between the IE-1 gene and the *'a'* sequences (Chou and Roizman, 1986, 1990; Ackermann *et al.*, 1986; Chou *et al.*, 1990). A similar region was reported to confer neurovirulence in HSV-2 strain HG52 (Taha *et al.*, 1988, 1989a, b, 1990). Therefore, the neurovirulence of the variant 1714 was determined and compared to that of the parental 1702 and strain 17^+ viruses by estimation of their LD_{50} values in BALB/c mice (see MacLean A. R. *et al.*, 1991a). It was found that the variant 1714 was at least 2×10^4-fold and 7×10^5-fold less neurovirulent than the parental 1702 and strain 17^+ viruses respectively. This provided evidence for the presence of a gene responsible for the neurovirulence phenotype in HSV-1 strain 17^+ R_L sequences. This gene has recently been identified, characterized and termed the RL_1 gene, both in HSV-1 strain 17^+ and HSV-2 strain HG52 genomes (McGeoch *et al.*, 1991; Dolan *et al.*, 1992). In HSV-1 strain 17^+, the RL_1 gene (ICP34.5) is located between nucleotides 125112 and 125856 (744 residues; 248 aa; McGeoch *et al.*, 1991). Therefore, most (86.42%) of the RL_1 coding sequences were removed from the variant 1714.

The production of a high titre virus stock (> 10^9 *pfu*/ml) and one-step growth experiments have demonstrated that the variant 1714 has similar growth characteristics to that of 1702 and strain 17^+ viruses (MacLean *et al.*, 1991a).

As the variant 1714 was derived from the variant 1702 and 1721 [see Section 3B:1 (a) (i)], it contained no *Xba I* restriction endonuclease sites [see Figure 42 (b), lane 3] and the virus was tk^- (data not shown). It was important therefore to see whether the avirulent phenotype of 1714 is retained

Figure 45. **Map of The Deletion In 1714**

(a) Structure of the HSV-1 genome in the prototype orientation.

(b) The *Hind III* restriction endonuclease map of the variant 1714 (identical to 1721).

(c) The expanded *Bam HI* ***k*** (***s+q***) fragment showing the position of the inverted "a" sequences and the DR1 elements. The endpoints of the 759 bp deletion in the variant 1714 are indicated. The positions of the RL1 (ICP34.5) ORF and the 5' terminus of the RL2 (IE110) gene are also indicated. The nucleotide numbers are based on the numbering system of the HSV-1 strain 17^+ DNA sequence (McGeoch *et al.*, 1988b).

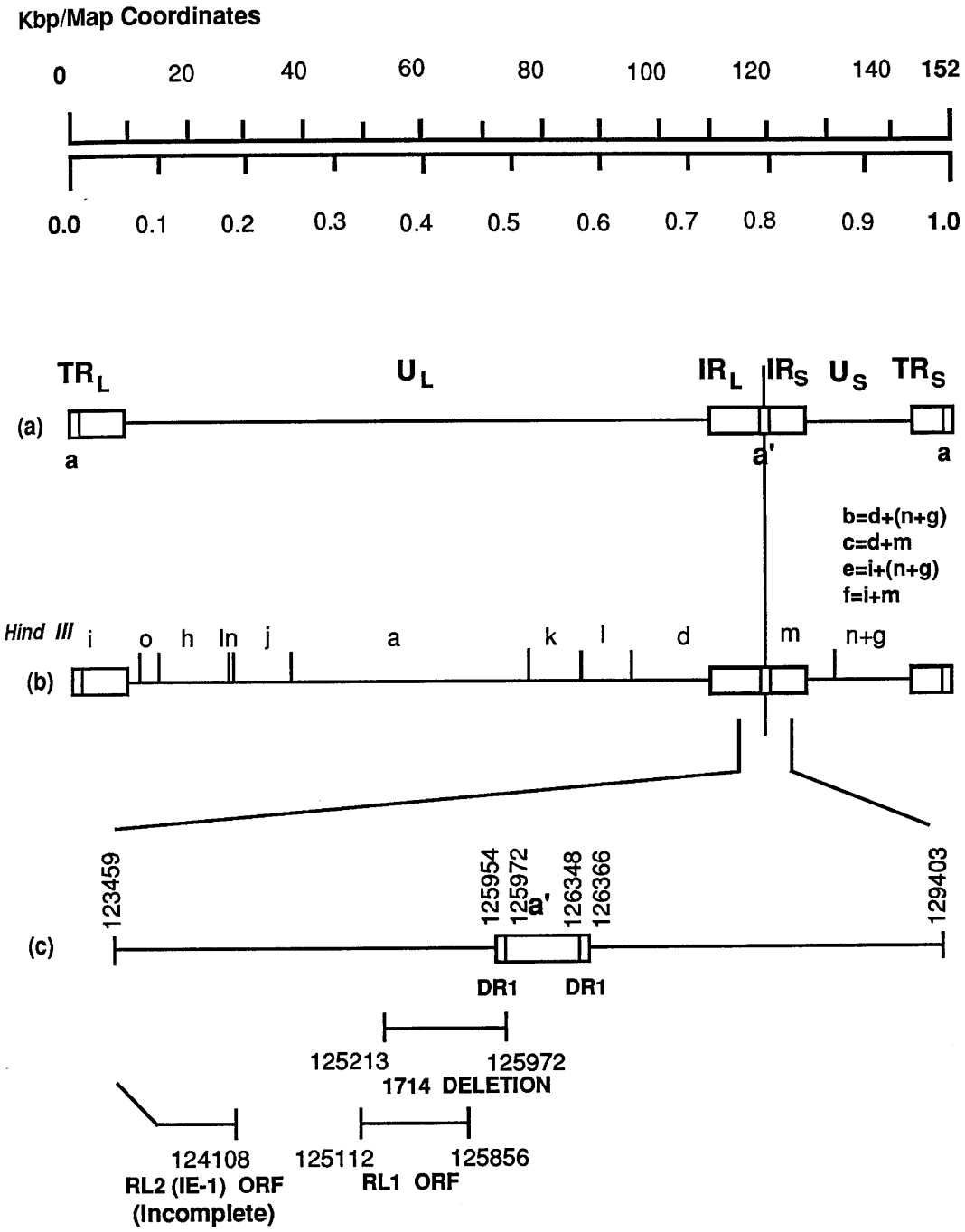

Figure 45. **Map of The Deletion In The HSV-1 (Strain 17+) Variant 1714**

in a wild-type background. This was achieved by transferring a fragment spanning the deletion in 1714 into an otherwise totally *wt* (strain 17+) genome. These experiments were carried out by Dr A R MacLean. A *wt* virus carrying the 1714 deletion was isolated and designated as 1716. An *Xba I* digest of 1716 DNA is shown in Figure 42 (b), lane 4. It can be seen that the variant 1716 contains all the *Xba I* bands (compare lanes 1 and 4) as that of the *wt* except that the *Xba I* ***g*** is reduced because of the presence of a 759 bp deletion. The characterisation of the variant 1716 has confirmed that the deleted sequences in 1714 were indeed responsible for the destruction of the neurovirulence phenotype in HSV-1 strain 17+ (MacLean A.R. *et al.*, 1991a). This virus is being developed as a prototype vaccine.

Several other variants carrying deletions similar to that of 1714 but smaller in size (approximately 400 to 550 bp) were isolated and preliminary characterisation of these variants by restriction endonuclease analysis was carried out (data not shown). Of these, four variants were plaque-purified a further three times and virus stocks were grown in 50mm petri dishes. These variants were then tested for neurovirulence by Dr L M Robertson. However, further characterisation was not carried out because their LD_{50} values were not significantly different from that of strain 17+ (data not shown).

(b) **Isolation of HSV-1 variants containing DNA deletions involving the repeat regions of the genome**

A large number of HSV-1 strain 17+ genomes with DNA deletions involving the repeat regions (TR_L, IR_L, IR_S, TR_S) have been isolated during the course of the work described in this thesis. These variants were isolated during the transfection and/or recombination experiments and have not shown any loss or gain of restriction endonuclease sites (for example, the *Hind III* or *Xba I* sites) on *Hind III*, *Bgl II-Hind III* and *Bgl II-Xba I* digestion of their DNAs. The DNA profiles of only two of these variants are described below.

(i) **DNA profile of 1721X193**

The *Hind III* restriction endonuclease DNA profile of 1721X193 is shown in Figure 46 (a), lane 3. Because of the absence of the 0.91 m.c. *Hind III* site, the *Hind III* ***g*** and ***n*** bands are missing and a novel ***g+n*** fused band is migrating below the ***f*** band [Figure 46 (a), lane 2; 1721 *Hind III* profile; see also Section 3B:1 (a) (i)]. The *Hind III* ***g*** containing joints ***b*** and ***e*** are migrating at the top of the gel track(***b'***) and between the ***c*** & ***d*** bands(***e'***) respectively [Figure 46 (a), lane 2]. In the *Hind III* profile of 1721X193 DNA,

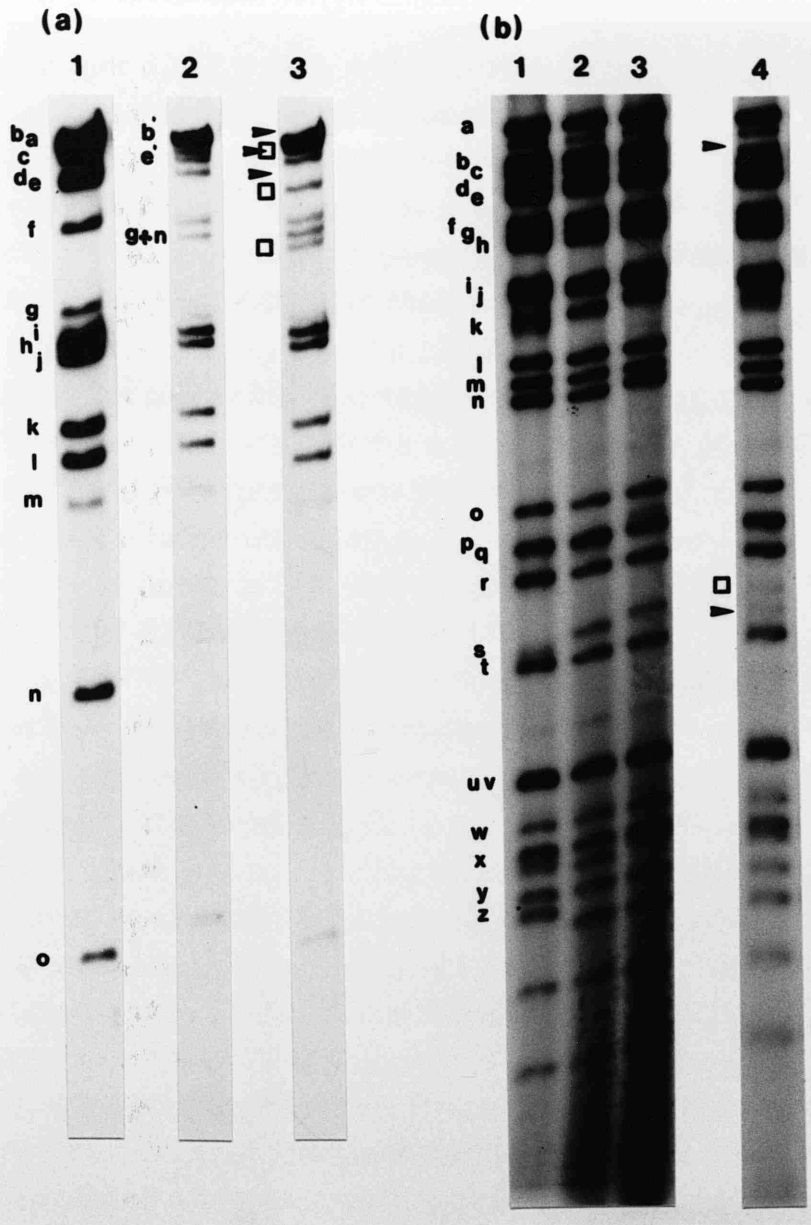

Figure 46. **The *Hind III* And *Bam HI* Profiles Of HSV-1 DNA**

(a) The *Hind III* restriction endonuclease profiles of HSV-1 strain 17+ (lanes 1), 1721 (lane 2) and 1721X193 (lane 3) DNAs labelled with ^{32}-P *in vivo* (0.5% agarose gel). The *wt Hind III* bands are labelled on left hand side. The missing bands are shown by filled arrowheads whereas the novel bands are indicated with open rectangles.

(b) The *Bam HI* restriction endonuclease profiles of HSV-1 strain 17+ (lane 1), 1702 (lane 2), 1721 (lane 3) and 1721X193 (lane 4) DNAs ^{32}-P-labelled *in vivo* (1.2% agarose gel). Letters refer to the *wt Bam HI* bands. The missing bands are indicated with filled arrowheads whereas the novel band is shown by an open rectangle.

the *Hind III* ***b'*** (Mr 30.51 x 10^6), ***c*** (Mr 22.75 x 10^6) and ***d*** (Mr 18.17 x 10^6) bands are missing and three novel bands of approximately 24.21 x 10^6 , 16.45 x 10^6 and 11.87 x 10^6 molecular weights are running with the ***a*** band, between the ***e'*** & ***f*** bands and below the ***g+n*** band respectively. These changes can be explained by a deletion of approximately 6.3 x 10^6 Mr (approximately 9,600 bp) within the *Hind III* ***d*** (U_L/IR_L) fragment, converting it to a ***d'*** band of approximately 11. 87 x 10^6 Mr. This will result in a reduction of 6.3 x 10^6 Mr in the size of the joint fragments ***b'*** [***d+(g+n)***] and ***c*** (***d+m***). The *Hind III* ***b'*** will now comigrate with the ***a*** band (below the *Hind III* ***a*** = 26.83 x 10^6 Mr); the ***c*** band will migrate below the normal ***d*** (18.17 x 10^6 Mr) band and the ***d*** band will run below the fused ***g+n*** band respectively, thus accounting for the three novel bands in the *Hind III* profile of 1721X193 DNA [Figure 46 (a), lane 3]. A *Bgl II-Hind III* digestion of 1721X193 DNA has shown that the *Bgl II* ***c*** (Mr 18.58 x 10^6), ***a'*** (Mr 17.45 x 10^6) and ***f*** (Mr 12.34 x 10^6) bands were missing and two novel bands of approximately 12.28 x 10^6 and 11.15 x 10^6 molecular weights were running above and below the ***d'*** (Mr 11.32 x 10^6) band respectively. The missing *Bgl II* ***f*** band was apparently reduced to a 6.04 x 10^6 Mr band, which was comigrating with the ***h'*** (Mr 6.20 x 10^6) band. The two novel bands have therefore accounted for the reduced ***f*** containing joint fragments ***c*** and ***a'*** respectively (data not shown; see Table 6 for the *Bgl II-Hind III* profile of 1721 DNA). Thus, the *Bgl II-Hind III* digestion has also indicated that the variant 1721X193 contains an approximate deletion of 6.3 x 10^6 Mr within the *Bgl II* ***f*** fragment [see Figure 48 (a)].

The 1721X193 DNA was then analysed with the *Bam HI* [Figure 46 (b)], *Hpa I* (Figure 47, lanes 1 to 4), *Kpn I* (Figure 47, lanes 5 to 8) and *Eco RI* (Figure 47, lanes 9 to 11) restriction endonucleases. On *Bam HI* digestion [see Figure 46 (b)], the 1721X193 DNA profile [Figure 46 (b), lane 4] differed from that of strain 17^+ [Figure 46 (b), lane 1], 1702 [Figure 46 (b), lane 2] and 1721 [Figure 46 (b), lane 3] DNAs in that the *Bam HI* ***b*** band (a faint *Bam HI* ***b*** band can be seen in Figure 46 (b), lane 4 because of contamination with approximately 20% of the parental 1721 DNA] was missing. Moreover, the intensity of the *Bam HI* ***s*** band was reduced and a novel band was migrating slightly above the ***s*** band. This suggested that the deletion starts from within the *Bam HI* ***b*** fragment (end of U_L) and ends within the *Bam HI* ***s*** fragment (IR_L). Therefore, the *Bam HI* ***b*** (Mr 6.66 x 10^6) was missing; one copy of the *Bam HI* ***s*** (Mr 1.91 x 10^6) was missing and the other copy was migrating normally as a 0.5 M band; and the fused ***b+s*** band was migrating above the

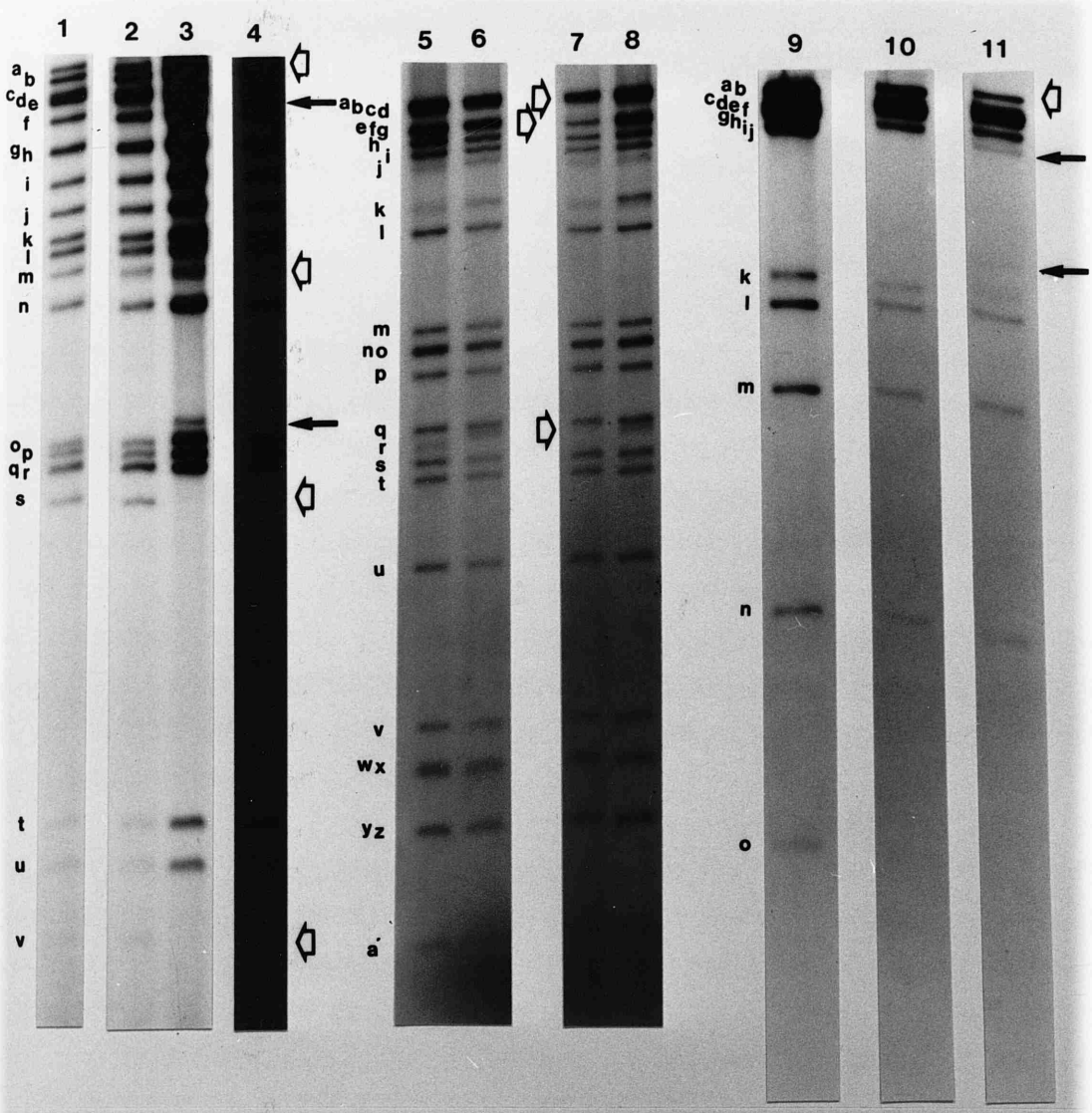

Figure 47. The *Hpa I*, *Kpn I* And *Eco RI* Restriction Endonuclease Profiles Of HSV-1 DNA

The *Hpa I* (lanes 1 to 4), *Kpn I* (lanes 5 to 8) and *Eco RI* (lanes 9 to 11) restriction endonuclease profiles of HSV-1 strain 17^+ (lanes 1, 5 & 9), 1702 (lane 6), 1721 (lanes 2, 8 & 10) and 1721X193 (lanes 3, 4, 7 & 11) DNAs ^{32}-P-labelled *in vivo* (0.8%, 0.6% and 0.7% agarose gels respectively). The *wt* restriction endonuclease bands are labelled on the left hand side of lanes 1, 5 and 9 respectively. The missing restriction endonuclease fragments are indicated with open arrowheads whereas the novel bands are shown by filled arrows.

normally migrating copy of the *Bam HI* ***s*** band. Thus, indicating a deletion of approximately 6.5 x 10^6 Mr involving the internal end of U_L and most of IR_L.

The *Hpa I* digestion of 1721 X 193 DNA (Figure 47, lanes 3 & 4) has indicated that *Hpa I* ***a*** (***a=m+c***;Mr 13.88 x 10^6), ***s*** (Mr 2.14 x 10^6) and ***v*** (Mr 0.82 x 10^6) were missing. In addition, one copy of *Hpa I* ***m*** (Mr 3.88 x 10^6) appeared to be missing and two novel bands were present : one migrating marginally faster than the comigrating ***c***, ***d*** & ***e*** bands (Mr 10.02 x 10^6, 10.87 x 10^6 and 9.95 x 10^6 respectively) and the other running above the ***o***, ***p***, ***q*** & ***r*** bands (Mr 2.33 x 10^6, 2.30 x 10^6, 2.38 x 10^6 and 2.16 x 10^6 respectively). The *Hpa I* profile of 1721X193 could be interpreted by considering a deletion starting from within the *Hpa I* ***s***, running through the ***v*** & ***r*** fragments and ending within the ***m*** fragment (see Figure 48). As *Hpa I* ***r*** is located between the ***v*** & ***m*** fragments (Figure 48) and comigrates with the ***o***, ***p*** & ***q*** bands, it's absence could not be detected in the DNA profile of 1721X193 (see Figure 47, lanes 3 & 4). The novel band with an approximate molecular weight of 2.5 x 10^6 (migrating above ***o***, ***p***, ***q*** & ***r*** bands; see Figure 47) would then be the fused *Hpa I* ***s+m*** band whereas the novel band migrating below the ***c***, ***d*** & ***e*** bands would be the reduced *Hpa I* ***a*** band, thus accounting for an approximate deletion of 6.5 x 10^6 Mr.

On *Kpn I* digestion (Figure 47, lanes 5 to 8), the profile of 1721X193 (lane 7) has shown that one copy of the *Kpn I* ***r*** (Mr 2.40 x 10^6) band was missing. The *Kpn I* ***r*** forms the joint fragments ***a*** (***r+j***) and ***e*** (***r+k***) which comigrate with the ***b***, ***c*** & ***d*** and ***f*** & ***g*** bands respectively. It can be seen that the intensity of the comigrating ***a***, ***b***, ***c*** & ***d*** bands is reduced (Figure 47, lane 7) possibly because of the absence of the ***a*** band. Similarly, the intensity of the comigrating ***e***, ***f*** & ***g*** bands is reduced suggesting the absence of the *Kpn I* ***g*** band (see Figure 48). The novel ***a'*** band and the fused ***g+r*** band could not be detected possibly because of their comigration with the ***i*** and ***p*** bands respectively.

The *Eco RI* restriction endonuclease digest of 1721X193 is shown in Figure 47, lane 11. Two novel bands can be seen : one migrating below the comigrating ***i*** & ***j*** bands and the other running above the ***k*** band. These novel bands can be explained as the reduced ***b*** and ***e*** bands respectively. The *Eco RI* ***e*** fragment (see Figure 48) is located at the internal end of U_L and contains the entire IR_L. If *Eco RI* ***e*** (Mr 10.42 x 10^6) is reduced by 6.5 x 10^6 Mr, it would run as a 3.92 x 10^6 Mr band above the ***k*** (Mr 3.66 x 10^6) band. The joint ***b*** (Mr 14.08 x 10^6) containing the ***e*** fragment would then be migrating as a 7.58 x 10^6 Mr band below the *Eco RI* ***j*** (Mr 8.27 x 10^6) band.

Figure 48. **The *Bam HI, Bgl II, Eco RI, Hind III, Hpa I* And *Kpn I* Restriction Endonuclease Maps Of HSV-1 DNA And Map Of The DNA Deletion In The Variant 1721X193**

(a) The *Hind III* restriction endonuclease map of 1721 (above the line) and the *Bgl II* map (below the line) for the DNA of HSV-1 strain 17^+.

(b) The *Hpa I* (above the line) and *Bam HI* (below the line) restriction endonuclease maps for the DNA of HSV-1 strain 17^+.

(c) The *Kpn I* (above the line) and *Eco RI* (below the line) restriction endonuclease maps for the DNA of HSV-1 strain 17^+.

(d) An expansion of UL/IR_L region of the HSV-1 genome. The positions of the *Bam HI* (B), *Bgl II* (Bg), *Eco RI* (E), *Hind III* (H), *Hpa I* (Hp) and *Kpn I* (K) restriction endonuclease cleavage sites are indicated. The approximate location of DNA deletion in the variant 1721X193 is also indicated.

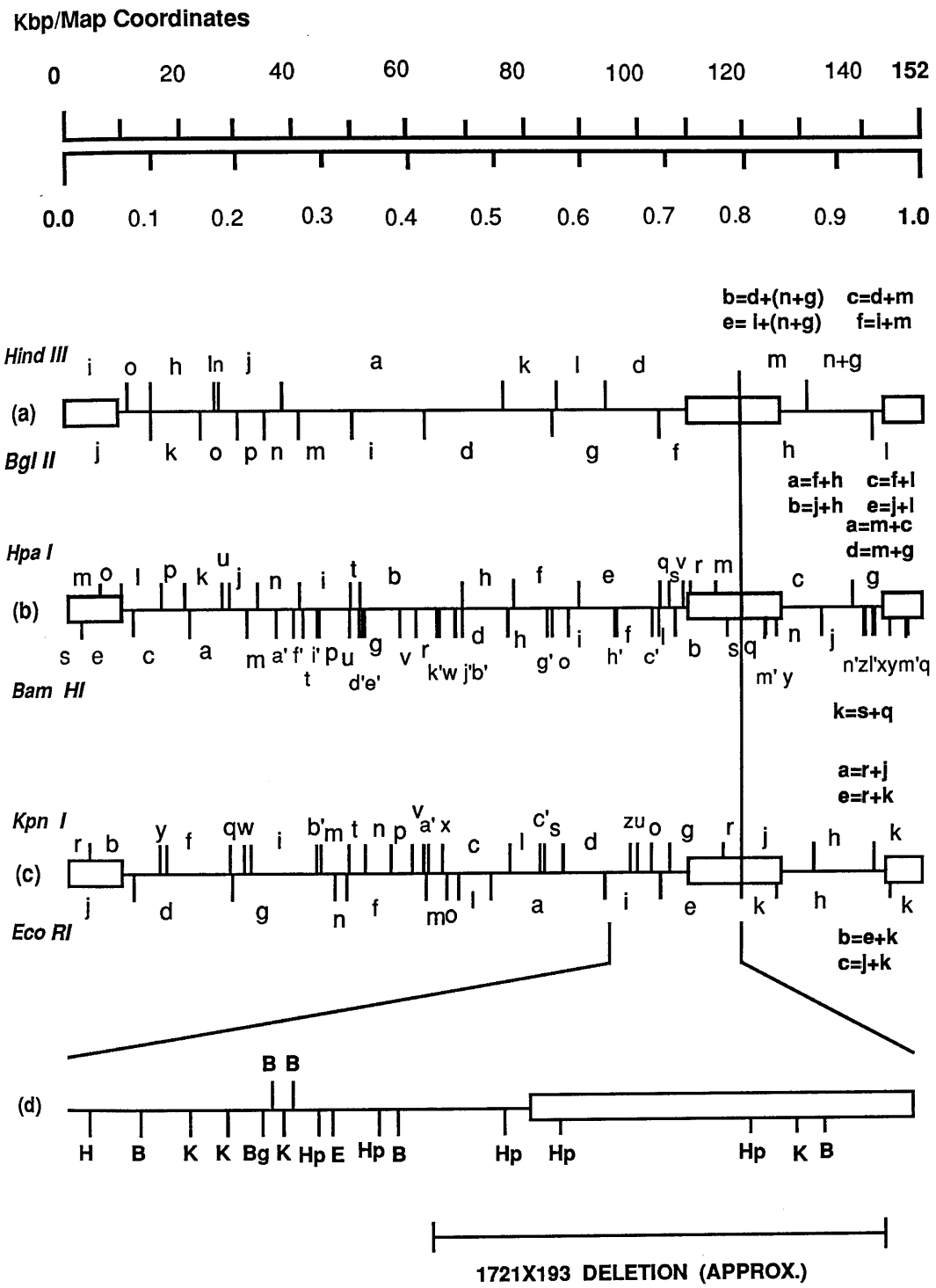

Figure 48. **Restriction Endonuclease Maps Of HSV-1 (Strain 17+) DNA And Map Of The Deletion In The Variant 1721X193**

It can be seen that the intensity of the comigrating ***a*** & ***b*** bands is reduced in the 1721X193 DNA profile (Figure 47, lane 11) suggesting that the *Eco RI* ***b*** band is missing. However, the absence of the *Eco RI* ***e*** band cannot be detected because of the comigrating ***f*** & ***g*** bands.

Taken together, the above restriction endonuclease analysis suggested that the variant 1721X193 contains a deletion of approximately 6.5 x 10^6 molecular weight (approximately 9.9 kbp) between 0.74 and 0.83 m.c.

(ii) **DNA profile of 1727X31**

This DNA deletion variant was isolated during the transfection experiments involving intact 1727 DNA and the plasmid pMF18 DNA [see Section 3B : 1 (a) (iv)]. The restriction endonuclease cleavage sites of 1727X31 were identical to that of the variant 1727 [see Section 3B : 1 (a) (iii)]. The variant (1727X31) has shown aberrant *Hind III* [Figure 49 (a)] as well as *Bgl II-Hind III* [Figure 49 (b)] DNA profiles compared to that of 1727 DNA. The mobility of the *Hind III* ***e'*** (Mr 30.72 x 10^6) and ***b'*** (Mr 30.51 x 10^6; see Table 5) bands was altered and the fused ***g+n*** (Mr 12.34 x 10^6) band was missing [Figure 49 (a), lane 4]. In addition, a novel band was migrating slightly above the *Hind III* ***j*** band [Mr 7.76 x 10^6; compare lanes 3 & 5 (1727 profile) with lane 4]. Comparison of the *Hind III* profile of 1727X31 with that of the parental viruses [Figure 49 (a)] has indicated that the fused ***g+n*** band was deleted by approximately 4.44 x 10^6 molecular weight, converting it (***g+n***) into a novel 7.9 x 10^6 Mr band. The ***g+n*** containing joint fragments ***e'*** and ***b'*** were also affected by the same deletion and therefore showed altered mobility.

A *Bgl II-Hind III* profile of 1727X31 DNA [Figure 49 (b), lane 4] was compared to that of 1727 DNA [Figure 49 (b), lanes 3 & 5] and showed that the *Bgl II* ***c*** (Mr 18.58 x 10^6), ***e*** (Mr 15.82 x 10^6) and ***l*** (Mr 6.24 x 10^6) bands were missing. However, no novel bands could be detected. Considering the *Hind III* restriction endonuclease profile of 1727X31 DNA (see above) with its *Bgl II-Hind III* profile, it appeared that the deletion was located within the *Hind III* ***g+n***/*Bgl II* ***l*** band between 0.93 and 1.00 m.c. If the estimation of the size of the deletion (4.44 x 10^6 Mr) was correct, then the missing *Bgl II* ***c***, ***e*** and ***l*** bands would comigrate with the ***b'***, ***d'*** and ***o'*** bands respectively [see the *Bgl II-Hind III* profile of 1727 DNA in Figure 49 (b), lanes 3 & 5; see also Table 6] and therefore could not be detected. To confirm this, it was necessary to analyse the 1727X31 DNA with various other restriction endonucleases. Therefore, 1727X31 DNA was subjected to the *Bam HI*, *Bgl II*, *Eco RI*, *Hpa I* and *Kpn I* restriction endonuclease digestion along with the DNAs of HSV-1 strain 17^+ (*wt*) and the variant 1727.

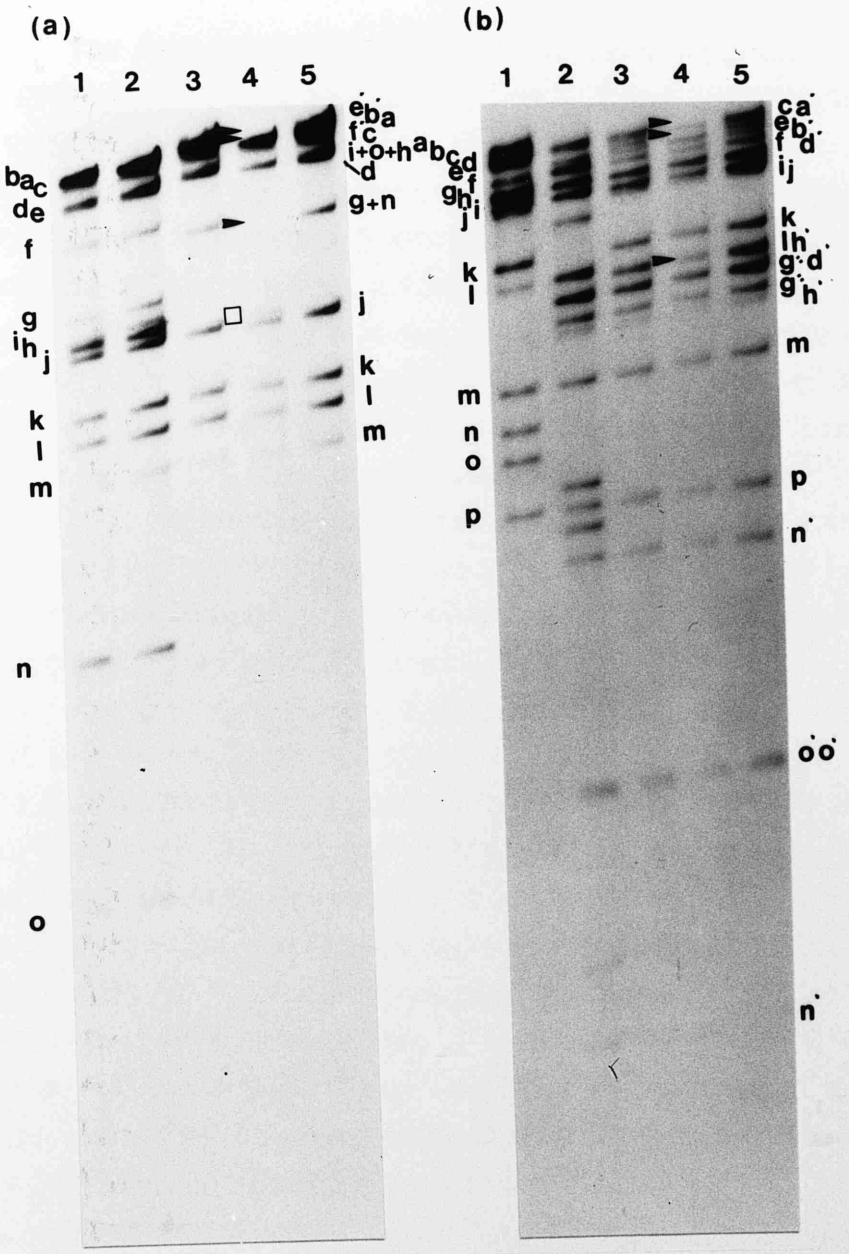

Figure 49. **The *Hind III* And *Bgl II-Hind III* Profiles Of HSV-1 DNA**

(a) The *Hind III* restriction endonuclease profiles of HSV-1 strain 17+ (lanes 1), 1702 (lane 2), 1727 (lanes 3 & 5) and 1727X31 (lane 4) DNAs ^{32}-P-labelled *in vivo* (0.5% agarose gel). Letters refer to the *wt Hind III* bands. The missing bands are shown by filled arrowheads and the novel band with an open rectangle.

(b) The *Bgl II-Hind III* restriction endonuclease digests of HSV-1 strain 17+ (lane 1), 1702 (lane 2), 1727 (lanes 3 & 5) and 1727X31 (lane 4) DNAs ^{32}-P-labelled *in vivo* (0.7% agarose gel). Letters refer to the *wt Bgl II* bands. The missing *Bgl II* bands are indicated with filled arrowheads.

The *Bam HI* restriction endonuclease profiles of the *wt*, 1727 and 1727X31 DNAs are shown in Figure 50, lanes 1, 2 and 3 respectively. It can be seen that the *Bam HI* ***x*** (Mr 1.28 x 10^6) and ***y*** (Mr 1.20 x 10^6) bands are missing (Figure 50, lane 3). Moreover, the intensity of the ***k*** band is reduced to half (compare the ***k*** band in lane 3 with that of lanes 1 & 2 in Figure 50) and a novel band (***k'***) of approximately 2.10 x 10^6 Mr is running between the ***p***, ***q*** and ***r*** bands. Thus, indicating that one copy of the ***k*** fragment is deleted by approximately 1.8 x 10^6 Mr. This can be interpreted as follows: the deletion starts within the *Bam HI* ***z*** or ***l'*** fragment (U_S) (see the *Bam HI* map of HSV-1 strain 17^+ in Figure 52), runs through the ***x***, ***y*** and ***m'*** fragments and ends within the ***q*** (TR_S) fragment, giving a total size of approximately 4.28 x 10^6 Mr. The novel *Bam HI* ***q*** fragment (approximate Mr 0.19 x 10^6) would have run off from the end of the agarose gel. However, no change in the mobility of *Bam HI* ***z*** was observed (see Figure 50, lanes 1 to 3).

The *Bgl II* restriction endonuclease digest of 1727X31 DNA (Figure 50, lane 6) has confirmed that the deletion was located towards the end of the S component of the genome involving U_S/TR_S. The *Bgl II* ***c*** (Mr 18.58 x 10^6), ***e*** (Mr 15.82 x 10^6) and ***l*** (Mr 6.24 x 10^6) bands were missing and two novel bands of approximately 14.28 x 10^6 Mr and 1.92 x 10^6 Mr were migrating in between the ***d*** & ***f*** bands and below the ***p*** band respectively. The novel 1.92 x 10^6 Mr band has accounted for the deleted ***l*** band with an estimated deletion of 4.32 x 10^6 Mr. The *Bgl II* ***l*** fragment forms the joint fragments ***c*** (***f+l***) and ***e*** (***j+l***). The deleted ***c*** (***c'***) fragment was migrating in between the ***d*** and ***f*** bands whereas the deleted ***e*** (***e'***) fragment (approximate Mr 11.5 x 10^6) would be comigrating with the ***g*** band (Mr 11.42 x 10^6) and, therefore, could not be detected.

On *Eco RI* digestion (Figure 50, lanes 7 to 9), the profile of 1727X31 (Figure 50, lane 9) showed missing ***c*** (Mr 11.93 x 10^6) and ***h*** (Mr 9.96 x 10^6) bands. In addition, the intensity of the ***k*** (Mr 3.66 x 10^6) band was reduced and a novel band was running above the ***i*** (Mr 8.76 x 10^6) band. This could be explained as : one copy of the ***k*** band was missing; both the missing ***h*** and ***k*** fragments were deleted and the fused ***h+k*** novel fragment (approximate Mr 9.3 x 10^6) was migrating above the ***l*** band; and, the ***k*** containing joint fragment ***c*** was reduced and comigrating with the ***e***, ***f*** and ***g*** bands.

On *Hpa I* digestion of 1727X31 DNA (Figure 51, lane 3), the *Hpa I* ***g*** (Mr 6.99 x 10^6) was missing and a novel band of approximately 2.67 x 10^6 Mr was migrating above the ***o*** band. The ***g*** containing joint fragment ***d*** (***g+m***)

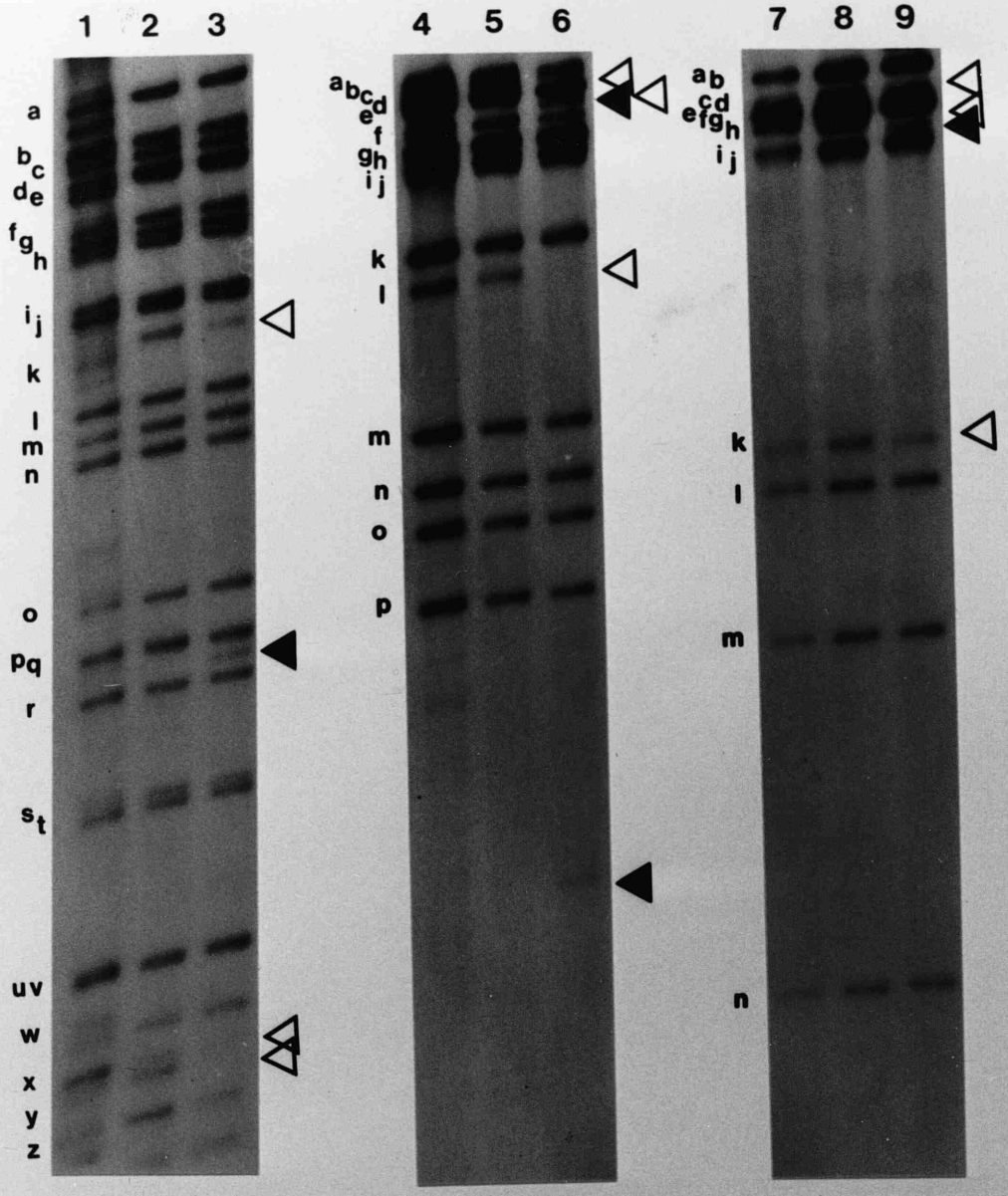

Figure 50. **The *Bam HI*, *Bgl II* And *Eco RI* Restriction Endonuclease Profiles Of HSV-1 DNA**

The ***Bam HI*** (lanes 1 to 3), ***Bgl II*** (lanes 4 to 6) and ***Eco RI*** (lanes 7 to 9) restriction endonuclease profiles of HSV-1 strain 17^+ (lanes 1, 4 & 7), 1727 (lanes 2, 5 & 8) and 1727X31 (lanes 3, 6 & 9) DNAs ^{32}-P-labelled *in vivo* (0.8% agarose gels). Letters refer to specific restriction endonuclease fragments. The missing bands are indicated with open triangles and the novel bands with filled triangles.

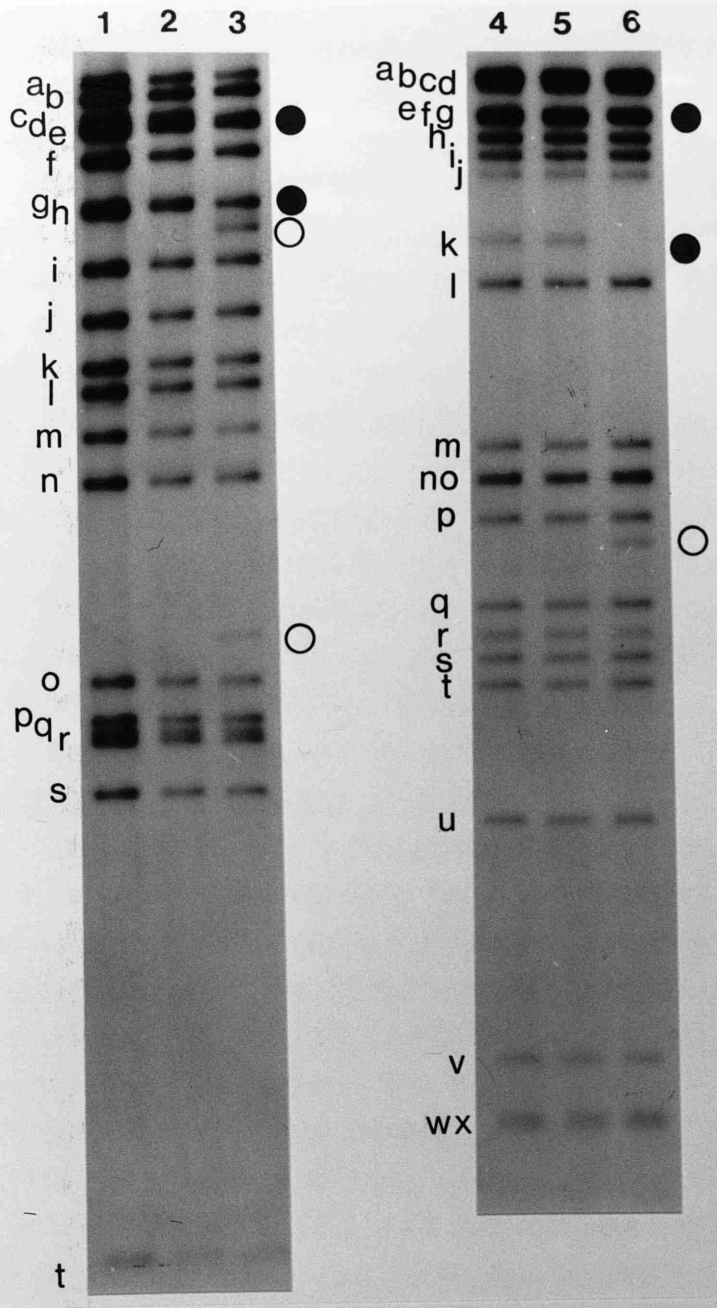

Figure 51. The *Hpa I* And *Kpn I* Restriction Endonuclease Profiles Of HSV-1 DNA

The *Hpa I* (lanes 1 to 3) and *Kpn I* (lanes 4 to 6) restriction endonuclease digests of HSV-1 strain 17^+ (lanes 1 & 4), 1727 (lanes 2 & 5), and 1727X31 (lanes 3 & 6) DNAs ^{32}-P-labelled *in vivo* (0.8% agarose gels). The *wt* restriction endonuclease bands are labelled on the left hand side of lanes 1 & 4 respectively. The missing bands are shown by filled circles whereas the novel bands are indicated with open circles.

was also missing and a novel band of approximately 6.55 x 10^6 Mr was running in between the ***h*** and ***l*** bands. Thus, suggesting a deletion of approximately 4.32 x 10^6 Mr within the *Hpa I* ***g*** fragment (see Figure 52).

The *Kpn I* digests of strain 17+, 1727 and 1727X31 DNAs can be seen in Figure 51, lanes 4, 5 and 6 respectively. The *Kpn I* ***k*** (Mr 4.84 x 10^6) and the ***k*** containing joint ***e*** (Mr 7.24 x 10^6) fragments were missing from the DNA profile of 1727X31 (Figure 51, lane 6). A novel band of approximate molecular weight of 2.77 x 10^6 was migrating below the *Kpn I* ***p*** (Mr 2.88 x 10^6) band. The *Kpn I* profile indicated that the *Kpn I* ***k*** was deleted by approximately 4.47 x 10^6 Mr resulting in a novel ***k'*** fragment of 0.37 x 10^6 Mr, which would have run off the agarose gel. Thus, the novel band observed below the ***p*** band represents the deleted ***e*** band.

The above described restriction endonuclease analysis indicated that the genome of 1727X31 is deleted within U_S/TR_S between 0.94 m.c. and 1.00 m.c. (see Figure 52). The size of the deletion was estimated to be between 6.5 and 6.8 Kbp (4.28 x 10^6 and 4.47 x 10^6 Mr).

(c) **Isolation of the variant 1740*In***

This variant was isolated during the recombination experiments between the variants 1708 (contains an additional *Xba I* site) and 1739 (contains an additional *Hind III* site but devoid of the *Xba I* sites) to generate a variant 1743 containing 11 *Hind III* (as opposed to the normally occuring 10 *Hind III* sites in strain 17+ genome) and 5 Xba I (compared to 4 *Xba I* sites in the *wt* genome) sites [see Section 3B:2 (b)]. The *Hind III* DNA profile (see Figure 53, lane 8) of 1740*In* was identical to that of 1739 (Figure 53, lane 7) and 1740 (data not shown), containing 11 *Hind III* sites at 0.08, 0.1, two at 0.18, 0.26, 0.374, 0.52, 0.58, 0.64, 0.88 and 0.91 m.c. The *Xba I* digest of 1740*In* DNA (Figure 53, lane 4) has shown that the viral genome was devoid of the 0.07 and 0.29 m.c. *Xba I* sites [identical to that of 1740 DNA; see Section 3B:2 (b)]. In addition, the *Bgl II-Hind III* (Figure 54, lanes 1 to 4) and *Bgl II-Xba I* (Figure 54, lanes 5 to 8) profiles of 1740*In* DNA have confirmed the presence of 11 *Hind III* and 3 *Xba I* (0.45, 0.63 and 0.74 m.c.) sites. However, the *Bgl II-Hind III* digest of 1740*In* DNA (Figure 54, lane 4) has shown that the *Bgl II* ***n'*** (Mr 1.06 x 10^6) band is missing and a novel band of approximately 1.32 x 10^6 Mr is running slightly above the *Bgl II* ***j'*** (Mr 1.23 x 10^6) band. Similarly, the *Bgl II-Xba I* profile of 1740*In* DNA (Figure 54, lane 7) has shown reduced intensity in the comigrating ***d'*** (Mr 3.79 x 10^6) and ***n*** (Mr 3.76 x 10^6) bands and a novel band of approximately 4.08 x 10^6 molecular weight was migrating in between the *Bgl II* ***m*** (Mr 4.21 x 10^6) and ***d'n*** bands. On *Bgl II* digestion of

Figure 52. **Restriction Endonuclease Maps Of HSV-1 DNA And Map Of The Deletion In 1727X31**

(a) The *Hind III* map of 1727 (above the line) and the *Bgl II* map (below the line) for the DNA of HSV-1 strain 17^+.

(b) The *Hpa I* (above the line) and *Bam HI* (below the line) restriction endonuclease maps for the DNA of HSV-1 strain 17^+.

(c) The *Kpn I* (above the line) and *Eco RI* (below the line) restriction endonuclease maps for the DNA of HSV-1 strain 17^+.

(d) An expansion of US/TR_S region of the HSV-1 strain 17^+ genome. The *Bam HI* (B), *Bgl II* (Bg), *Eco RI* (E), *Hind III* (H), *Hpa I* (Hp) and *Kpn I* (K) restriction endonuclease cleavage sites are indicated. Also shown is the approximate DNA deletion in the variant 1727X31.

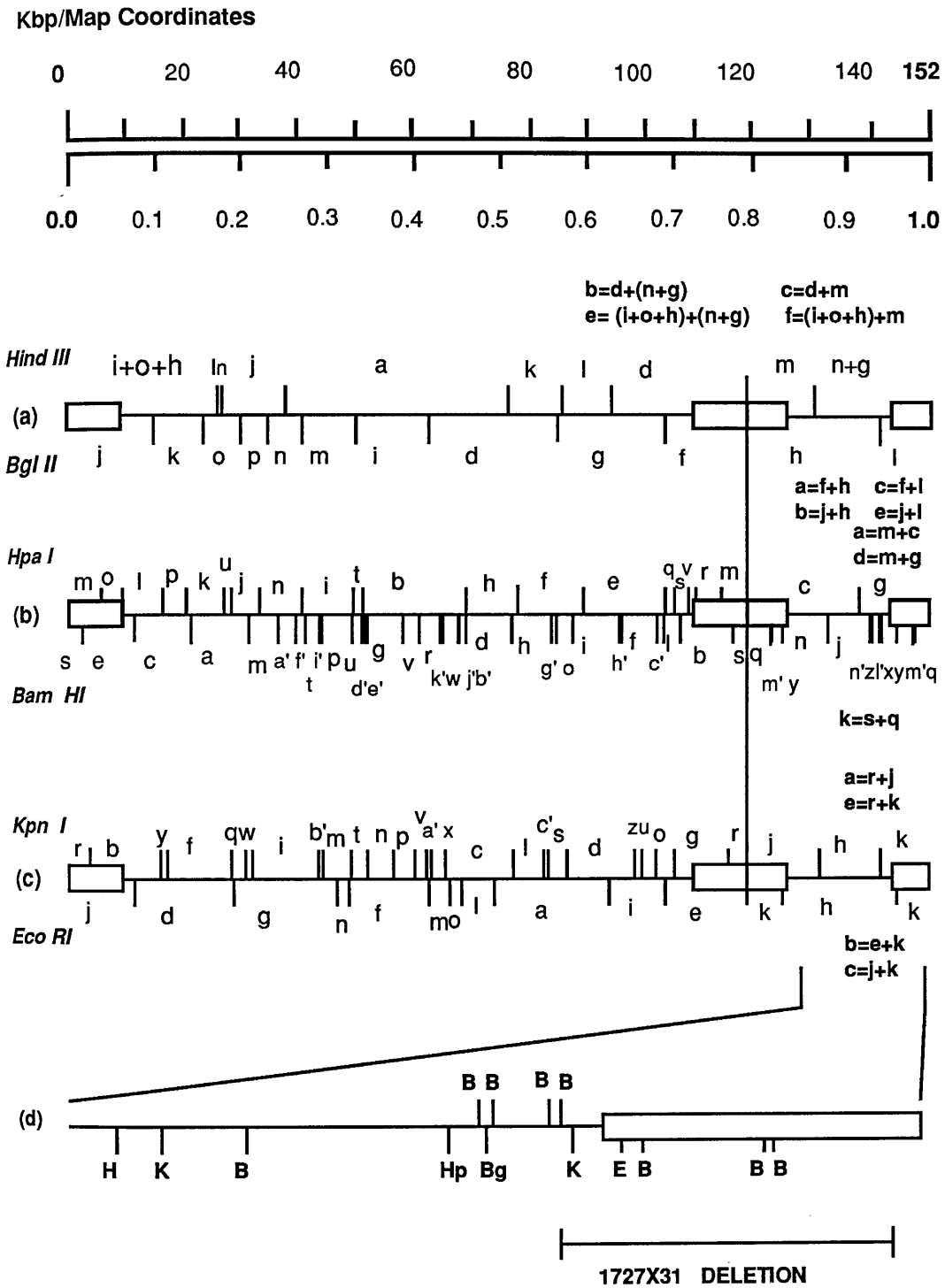

Figure 52. **Restriction Endonuclease Maps Of HSV-1 (Strain 17±) DNA And Map Of The Deletion In The Variant 1727X31**

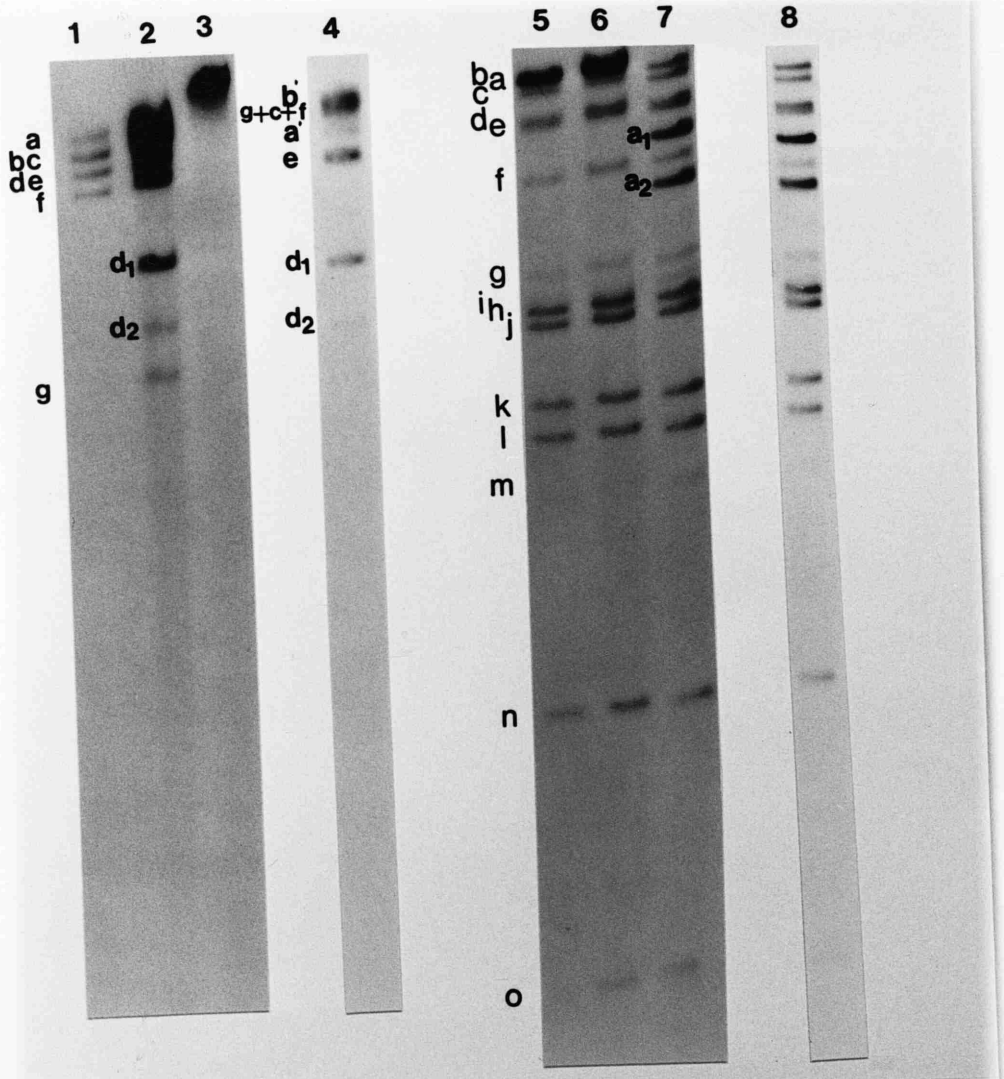

Figure 53. **The *Xba I* And *Hind III* Restriction Endonuclease Profiles Of HSV-1 Strain 17±, 1708, 1739 And 1740*In* DNA**

The *Xba I* (lanes 1 to 4) and *Hind III* (lanes 5 to 8) restriction endonuclease profiles of HSV-1 strain 17+ (lanes 1 & 5), 1708 (lanes 2 & 6), 1739 (lanes 3 & 7) and 1740*In* (lanes 4 & 8) DNAs ^{32}-P-labelled *in vivo* (0.5% agarose gels). The *wt Xba I* and *Hind III* bands are labelled on the left hand side of lanes 1 and 5 respectively. The additional *Xba I* site at 0.74 m.c. cleaves the *Xba I d* band into two smaller d_1 and d_2 bands (lanes 2 & 4; MacLean and Brown, 1987c) whereas the additional *Hind III* site at 0.374 m.c. cleaves the *Hind III a* fragment into two smaller a_1 and a_2 bands (lanes 7 & 8).

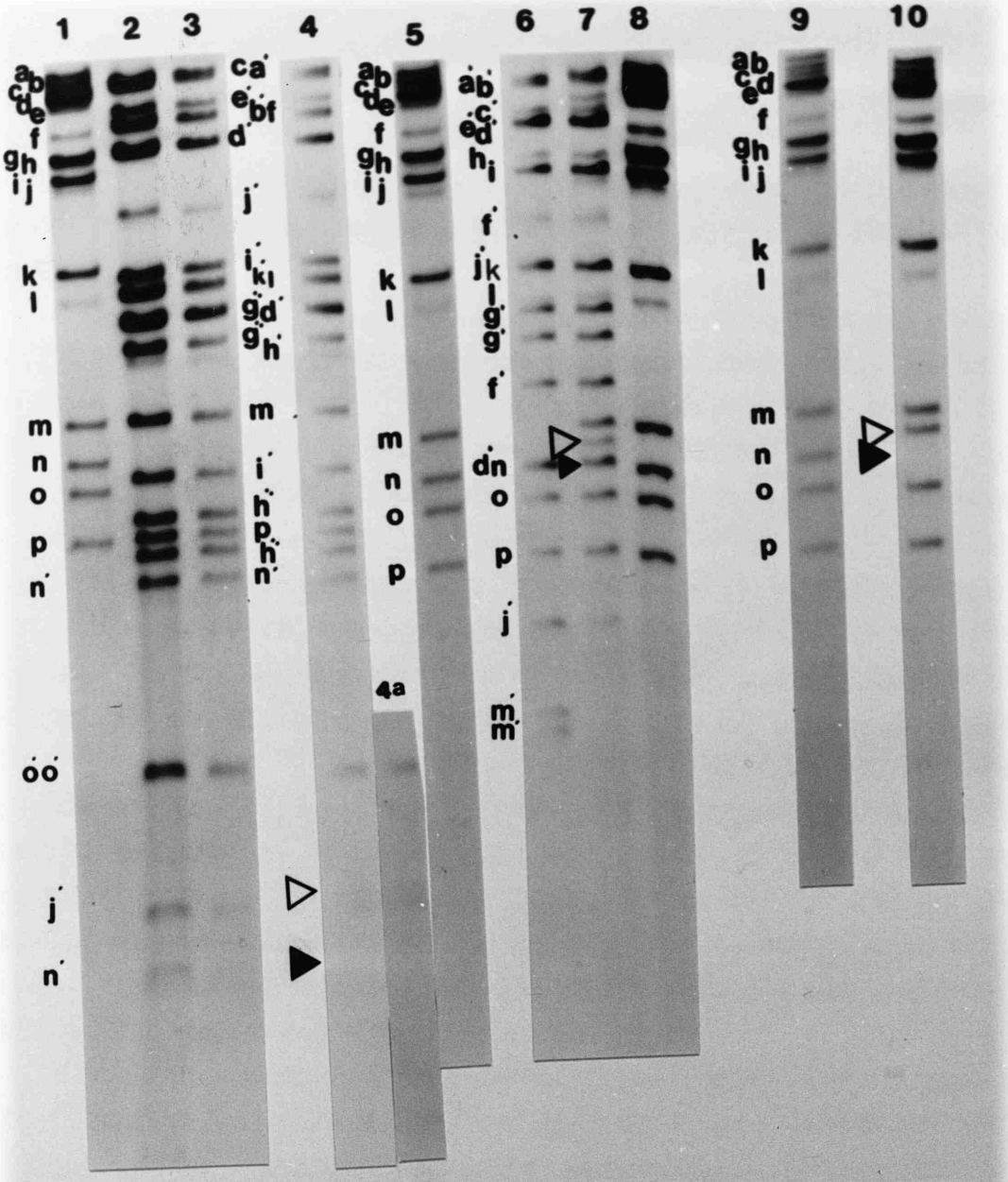

Figure 54. **The *Bgl II*, *Bgl II-Hind III* And *Bgl II-Xba I* Profiles Of HSV-1 DNA**

The *Bgl II* (lanes 1, 5, 9 & 10), *Bgl II-Hind III* (lanes 2, 3 & 4) and *Bgl II-Xba I* (lanes 6, 7 & 8) restriction endonuclease profiles of HSV-1 strain 17^+ (lanes 1, 5 & 9), 1708 (lane 6), 1739 (lanes 2 & 8), 1740 (lane 3) and 1740*In* (lanes 4, 7 & 10) DNAs ^{32}-P-labelled *in vivo* (0.5% agarose gels). Lane 4a (between lanes 4 & 5) represents a slightly longer exposure of the low molecular weight bands in lane 4. The *wt Bgl II* bands are labelled on the left hand side of lanes 1, 5, 6 and 9 as well as on the right hand side of lane 3. The missing bands are indicated with a filled triangle and the novel bands with an open triangle.

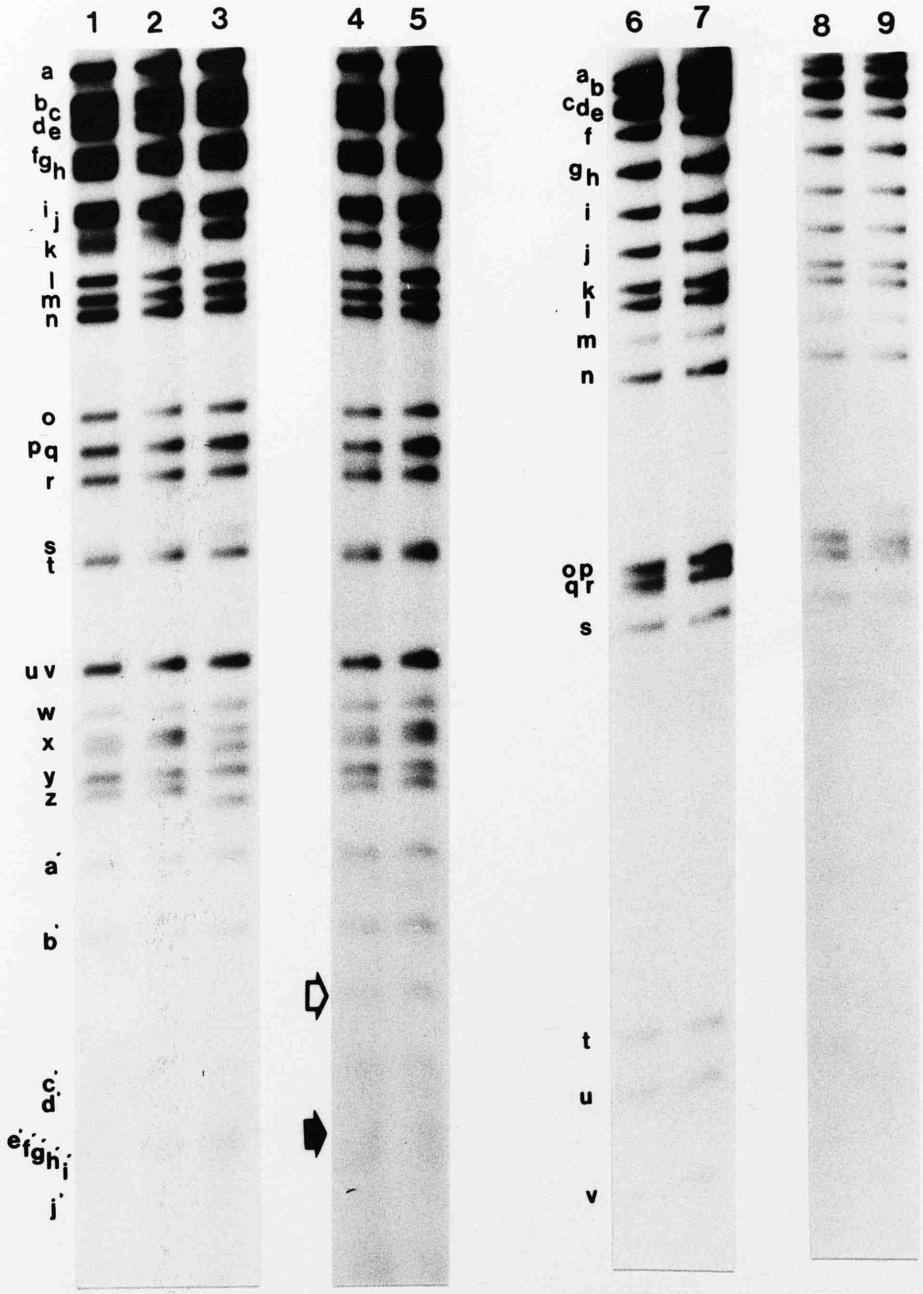

Figure 55. **The *Bam HI* And *Hpa I* Profiles Of HSV-1 DNA**

The *Bam HI* (lanes 1 to 5) and *Hpa I* (lanes 6 to 9) restriction endonuclease profiles of HSV-1 strain 17+ (lanes 1 & 6), 1721 (lanes 2 & 7), 1740 (lanes 3 & 8) and 1740*In* (lanes 4, 5 & 9) DNAs ^{32}-P-labelled *in vivo* (1.2% and 0.9% agarose gels respectively). The *wt Bam HI* bands are labelled on the left hand side of lane 1 whereas the *wt Hpa I* bands are labelled on the left hand side of lane 6. The missing *Bam HI f'* band (cannot be seen because of comigration with the *e'g'h'&i'* bands) is indicated with a filled arrowhead and the novel band with an open arrowhead.

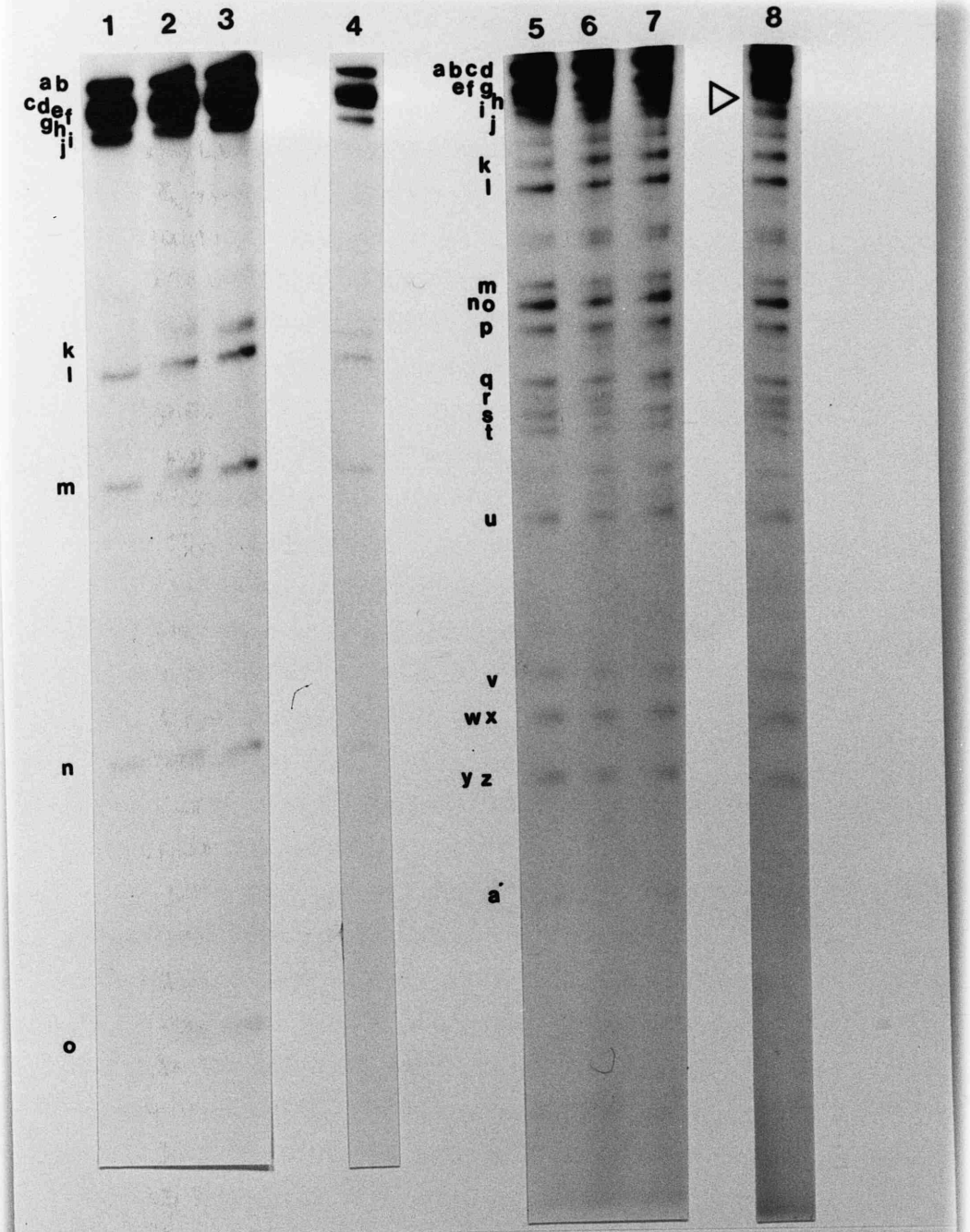

Figure 56. The *Eco RI* And *Kpn I* Restriction Endonuclease Profiles Of HSV-1 DNA

The *Eco R I* (lanes 1 to 4) and *Kpn I* (lanes 5 to 8) restriction endonuclease profiles of HSV-1 strain 17+ (lanes 1 & 5), 1721 (lanes 2 & 6), 1740 (lanes 3 & 7) and 1740In (lanes 4 & 8) DNAs ^{32}-P-labelled *in vivo*. The *wt* restriction endonuclease bands are labelled on the left hand side of lanes 1 and 5 respectively. The altered mobility of the *Kpn I i* band is indicated with an open triangle.

As discussed above, the DNA insert was located within the *Bgl II n* (n. 35729 to 41448) and *Bam HI f'* (n. 40778 to 41545) fragments, that is, between nucleotides 40778 and 41448. On the genome of HSV-1 strain 17+, the UL19 and UL20 ORFs are located between nucleotides 36406 & 40528 and 40822 & 41488 respectively (McGeoch *et al.*, 1988b). The UL19 gene encodes the major capsid protein, an essential component for capsid formation. The promoter region of the UL19 gene extends 30 to 80 bp into the 3' terminus of the leftward oriented UL20 ORF (Costa *et al.*, 1985). If the DNA insert in 1740*In* was located in between the UL19 and UL20 ORFs, it would have interrupted the UL19 promoter region. Therefore, it was apparent that the insert was located within the UL20 ORF, whose function and essential/nonessential nature were not known. To find out whether 1740*In* produces normal levels of the UL20 gene product, immunoprecipitations have been carried out by Dr C A MacLean (Figure 57). The 22K protein product of the UL20 gene was specifically precipitated from extracts of cells infected with HSV-1 strain 17+ (lane 10), 1708 (lane 16), 1739 (lane 14) and 1740*In* (lane 12). It can be seen that 1740*In* produced similar levels of the UL20 gene product compared to that of the parental 17+, 1708 and 1739 viruses, suggesting that the DNA insert in 1740*In* has not affected either the size or production of the 22K protein.

DNA sequence of the *Bam HI f'* fragment from the genome of 1740*In* was determined by Dr Amina Fu. The *Bam HI f'* fragment was eluted out, purified and cloned into the *Bam HI* site of the plasmid vector pGEM. The *Bam HI f'* fragment was cloned into M13mp19 and dideoxynucleotide sequencing of this fragment was carried out (data not shown; Dr Amina Fu, personal communication). DNA sequence of the *Bam HI f'* fragment was determined from both orientations and more than 400 bp sequence was revealed from both ends, i.e., from nucloetide numbers 40778 and 41545. However, because of the large size (approximately 1140 bp; see above) of the fragment, sequence from the middle region could not be determined. It was therefore necessary to find suitable restriction endonuclease subfragments of the *Bam HI f'* fragment to determine the sequence of the remaining region. However, sequence determination (as above) of more than 800 bp of the *f'* fragment revealed the presence of an additional *Hpa I* site. Therefore, the *Hpa I* site between the *Hpa I n/i* fragments (n. 41134) and the additional *Hpa I* site have generated a novel *Hpa I* fragment, which was cloned into M13mp19. The size of the novel *Hpa I* fragment was estimated to be around 350 bp. DNA sequence of the novel *Hpa I* fragment was determined (data not shown).

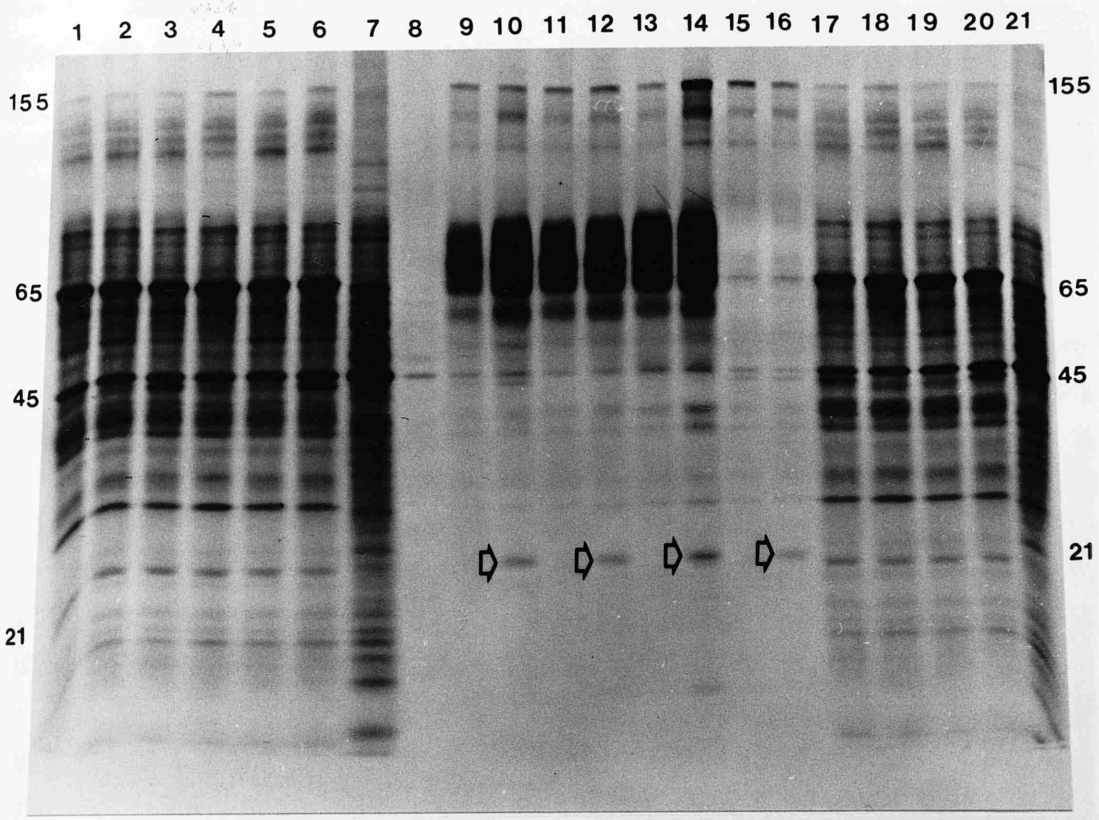

Figure 57. **Immunoprecipitation For The Variant 1740*In***

Immunoprecipitation of the UL20 gene product. Antiserum was raised against a synthetic oligopeptide, NH_2-Gln-Met-Leu-Pro-Pro-Thr-Asp-Pro-Leu-Arg-Thr-Arg-Tyr-COOH, representing amino acids 168 to 179 of the UL20 ORF with a carboxy-terminal tyrosine residue added to facilitate coupling to BSA (MacLean, C.A. *et al.*, 1991). Proteins from HSV-1 (strain 17^+)-infected (lanes 9 & 10), 1740*In*-infected (lanes 11 & 12), 1739-infected (lanes 13 & 14), 1708-infected (lanes 15 & 16) or Mock-infected (lane 8) cell extracts labelled with [^{35}S]methionine were precipitated using immune serum in the presence of 10 μg of the peptide against which the serum was raised (lanes 9, 11, 13 & 15) or an unrelated control peptide (lanes 8, 10, 12, 14 & 16), and analysed on 5 to 12.5% SDS-PAGE (see Methods : Section 2B). A band which appeared to be specifically precipitated is shown with an open arrowhead. Molecular weights x 10^3 are shown on both sides of the figure. HSV-1 (strain 17^+)-infected (lanes 6 & 20), 1740*In*-infected (lanes 1, 2, 5 & 19) 1739-infected (lanes 4 & 18), 1708-infected (lanes 3 & 17) and Mock-infected (lanes 7 & 21) cell extracts labelled with [^{35}S]methionine are also shown.

The *Hpa I* restriction endonuclease analysis of 1740*In* DNA showed no change in the mobility of the *Hpa I I* band (see above; Figure 55, lane 9). Because of the presence of an additional *Hpa I* site (as above), a novel *Hpa I* fragment was generated. The size of the novel *Hpa I* fragment was approximately equal to that of the DNA insert. This 350 bp *Hpa I* fragment would have run off the agarose gel and could not be observed in the *Hpa I* profile of 1740*In* DNA (Figure 55, lane 9).

Alignment of the determined DNA sequences (as above) and the HSV-1 strain 17^+ DNA sequence (McGeoch *et al.*, 1988b) revealed that the DNA insert in the insertion variant 1740*In* was a result of duplication of a 356 bp DNA sequence between nucleotides 40801 and 41157 (see Figure 58). This 356 bp DNA was inserted between nucleotides 40801 and 40802, 21 bp downstream of 3' end of the UL20 ORF. Because of duplication of these sequences, neither the UL20 ORF nor the UL19 promoter region was interrupted and therefore, no effect was observed on the production of the UL20 gene product (see above) or the viability and growth properties (see below) of the variant 1740*In*.

To determine the growth pattern of the variant 1740*In*, one-step growth experiments were carried out in BHK21/C13 cells at 37°C. The growth curves of HSV-1 strain 17^+, 1740 and 1740*In* are shown in Figure 59. It can be seen that the variant 1740*In* grew as well as the parental 1740 and strain 17^+ viruses. High titre (2.4×10^{10} pfu/ml) virus stocks of 1740*In* have been obtained. In addition, the plaque morphology of 1740*In* in BHK21/C13 cells was identical to that of the parental viruses (data not shown).

(d) **Isolation of HSV-1 DNA insertion variants**

Towards the end of the project, several HSV-1 (strain 17^+) variants containing large DNA inserts were isolated. The genomes of these insertion variants were analysed with the *Bgl II* (Figure 60), *Bgl II-Hind III*, *Bgl II-Xba I*, *Hind III*, *Hpa I*, *Kpn I*, *Xba I* (data not shown), *Eco RI* (Figure 61), *Bam HI* (Figure 62, lanes 1 to 4) and *Pvu II* (Figure 62, lanes 5 to 8) restriction endonucleases and compared with those of the parental viruses including the *wt* (strain 17^+) virus. The estimated sizes of DNA inserts varied from one variant to another ranging from approximately 3.9×10^6 Mr to more than 20.0×10^6 Mr (approximately 6.00 Kbp to >30 Kbp). However, the location of these DNA inserts within the genomes of the insertion variants could not be determined by RE analysis. Further analysis such as Southern blot hybridization experiments using DNA inserts as probes would be required to determine approximate locations of these inserts whereas cloning and

Figure 58. **Map Of DNA Insertion In 1740*In***

(a) The structure of HSV-1 strain 17^+ genome in the prototype orientation. The UL19, UL20 and UL21 genes are shown below the solid line (U_L). Arrowheads represent the relative orientation of the genes.

(b) The *Hind III* (above the line) map of 1740*In* (identical to that of 1739 to 1743) and *Bgl II* (below the line) map for the DNA of HSV-1 strain 17^+.

(c) An expanded section (*Bam HI* ***f'*** fragment) of U_L. The positions of the *Hpa I* site, UL20 ORF and the 356 bp DNA insert in 1740*In* are indicated. The nucleotide numbers are from the complete HSV-1 strain 17^+ DNA sequence as numbered by McGeoch et al.(1988b).

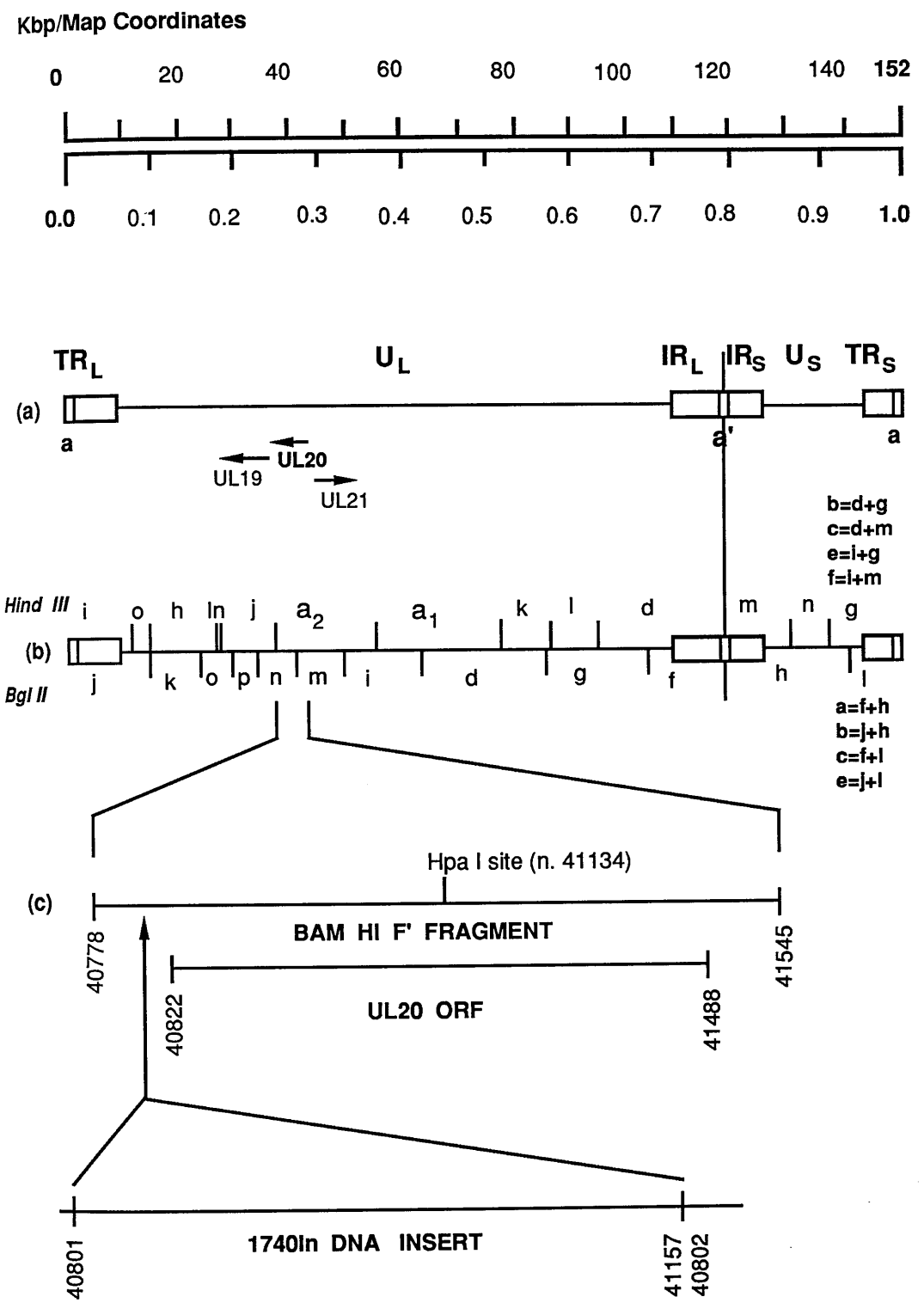

Figure 58. **Structure Of The Variant 1740*ln***

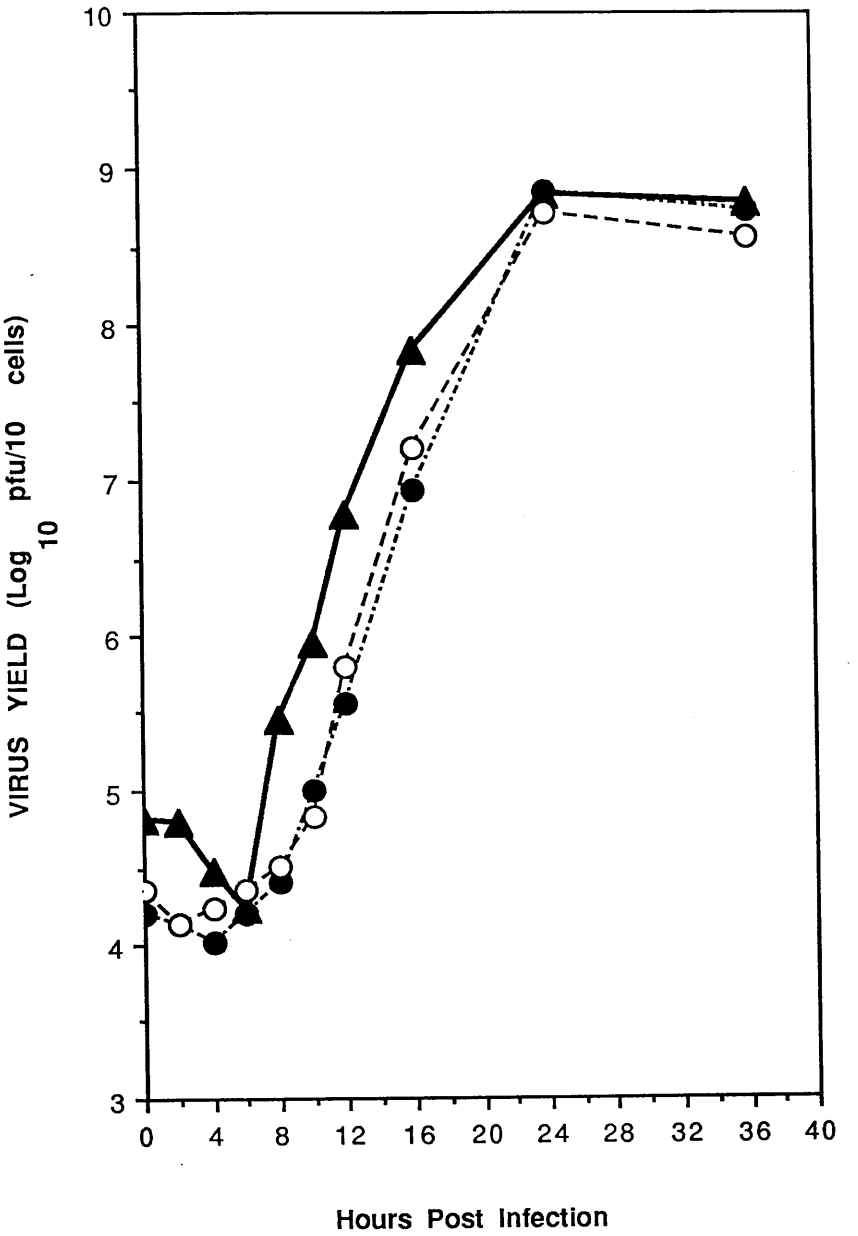

Figure 59. **Single-Cycle Growth Curves Of HSV-1 Strain 17+, 1740 And 1740*In***

One-step growth curves of HSV-1 strain 17+ (—▲—), 1740 (--●---) and 1740*In* (--○--). BHK21/C13 cells were infected at an *moi* of 5 pfu/cell, the cell monolayers were washed twice with PBS-calf, overlaid with ETC5 or ETC10 and incubated at 37°C. Cells were harvested at 0, 2, 4, 6, 8, 10, 12, 16, 24 and 36 h *pi* and the progeny virus titrated on BHK21/C13 cells (see Section 2B: Methods).

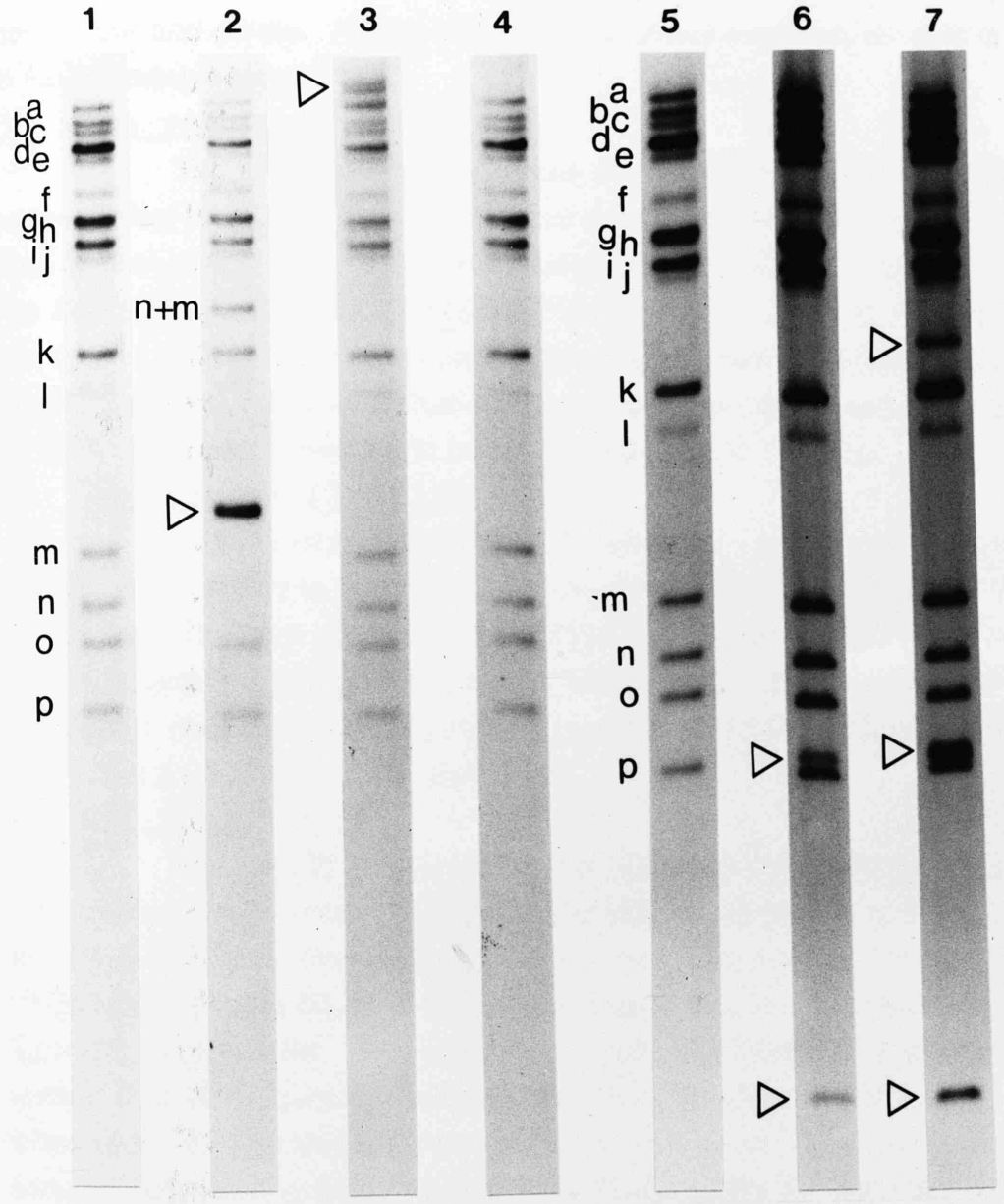

Figure 60. The *Bgl II* Profiles Of HSV-1 DNA

The *Bgl II* restriction endonuclease profiles of HSV-1 strain 17+ (lanes 1 & 5), 1738X7 (lane 2), 1743X138 (lane 3), R-582 (lane 4), 1721X23 (lane 6) and 1721X71 (lane 7) DNAs ^{32}-P-labelled *in vivo* (0.5% agarose gels). Letters refer to the *wt Bgl II* restriction endonuclease fragments. The novel bands suggesting the presence of DNA inserts are indicated with open triangles.

subsequent sequence determination would be essential to know their precise nature and end points. A brief description of these insertion variants is given in the following sections.

(i) **The variant 1738X7**

The restriction endonuclease profiles of the variant 1738X7 DNA were identical to that of 1738 in that it was devoid of the 0.272 m.c. *Bgl II* site, 7 *Hind III* sites plus the four normally occuring *Xba I* sites [see Section 3B:1 (c)]. The *Bgl II* (Figure 60, lane 2), *Eco RI* (Figure 61, lane 2), *Bam HI* (Figure 62, lane 2) and various other restriction endonuclease (data not shown) profiles of 1738X7 DNA have indicated that it contains a large DNA insert. The size of the DNA insert was estimated to be between 6400 and 6850 bp.

(ii) **The variant 1743X138**

The variant 1743X138 showed identical restriction endonuclease profiles to those of 1743 [see Section 3B:2 (b)]. The *Bgl II*, *Eco RI* and *Bam HI* DNA profiles of 1743X138 are shown in Figure 60, lane 3, Figure 61, lane 3 and Figure 62, lane 3 respectively. These and other (data not shown) restriction endonuclease profiles of 1743X138 indicated the presence of a >20.00 x 10^6 Mr DNA insert.

(iii) **The variant R-582**

The *Hind III*, *Bgl II-Hind III*, *Bgl II-Xba I* and *Xba I* (data not shown) DNA profiles of the variant R-582 indicated that it was devoid of the 0.08, 0.1 and 0.91 m.c. *Hind III* plus the 0.07 and 0.74 m.c. *Xba I* sites. The *Bgl II* profile of R-582 DNA (Figure 60, lane 4) was found to be identical to that of strain 17^+ (Figure 60, lane 1) DNA. However, the *Hind III*, *Bgl II-Hind III*, *Kpn I* (data not shown), *Eco RI* (Figure 61, lane 4) and *Bam HI* (Figure 62, lane 4) DNA profiles of R-582 revealed the presence of a DNA insert. The size of the DNA insert was estimated to be between 3.9 x 10^6 and 4.00 x 10^6 molecular weight.

(iv) **The variant 1721X23**

This variant demonstrated identical restriction endonuclease patterns to those of 1721 [data not shown; see Section 3B:1 (a) (i)]. The restriction endonuclease DNA profiles of 1721X23 including the *Bgl II* (Figure 60, lane 6), *Eco RI* (Figure 61, lane 6) and *Pvu II* (Figure 62, lane 6) indicated that the genome of 1721X23 contains a DNA insert of approximately more than 6.00 x 10^6 Mr.

(v) **The variant 1721X71**

The restriction endonuclease profiles of the variant 1721X71 DNA were identical to those of the variant 1721 (data not shown). These profiles were also identical to those of 1721X23 except that an extra band was

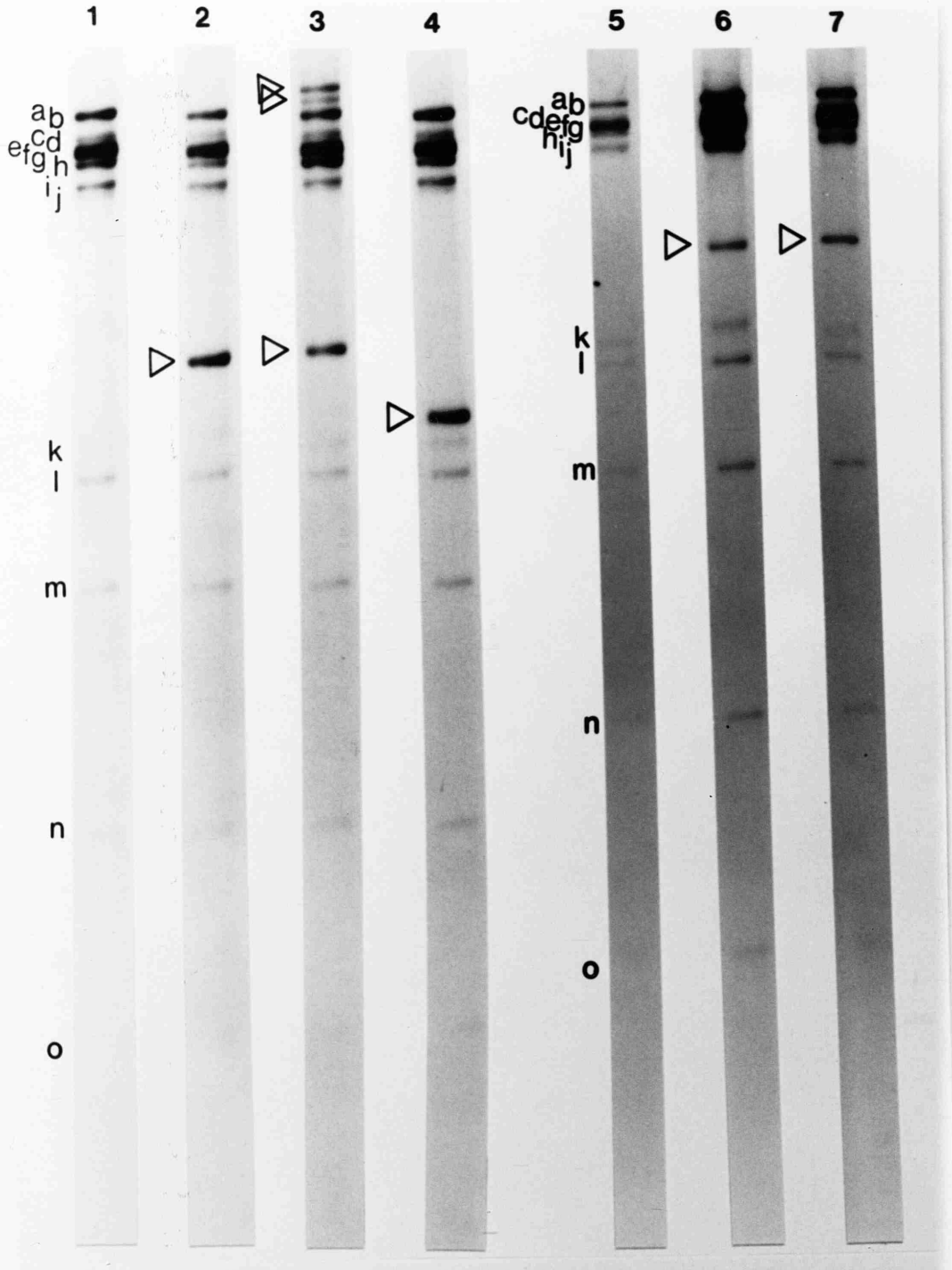

Figure 61. The *Eco RI* Profiles Of HSV-1 DNA

The *Eco RI* restriction endonuclease profiles of HSV-1 strain 17+ (lanes 1 & 5), 1738X7 (lane 2), 1743X138 (lane 3), R-582 (lane 4), 1721X23 (lane 6) and 1721X71 (lane 7) DNAs ^{32}-P-labelled *in vivo* (0.6% agarose gels). Letters refer to the *wt Eco RI* fragments. The novel bands suggesting the presence of DNA inserts are indicated with open triangles.

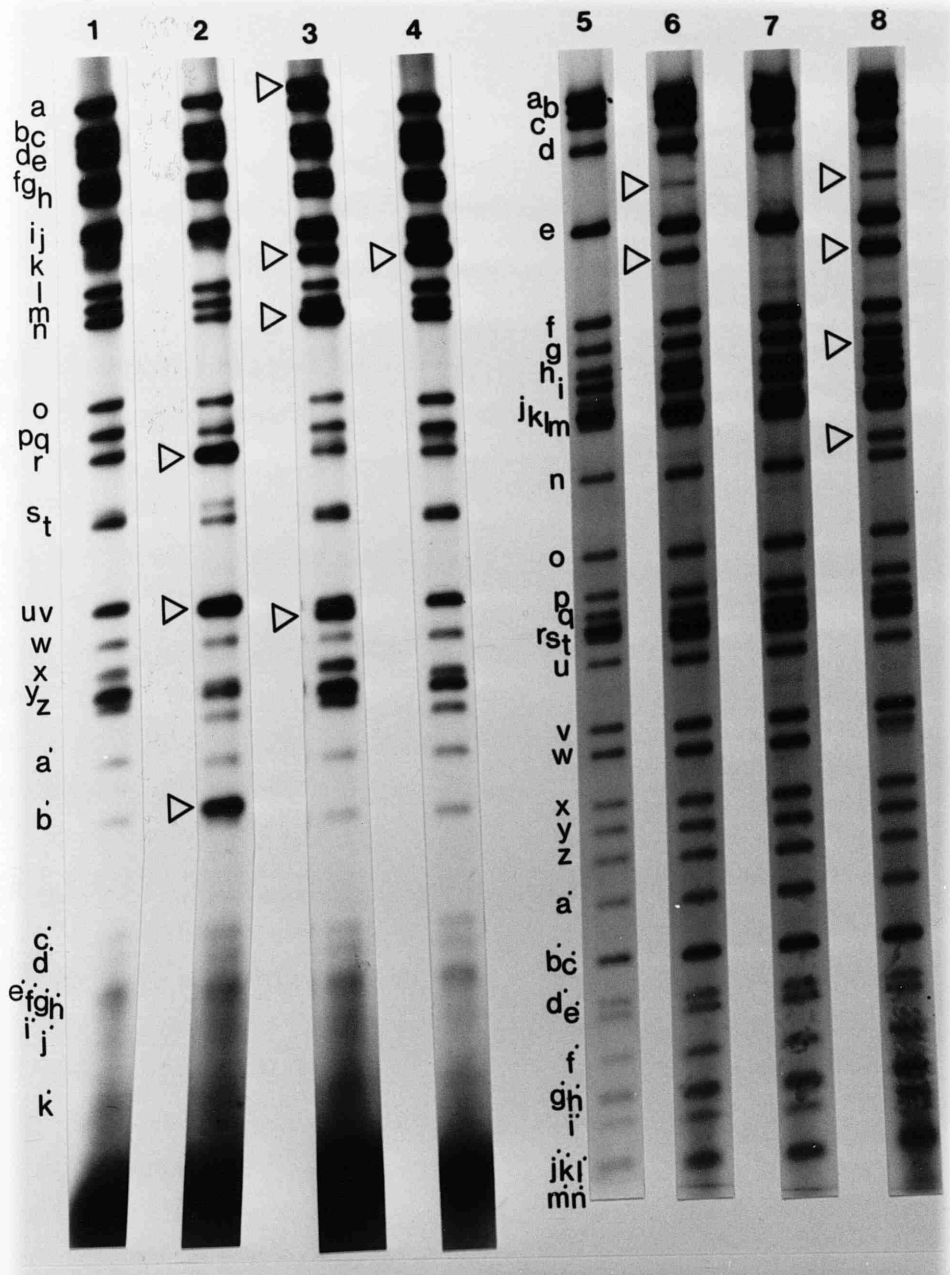

Figure 62. **The *Bam HI* And *Pvu II* Restriction Endonuclease Profiles Of HSV-1 DNA**

The *Bam HI* (lanes 1 to 4) and *Pvu II* (lanes 5 to 8) restriction endonuclease profiles of HSV-1 strain 17+ (lanes 1, 5 & 7), 1738X7 (lane 2), 1743X138 (lane 3), R-582 (lane 4), 1721X23 (lane 6) and 1721X71 (lane 8) DNAs ^{32}-P-labelled *in vivo* (1.2% agarose gels; Davison, 1981). Letters refer to the *wt* restriction endonuclease fragments. The novel bands are shown by open triangles.

observed (see Figures 60 & 61, lane 7; see also Figure 62, lane 8), suggesting that it contains a larger DNA insert. The size of the DNA insert was estimated to be around 16.00 x 10^6 Mr.

SECTION 3C : HSV-1 STRAIN 17± INTRASTRAIN RECOMBINATION STUDIES

With the aim of using the restriction endonuclease sites as multiple unselected markers in HSV-1 (strain 17^+) intrastrain recombination studies, HSV-1 genomes lacking the *Xba I* (Brown *et al.*, 1984; MacLean and Brown, 1987a), *Hind III* [see Section 3B:1 (a)] and *Bgl II* (see Section 3B:1 (b) & (c)] restriction endonuclease cleavage sites were generated. In addition, HSV-1 genomes containing an additional *Xba I* site (MacLean and Brown, 1987c) and an additional *Hind III* site [see Section 3B:2 (a)] were isolated. The variants containing additional *Xba I* and *Hind III* restriction endonuclease cleavage sites were then recombined to generate an HSV-1 genome containing both the additional *Xba I* and *Hind III* sites [see Section 3B:2 (b)] prior to carrying out the intrastrain recombination experiments (see below).

1. RECOMBINATION EXPERIMENTS

Non-selective recombination experiments were carried out in BHK21/C13 cells at 37°C (see Section 2B:16) using the variants 1738 and 1743 as the two parents. The variant 1743 was designated as parent 1 (P1) and contained 12 *Bgl II*, 11 *Hind III* and 5 *Xba I* restriction endonuclease cleavage sites [see Section 3B:2 (b)]. Similarly, the variant 1738 was designated as parent 2 (P2) and contained 11 *Bgl II*, 3 *Hind III* and no *Xba I* sites [see Section 3B:1 (c)]. The *Bgl II*, *Hind III* and *Xba I* maps of HSV-1 strain 17^+, 1743 and 1738 are shown in Figure 63. It can be seen that the variants 1743 and 1738 differ in 14 unselected markers (restriction endonuclease sites).

To determine the best parental input ratio (P1:P2) to give a 1:1 output ratio, preliminary recombination experiments involved infection of BHK21/C13 cell monolayers at a total *moi* of 10 *pfu*/cell at 1:1, 1:3 and 3:1 P1:P2 ratios followed by incubation at 37°C. At 24 h *pi*, cells were harvested, sonicated and the progeny titrated. The resultant progeny DNA from each parental ratio (input) was screened for the distribution of unselected markers. Twenty-four single, well-separated plaques were isolated from each parental

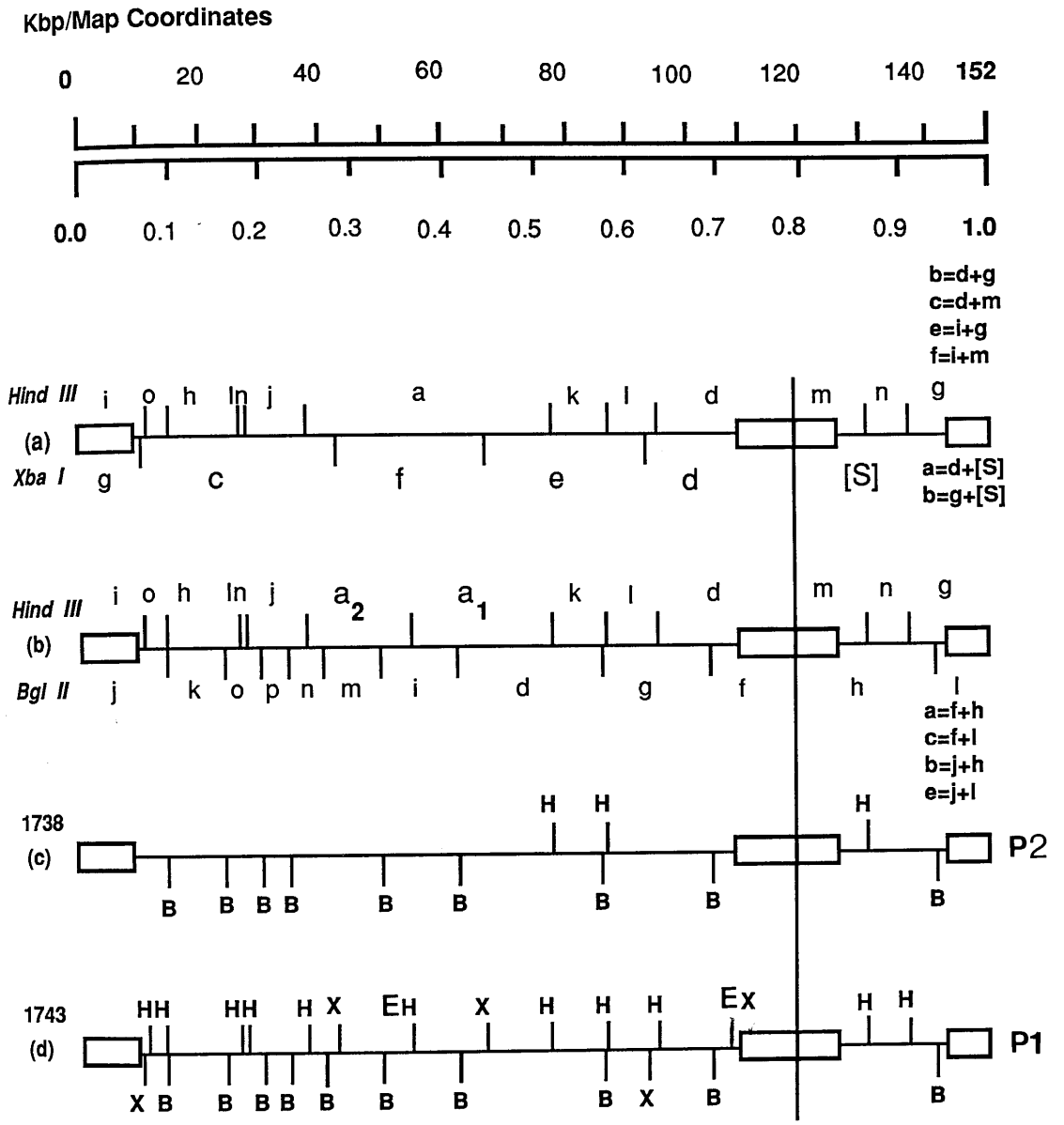

Figure 63. **The *Bgl II*, *Hind III* And *Xba I* Maps Of HSV-1 Strain 17+, 1738 And 1743**

(a) The *Hind III* (above the line) and *Xba I* (below the line) maps of HSV-1 strain 17+.

(b) The *Hind III* (above the line) map of 1739 and *Bgl II* (below the line) map of HSV-1 strain 17+.

(c) The *Bgl II*, *Hind III* and *Xba I* maps of the variant 1738.

(d) The *Bgl II*, *Hind III* and *Xba I* maps of the variant 1743.

B=*Bgl II*; H=*Hind III*; X=*Xba I*. ; E=Extra RE site.

cross, grown into virus stocks and their ^{32}-P-labelled DNAs [see Section 2B:4 (f)] were examined by *Bgl II-Hind III* and *Bgl II-Xba I* double digests. Each plaque isolate was scored for the presence or absence of each of the *Bgl II*, *Hind III* and *Xba I* restriction endonuclease site.

The 24 progeny from an input parental ratio of 1:1 P1:P2 contained 01 P1, 08 P2 and 15 recombinants. With an input parental ratio of 1:3 P1:P2, the progeny contained no P1, 18 P2 and 06 recombinants. The 3:1 P1:P2 parental ratio produced a 24 progeny consist of 11 P1, 03 P2 and 10 recombinants. On the basis of this preliminary data, the input ratio of 1:1 P1:P2 was selected to pick another 1008 single, well-separated progeny plaques. Linbro-well stocks were grown from these isolates and their ^{32}-P-labelled DNA prepared and analysed for the distribution of *Bgl II*, *Hind III* and *Xba I* sites.

2. ANALYSIS OF RECOMBINANTS

Because of the deleted *Hind III* or *Xba I* sites, larger (high molecular weight) *Hind III* or *Xba I* fused fragments were generated by the respective restriction endonuclease alone and it was therefore not possible to distinguish between the cleaved high molecular weight bands. Double digests by the *Bgl II-Hind III* as well as the *Bgl II-Xba I* restriction endonucleases were carried out to determine the presence or absence of a specific restriction endonuclease site within the genomes of the 1008 single plaque isolates screened. In addition, determination of the presence or absence of the 0.272 m.c. *Bgl II* site was achieved by using the same combination of restriction endonucleases. For determination of the presence or absence of specific restriction endonuclease sites, refer to the *Bgl II-Hind III* profiles of the variants 1721 to 1739 (Figures 23, 29, 34 & 36; see also Tables 6 & 8) and the *Bgl II-Xba I* profiles of 1740 to 1743 (Figure 40; Table 11).

As mentioned earlier, the total number of unselected markers was fourteen i.e. 1 *Bgl II*, 8 *Hind III* and 5 *Xba I* restriction endonuclease cleavage sites (see Figure 63). The *Bgl II* site at 0.272 m.c., the *Hind III* sites at 0.08, 0.1, two at 0.18, 0.26, 0.374, 0.64 and 0.91 m.c. and the *Xba I* sites at 0.07, 0.29, 0.45, 0.63 and 0.74 m.c. were present in P1 whereas the genome of P2 was devoid of these sites. These restriction endonuclease sites were scored as being present or absent within the genomes of the 1008 progeny plaques. If a genome structure was not found to be identical to either of the parents (using RE analysis; see above), it was considered a recombinant. The genome structures of the recombinants suggested that the two parental

molecules (or two progeny molecules at a later stage during the process of recombination) must have recombined at an arbitrary point between at least one pair of unselected markers. For instance, as a result of a single crossover between the 0.07 m.c. *Xba I* site and the 0.08 m.c. *Hind III* site, two different but reciprocal genome structures are possible. The first recombinant would have an identical genome structure to that of parent 1 except that the 0.07 m.c. *Xba I* site would be absent whereas the second recombinant would have a vice versa genome structure. Similarly, recombination between the 0.08 and 0.1 m.c. *Hind III* sites, 0.1 and 0.18 m.c. *Hind III* sites, 0.18 and 0.26 m.c. *Hind III* sites, 0.26 m.c. *Hind III* and 0.272 m.c. *Bgl II* sites, 0.272 m.c. *Bgl II* and 0.29 m.c. *Xba I* sites, 0.29 m.c. *Xba I* and 0.374 m.c. *Hind III* sites, 0.374 m.c. *Hind III* and 0.45 m.c. *Xba I* sites, 0.45 and 0.63 m.c. *Xba I* sites, 0.63 m.c. *Xba I* and 0.64 m.c. *Hind III* sites, 0.64 m.c. *Hind III* and 0.74 m.c. *Xba I* sites, and 0.74 m.c. *Xba I* and 0.91 m.c. *Hind III* sites was determined. It should, however, be noted that recombination, if any, between the two *Hind III* sites at 0.18 m.c. could not be determined because these sites are located very close to each other (52 bp apart) and the resultant 52 bp *Hind III* fragment cannot be observed on an ordinary agarose gel. Therefore, only twelve regions (as opposed to thirteen) were determined to have an odd number of crossovers between pairs of restriction endonuclease sites. Using this criterion for analysis of the plaque isolates, each of the 1008 progeny was scored as P1, P2 or a recombinant. The progeny contained 75 P1, 465 P2, 17 unscorable genomes (mixed genomes) and 451 recombinants. Thus, the overall recombination frequency was 44.74%

With fourteen scorable markers, 2^{14} or 16,384 scorable genome structures including the two parental structures are possible. However, as the two markers at 0.18 m.c. (*Hind III* sites; see above) were scored as one, the total number of possible scorable genome structures would reduce to 8,192 (4,095 reciprocals). For example, the genome structures 2 and 12 in Figure 64 show a single crossover between the 0.1 and 0.18 m.c. *Hind III* sites (region 3 in Figure 64). Similarly, genomes 6 and 18 represent a single crossover between the 0.45 and 0.63 m.c. *Xba I* sites (region 9; Figure 64). Similar structures can also be seen in genomes representing multiple crossovers between pairs of markers (Figures 65 to 69). These figures (Figures 64 to 69) were kindly prepared by Dr J E Harland.

Of 451 scorable recombinant genomes among the 1008 progeny, 109 had single crossovers, 175 showed double crossovers, 68 had triple crossovers, 66 four crossovers, 24 five crossovers, 08 six crossovers and

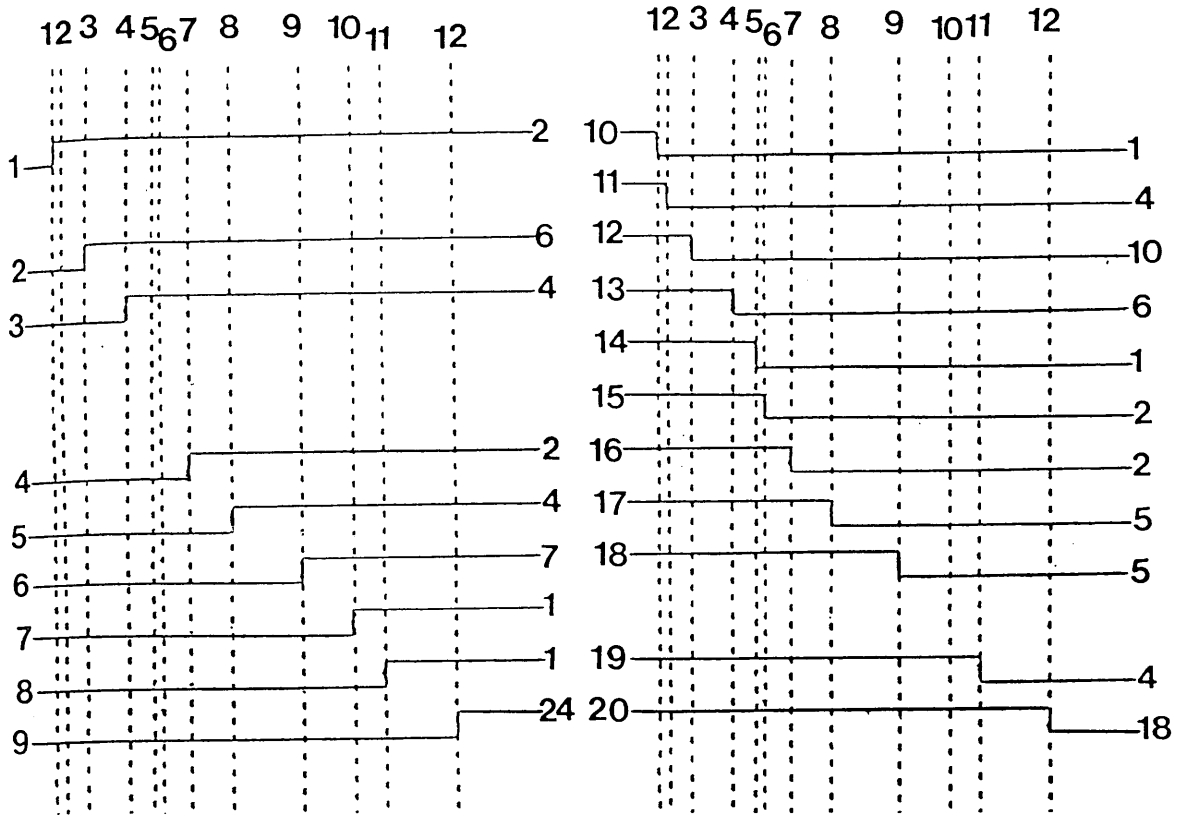

Figure 64. **Structures Of Recombinants With Single Crossovers**

Genome structures of the recombinants which have shown single crossovers at an arbitrary point between any one pair of non-selected markers (restriction endonuclease sites). There were twelve regions of recombination : between the 0.07 m.c. *Xba I* and the 0.08 m.c. *Hind III* sites (region 1); between the 0.08 and 0.1 m.c. *Hind III* sites (region 2); between the 0.1 and 0.18 m.c. *Hind III* sites (region 3); between the 0.18 and 0.26 m.c. *Hind III* sites (region 4); between the 0.26 m.c. *Hind III* and the 0.27 m.c. *Bgl II* sites (region 5); between the 0.27 m.c. *Bgl II* and the 0.29 m.c. *Xba I* sites (region 6); between the 0.29 m.c. *Xba I* and the 0.37 m.c. *Hind III* sites (region 7); between the 0.37 m.c. *Hind III* and the 0.45 m.c. *Xba I* sites (region 8); between the 0.45 and 0.63 m.c. *Xba I* sites (region 9); between the 0.63 m.c. *Xba I* and the 0.64 m.c. *Hind III* sites (region 10); between the 0.64 m.c. *Hind III* and the 0.74 m.c. *Xba I* sites (region 11); and between the 0.74 m.c. *Xba I* and the 0.91 m.c. *Hind III* sites (region 12). The dotted lines represent the arbitrary point of recombination within these regions. Within a single genome structure, the upper horizontal lines represent regions from the variant 1743 (P1) whereas the lower lines from the variant 1738 (P2). The short vertical lines joining the horizontal lines represent an arbitrary point where genomes have crossed over. Figures on the left are serial numbers of the genome structures whereas figures on the right represent actual number of genome structures isolated during this study.

Figure 65. **Genome Structures Of Recombinants With Double Crossovers**

The genome structures of recombinants with two crossovers are shown in a serial order. Within a single genome structure, the upper horizontal lines represent regions from the variant 1743 (P1) whereas the lower lines from the variant 1738 (P2). The short vertical lines joining the horizontal lines represent an arbitrary point where genomes had crossed over. The dotted lines are the arbitrary point of recombination in the following twelve regions : between the 0.07 m.c. *Xba I* and the 0.08 m.c. *Hind III* sites (region 1); between the 0.08 and 0.1 m.c. *Hind III* sites (region 2); between the 0.1 and 0.18 m.c. *Hind III* sites (region 3); between the 0.18 and 0.26 m.c. *Hind III* sites (region 4); between the 0.26 m.c. *Hind III* and the 0.27 m.c. *Bgl II* sites (region 5); between the 0.27 m.c. *Bgl II* and the 0.29 m.c. *Xba I* sites (region 6); between the 0.29 m.c. *Xba I* and the 0.37 m.c. *Hind III* sites (region 7); between the 0.37 m.c. *Hind III* and the 0.45 m.c. *Xba I* sites (region 8); between the 0.45 and 0.63 m.c. *Xba I* sites (region 9); between the 0.63 m.c. *Xba I* and the 0.64 m.c. *Hind III* sites (region 10); between the 0.64 m.c. *Hind III* and the 0.74 m.c. *Xba I* sites (region 11); and between the 0.74 m.c. *Xba I* and the 0.91 m.c. *Hind III* sites (region 12). The recombinant structure 24 was not represented; the recombinant structures 2, 3, 5, 8, 9, 10, 17, 18, 20, 21, 26, 35, 36, 37, 38, 39, 40, 42, 46, 47, 51, 54, 56 & 59 were represented only once; the recombinant structures 1, 14, 15, 16, 30, 34, 44, 45, 48, 49, 50 & 60 were represented twice; the recombinant structures 7, 13, 25, 41, 43, 53 & 55 were represented three times; the recombinant structures 4, 11, 12, 22, 23, 28, 52 & 57 were represented four times; the recombinant structures 6, 19 & 29 were represented five times; the recombinant structure 58 was represented nine times; the recombinant structure 32 was represented ten times; the recombinant structures 27 & 31 were represented thirteen times; and the recombinant structure 33 was represented fourteen times within the 1008 progeny.

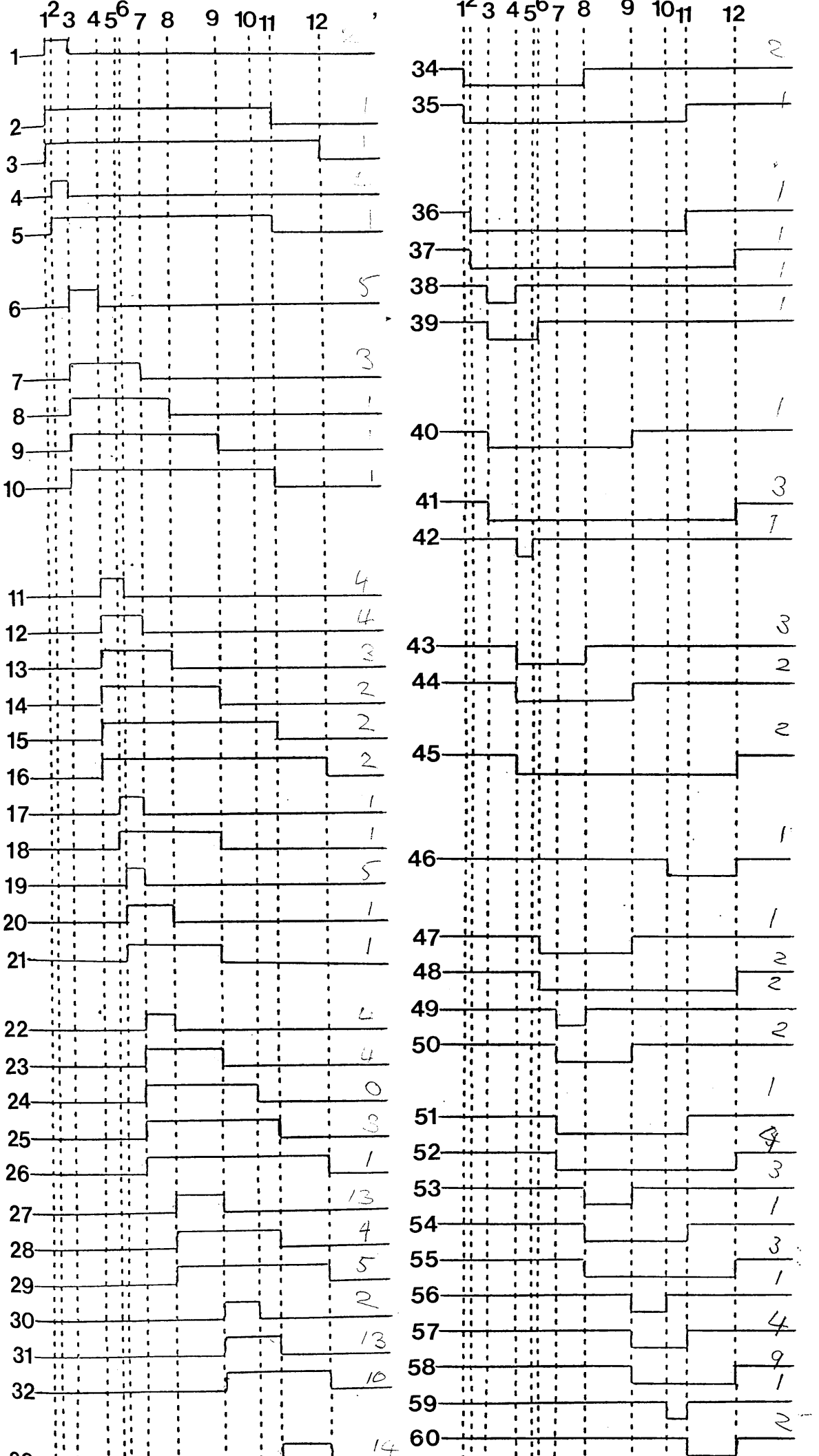

FIGURE 65

01 seven crossovers (see Table 12). No recombinant showing more than 7 crossovers was identified. This data showed a decrease in the number of recombinants with an increase in the number of detectable crossovers. However, the number of recombinants with double crossovers is an exception, indicating a possibility that the recombinants with two crossovers might be the most common forms generated during the recombination process.

Of the 109 recombinants showing a single crossover, 20 different genome structures were obtained (see Figure 64). Similarly, the 175 recombinants with double crossovers gave 59 genome structures (Figure 65); the 68 recombinants with triple crossovers gave 56 genome structures (Figure 66); the 66 recombinants with four crossovers gave 54 genome structures (Figure 67); the 24 recombinants having five crossovers gave 24 genome structures (Figure 68); the 8 recombinants with six crossovers gave 7 genome structures and one genome structure for the recombinant which had seven crossovers (Figure 69), giving a total of 221 (or 49% of the total number of recombinants) different genome structures (see Table 13). To obtain all the 8,192 different recombinant genome structures (see above) was beyond the scope of this study.

Of the 221 recombinant genome structures found, the most common structures were the reciprocal genomes 9 and 20 (shown in Figure 64; 4.17% of the total progeny). These recombinants had a single crossover between the 0.74 m.c. *Xba I* and 0.91 m.c. *Hind III* sites, i.e., between the long (L) and short (S) components of the genome. Similarly, the most abundant crossover in recombinants with multiple crossovers was found to be between the L and S components of the HSV-1 genome (see Figures 65 to 69). The most abundant recombinant structures containing crossovers within the long (L) component of the genome were 2 & 12 (shown in Figure 64; 1.59% of the total progeny) and 31 & 57 (shown in Figure 65; 1.69% of the total progeny). The recombinants 2 & 12 (Figure 64) had a single crossover between the 0.1 and 0.18 m.c. *Hind III* sites. On the other hand, recombinants 31 & 57 (Figure 65) had two crossovers : between the 0.45 & 0.63 m.c. *Xba I* sites and 0.64 m.c. *Hind III* & 0.74 m.c. *Xba I* sites respectively.

Table 14 summarizes the number of recombinants between pairs of markers and their percentage in the total progeny. It can be seen that there is an average of 20.14% recombination between any one pair of markers ranging from 5.76% to 41.46% . The average percentage of recombination

TABLE 12

A NUMBER OF CROSS-OVERS	B NUMBER OF RECOMBINANTS	C % OF TOTAL RECOMBINANTS[1]
1	109	24.17
2	175	38.80
3	068	15.08
4	066	14.63
5	024	05.32
6	008	01.77
7	001	00.22
> 8	000	00.00
	451	99.99%

< 0.01% had > 7 cross-overs

Table 12. **Distribution Of Recombinant Progeny In Relation To The Number Of Crossovers**

1. (Number of recombinants/total number of recombinants) x 100.

Figure 66. **Genome Structures Of The Recombinants With Three Crossovers**

The genome structures of recombinants which had three crossovers are diagrammatically represented in a serial order. The twelve regions of recombination are shown with vertical dotted lines (see Figures 64 & 65 for detailed description). The genome structures 16, 34, 36, 47 & 54 were not represented; the genome structures 12, 48 & 61 were represented twice; the genome structures 18, 33 & 58 were represented three times; the genome structure 59 was represented four times (indicated on the right hand side of these structures); whereas the remaining structures were represented only once (not indicated in the figure) among the 451 recombinants isolated during this study.

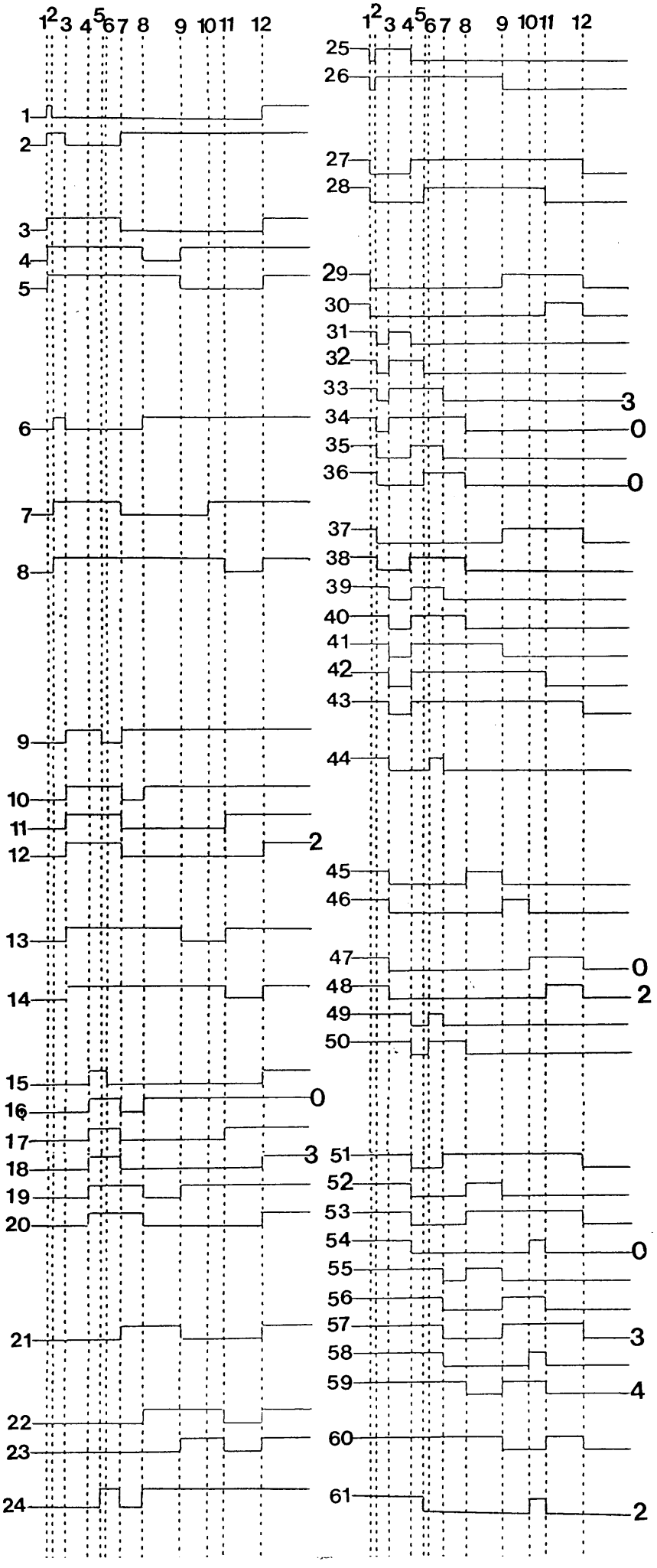

Figure 66. **Genome Structures With Three Crossovers**

Figure 67. **Structures Of The Recombinants With Four Crossovers**

The genome structures of recombinants with four crossovers are shown in a serial order. Structures 11, 13, 18, 21, 25, 40, 45 & 56 were not represented among the recombinants. The genome structures 19, 22, 32, 35, 48, 51, 52 & 59 were represented twice, the genome structures 12 & 50 were represented three times and the remaining 44 genome structures were represented only once among the 1008 progeny. The figures at the top and the dotted lines below them represent the twelve regions of recombination (see Figures 63, 64 & 65).

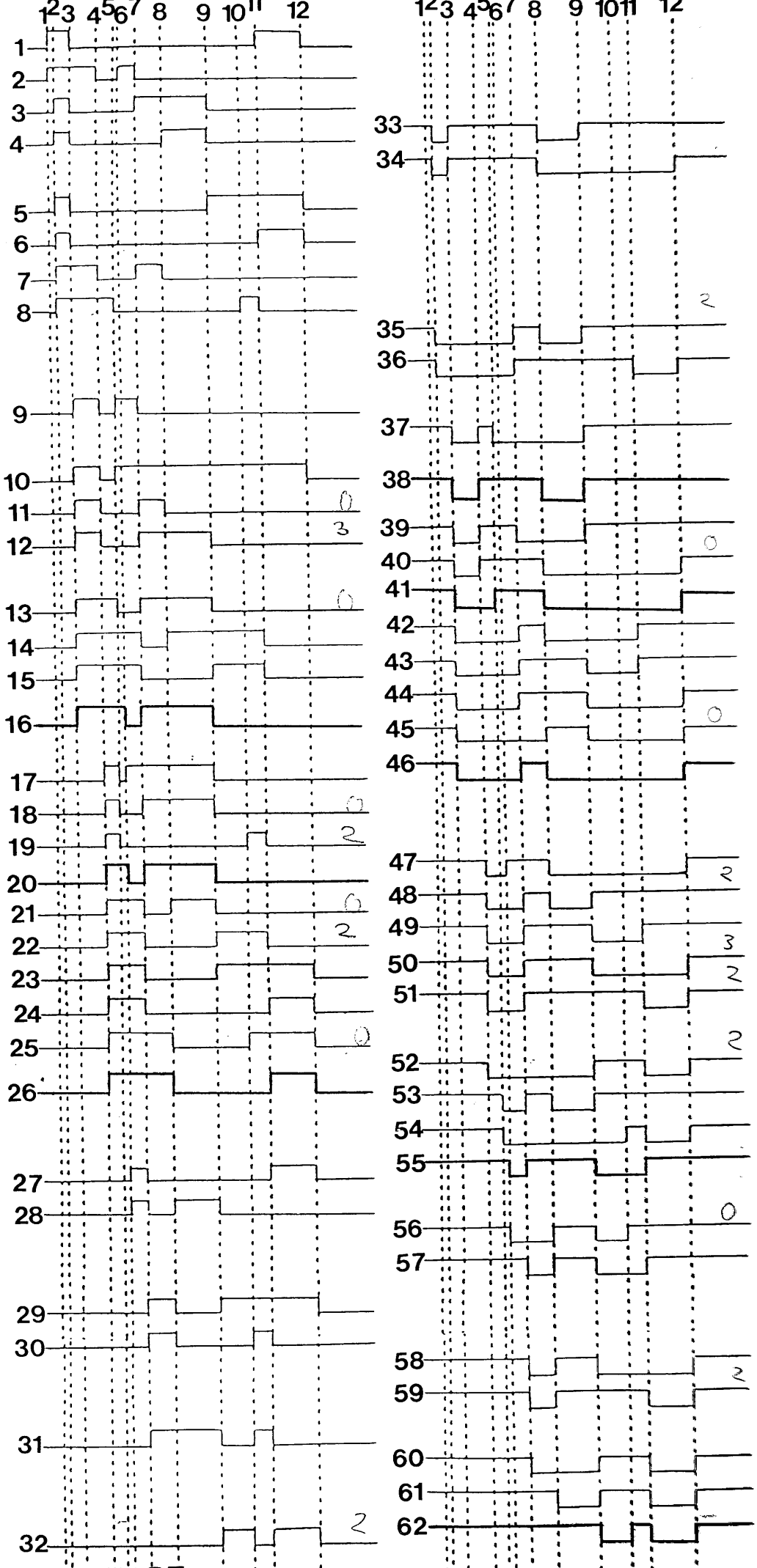
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FIGURE 67

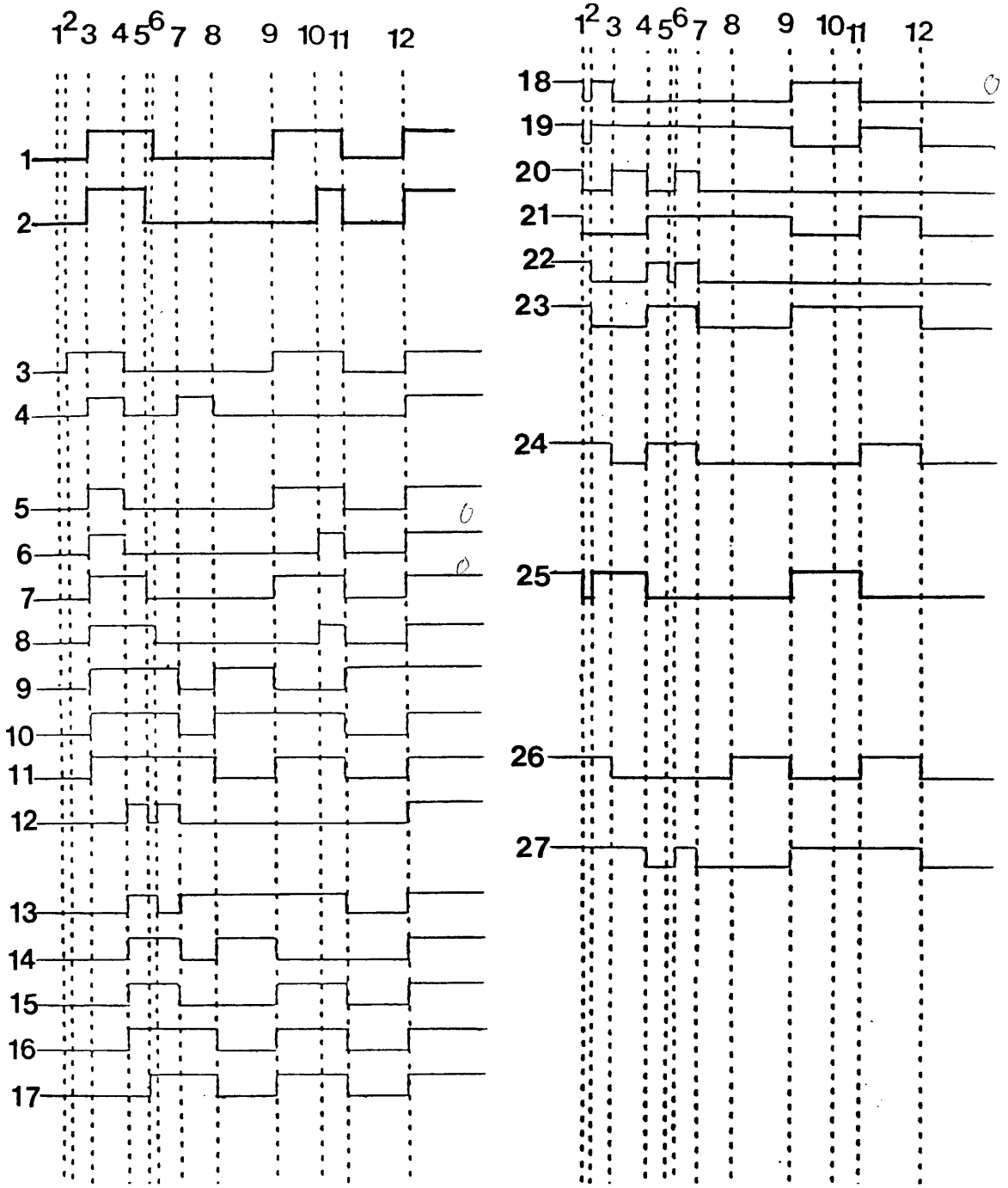

Figure 68. **Structures Of Recombinants With Five Crossovers**

The genome structures of recombinants with five crossovers are illustrated in a serial order. The recombinant structures 6, 7 & 18 were not represented among the 451 recombinants isolated during this study. Only one genome structure of each of the remaining recombinants was isolated. See legends of Figures 64 & 65 for description regarding the regions of recombination. See Figure 63 for the parental viruses used in these experiments.

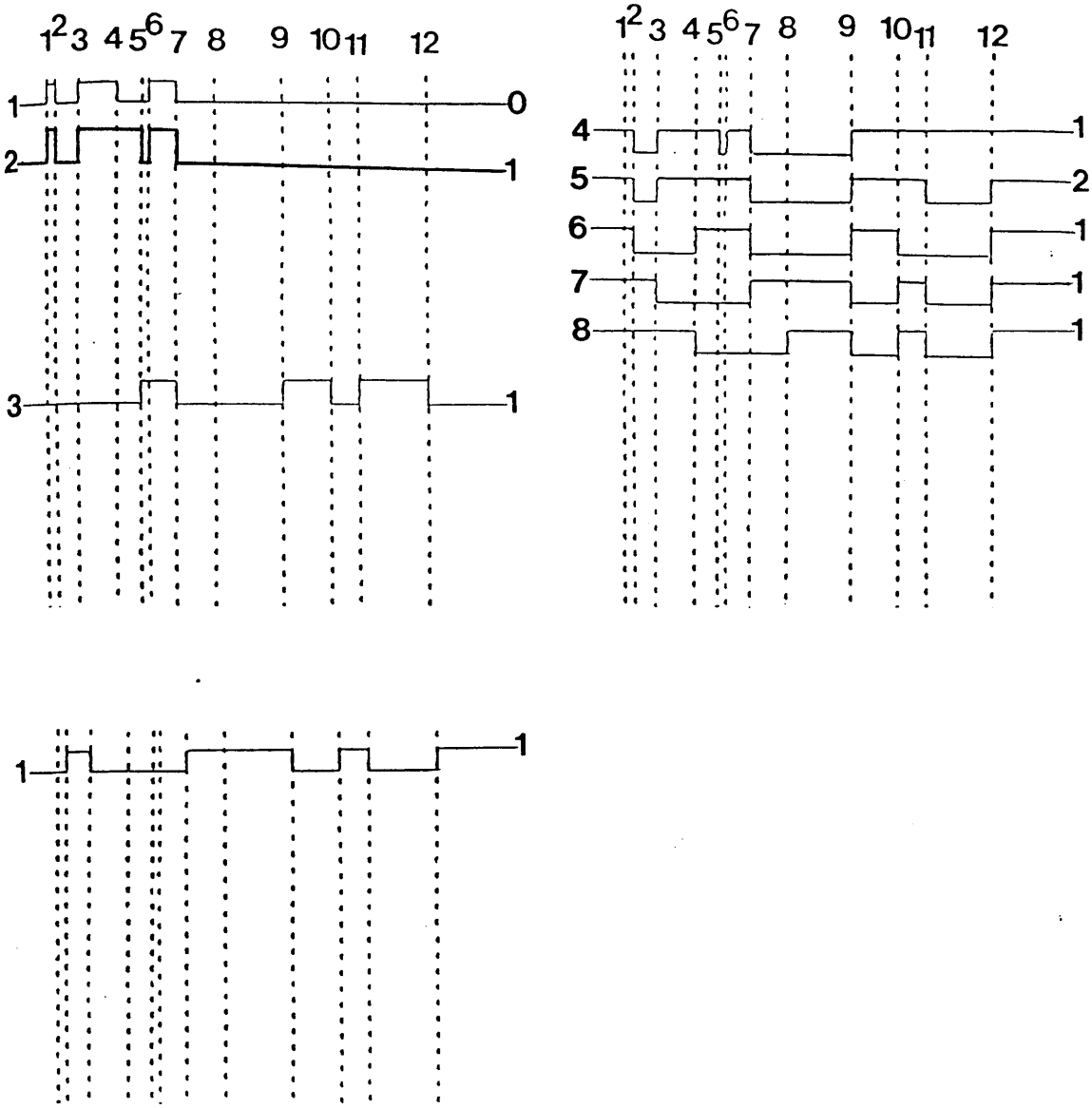

Figure 69. **Genome Structures Of Recombinants With Six And Seven Crossovers**

Structures of recombinants whose genomes had crossed over six or seven times at an arbitrary point between the unselected markers. Figures at the top represent regions of recombination between the thirteen markers (see Figure 63). The dotted lines show an arbitrary point of recombination within these regions. For detailed description, see legends of Figures 64 and 65.

TABLE 13

A	B	C	D
NUMBER OF CROSS-OVERS	NUMBER OF RECOMBINANTS	NUMBER OF DIFFERENT GENOME STRUCTURES	TOTAL NUMBER OF POSSIBLE GENOME STRUCTURES[1]
0	000	00	001
1	109	20	012
2	175	59	066
3	068	56	220
4	066	54	495
5	024	24	792
6	008	07	924
7	001	01	792
8	000	00	495
9	000	00	220
10	000	00	066
11	000	00	012
12	000	00	001
TOTAL :	451	221	4096
		RECIPROCALS	4096
		TOTAL	8192

Table 13. **Distribution Of Genome Structures In Relation To The Number Of Crossovers**

1. $\frac{(12-n)!}{n!}$ where, !=factorial

TABLE 14

A	B	C
CROSS-OVER[1]	NUMBER OF RECOMBINANTS	% RECOMBINANTS (B*/451 X 100)
01 x 02	026	05.76
02 x 03	047	10.42
03 x 04	102	22.61
04 x 06	111	24.61
06 x 07	029	06.43
07 x 08	037	08.20
08 x 09	125	27.71
09 x 10	106	23.50
10 x 11	163	36.14
11 x 12	029	06.43
12 x 13	128	28.38
13 x 14	187	41.46

Table 14. **Frequency Of Recombinant Progeny Between Pairs Of Unselected Markers**

1. Unselected pairs of markers *i.e.* restriction endonuclease cleavage sites :

   01=0.07 m.c. *Xba I* site
   02=0.08 m.c. *Hind III* site
   03=0.1 m.c. *Hind III* site
   04 & 05=0.18 m.c. *Hind III* sites
   06=0.26 m.c. *Hind III* site
   07=0.272 m.c. *Bgl II* site
   08=0.29 m.c. *Xba I* site
   09=0.374 m.c. Hind III site
   10=0.45 m.c. *Xba I* site
   11=0.63 m.c. Xba I site
   12=0.64 m.c. *Hind III* site
   13=0.74 m.c. *Xba I* site
   14=0.91 m.c. *Hind III* site

*. B = number of recombinants.

between pairs of markers within the L component is 18.19 with a range from 5.76 to 36.14. As there are thirteen markers (twelve scorable markers) within the L component of the genome and only one within the S component, it was not possible to compare the percentage of recombinants between markers within the S component to those of L component. However, it can be seen that the percentage of recombinants between the 0.74 m.c. *Xba I* site (marker 13; L component) and 0.91 m.c. *Hind III* site (marker 14; S component) is the highest (18.55%) among any pair of markers. In order to understand why the percentage of recombinants varied markedly between different pairs of markers, it was scored as a function of genome length (Table 15). It can be seen that there is a gradual decrease in the percentage of recombinants with increase in distance between the markers i.e. when the genome length (GL) is 1%, the average recombination frequency (RF) is 2.77%; 2% of GL gave an RF of 2.08%; the distance of 8% GL gives an RF of 1.37%; when the GL is 10%, the RF is 1.26%; when the GL is 18%, the RF is 0.89%; and, when the genome length is increased to 50%, the RF decreased to 0.37%. The average recombination frequency of 1.70% with a range from 0.37% to 2.87% (Table 15) reflects the same range of variation between pairs of markers. Furthermore, the recombination frequency between markers 13 (L component) and 14 (S component) is the lowest (0.37%) compared to that of every other pair of markers, indicating a marked difference in recombination frequency because of 50% of the genome length. The pairs of markers within the long (L) component of the genome have shown a range from 0.89% to 2.87% recombination per unit length, giving an average of 1.83%. As expected, the lowest recombination frequency of 0.89% is between the markers whose distance apart is 18% of the genome length whereas when the distance between a pair of markers is 1% of the genome length, the recombination frequency ranges from 2.57% to 2.87% (see Table 15).

Because of inversion of the L or S components of the HSV genome during the formation of four possible isomers (see Section 1B:2), the distance between any marker in L and any marker in S is 50% of the genome length (see Brown *et al.*, 1992). Therefore, the recombination frequency between every marker within the L component and the marker in S component was scored to find out the role, if any, of the four isomeric forms of the genome in the recombination process. Table 16 summarize the number and percentage of recombinants between such pairs of markers along with the recombination frequency in relation to the genome length. It can be seen that the values representing the percentage of recombinants are very similar for all

TABLE 15

A	B	C	D
CROSS-OVER[1]	%GENOME LENGTH[2]	%RECOMBINANTS[3]	%RECOMBINANTS/ GENOME LENGTH
0.07-0.08	1	02.57	2.57
0.08-0.10	2	04.66	2.33
0.10-0.18	8	10.11	1.26
0.18-0.26	8	11.00	1.37
0.26-0.27	1	02.87	2.87
0.27-0.29	2	03.67	1.83
0.29-0.37	8	12.40	1.55
0.37-0.45	8	10.50	1.30
0.45-0.63	18	16.17	0.89
0.63-0.64	1	02.87	2.87
0.64-0.74	10	12.69	1.26
0.74-0.91	50(17)	18.55	0.37

Table 15. **Frequency Of Recombinant Progeny In Relation To Genome Length**

1. Restriction endonuclease sites at map coordinates (m.c.)
2. Distance between the markers (RE sites) as a function of the total genome length
3. (Number of recombinants/total number of progeny) x 100.

TABLE 16

A	B	C	D
CROSS-OVER[1]	NUMBER OF RECOMBINANTS[2]	%RECOMBINANTS[3] (B/451 X 100)	%RECOMBINANTS/ MAP UNIT [4]
01 x 14	202	44.78	0.89
02 x 14	194	43.00	0.86
03 x 14	203	45.00	0.90
04 x 14	189	41.90	0.83
06 x 14	201	44.56	0.89
07 x 14	199	44.12	0.88
08 x 14	214	47.45	0.94
09 x 14	206	45.67	0.91
10 x 14	210	46.56	0.93
11 x 14	203	45.00	0.90
12 x 14	191	42.35	0.85
13 x 14	197	43.68	0.87

Table 16. **Frequency Of Recombination In Relation To 50% Of The Genome Length**

1. Unselected pairs of markers (RE sites). See Table 12
2. Number of recombinants with a crossover between the markers in column A.
3. (Number of recombinants (B)/total number of recombinants) x 100
4. %Recombinants/50% of the genome length

TABLE 17

SITE POSITION[1] POSITIVE IN RECOMBINANTS	NUMBER OF RECOMBINANTS	% IN TOTAL RECOMBINANTS[2]
0.07	201	44.56
0.08	203	45.00
0.10	198	43.90
0.18	190	42.12
0.26	202	44.78
0.27	199	44.12
0.29	203	45.00
0.37	171	37.90
0.45	173	38.30
0.63	185	41.00
0.64	186	41.20
0.74	165	36.58
0.91	186	41.20

Table 17. **Distribution Of Recombinant Progeny In Relation To The Restriction Endonuclease Sites (Non-Selected Markers)**

1. The *Bgl II* (0.27), *Hind III* (0.08, 0.1, 0.18, 0.26, 0.37, 0.64 & 0.91) and *Xba I* (0.07, 0.29, 0.45, 0.63 & 0.74) restriction endonuclease cleavage sites at map coordinates (m.c.)
2. (Number of recombinants/total number of recombinants) x 100.

pairs of markers with an average of 44.51% ranging from 41.90% to 47.45%. Similarly, the average recombination frequency in relation to the 50% genome length is 0.88 per map unit with a range from 0.83 to 0.94 per map unit.

The recombinants were also scored for the presence or absence of a specific restriction endonuclease site in order to see whether any particular marker is prominently represented among them. It can be seen from Table 17 that the number of recombinants representing the various unselected markers (RE sites) are very similar with an average of 189 per marker. Similarly, the percentage of these recombinants per marker varies from 36.58% to 45% with an average of 41.97%. Therefore every marker is almost equally represented among the recombinants and none of the markers had any advantage over the others during the process of recombination.

CHAPTER 4:

DISCUSSION

DISCUSSION

The experiments described in this thesis were designed to investigate the factors involved in the process of herpes simplex virus recombination. Restriction endonuclease cleavage sites were chosen as unselected markers to study the phenomenon in terms of: the possibility of existence of certain "hot spots" of recombination; the identification of genes which might be involved directly or indirectly in the process; the contribution of parental and progeny molecules; the contribution of the four HSV isomers in recombination; the role of cellular factors during the recombination process; and the role of genomic alignment in recombination. Therefore, Brown *et al.* (1984) and subsequently MacLean and Brown (1987a) have generated an HSV-1 strain 17^+ variant, 1702, devoid of the four normally occuring *Xba I* sites at 0.07, 0.29, 0.45 and 0.63 map coordinates (m.c.). This variant (1702) was used as the parental virus to delete the more frequently occuring *Hind III* restriction endonuclease cleavage sites. Out of the 10 *Hind III* sites located at 0.08, 0.1, two at 0.18, 0.26, 0.52, 0.58, 0.64, 0.88 and 0.91 m.c. on the *wild-type* HSV-1 genome, seven (the *Hind III* sites at 0.08, 0.1, 0.18, 0.26, 0.64 & 0.91 m.c.) were deleted during the course of the project described herein. In addition, an HSV-1 virus lacking the 0.272 m.c. *Bgl II* site with no other detectable deletion and/or insertion, termed 1734, was spontaneously isolated. The deleted 0.272 m.c. *Bgl II* site in 1734 was then introduced into the genome of 1733 (lacking the 7 *Hind III* sites) to construct the variant 1738. Furthermore, another virus (1739) containing an additional *Hind III* site around 0.374 m.c. was spontaneously isolated. This additional *Hind III* site was introduced into the genome of the variant 1708 (MacLean and Brown, 1987c) to construct the virus 1743. Thus, the virus 1738 containing no *Xba I*, 3 *Hind III* and 11 *Bgl II* restriction endonuclease cleavage sites differed markedly from the virus 1743 containing 5 *Xba I*, 11 *Hind III* and 12 *Bgl II* sites. These two viruses (1738 and 1743) were used in intrastrain recombination experiments.

During the process of isolating the HSV-1 genomes devoid of the *Hind III/Bgl II* sites or generating the genomes containing additional restriction endonuclease sites, several genomes with DNA deletions/insertions were isolated. Only preliminary characterization of these viruses was carried out. The isolation of restriction endonuclease site-deletion/insertion variants is discussed in Section 4A. The recombination experiments using the variants

1743 and 1738 as parental viruses are considered in Section 4B. Section 4C discusses briefly the isolation of DNA deletion/insertion variants whereas future prospects regarding the work described herein are given in Section 4D.

SECTION 4A : ISOLATION OF HSV-1 RESTRICTION ENDONUCLEASE CLEAVAGE SITE DELETION/INSERTION VARIANTS

Following the suggestion that an essential part of HSV recombination analysis should be a study of the segregation of unselected markers among recombinant progeny rather than measurements of frequencies of recombination between selected markers (Honess *et al.*, 1980), Brown *et al.* (1984) decided to use restriction endonuclease (RE) cleavage sites as unselected markers in HSV recombination studies. In order to obtain HSV-1 (strain 17^+) as well as HSV-2 (strain HG52) genomes lacking RE sites, an enrichment selection technique was used since the complete sequence of strain 17^+ or HG52 was unavailable at the start of this project. The selection enrichment technique was first described by Jones and Shenk (1978), who used the restriction endonuclease *Eco RI* to isolate a series of deletion and substitution mutants of adenovirus type 5. The procedure involves cleavage of viral DNA with a restriction endonuclease, rejoining the resulting fragments by incubating with DNA ligase and transfecting the modified DNA onto cell monolayers. However, the ligation step was omitted from the procedure by Brown *et al.* (1984) because no increase in the isolation frequency of deletion mutants of HSV was observed by including it. The use of an excess of a particular restriction endonuclease to digest viral DNA makes it possible to enrich the naturally occuring virus genomes within a population of molecules which lack that particular RE site . The virus genomes lacking one or more sites of a particular restriction endonuclease would have a greater survival probability compared to the *wild-type* virus after subjecting their DNA to digestion by the same restriction enzyme. In addition, such viral genomes would have a better chance of establishing infection when the digested DNA molecules are transfected onto cell monolayers. Using this criterion, HSV-1 (Brown *et al.*, 1984; MacLean and Brown, 1987a) and HSV-2 (Harland and Brown, 1988) genomes lacking *Xba I* sites were isolated.

Although three out of four *Xba I* sites from HSV-1 strain 17^+ (Brown *et al.*, 1984; MacLean and Brown, 1987a) and the four normally

occuring *Xba I* sites from HSV-2 strain HG52 (Harland and Brown, 1988) were deleted by enrichment selection, the isolation frequency of the various *Xba I* site-deletion variants differed considerably. The 0.07 and 0.63 m.c. *Xba I* sites of HSV-1 were removed easily with an isolation frequency of 3.6% (Brown *et al.*, 1984) while the 0.45 m.c. *Xba I* site was deleted after several unsuccessful attempts and seven rounds of serial selection enrichment (MacLean, 1988). However, the frequency of isolation of the 0.45 m.c. *Xba I* site-deletion variant (1701) after the seventh round of serial selection enrichment was 7.14% (the overall frequency was approximately 0.1%). On the other hand, the 0.29 m.c. *Xba I* site could not be deleted despite the analysis of over 2,000 plaque isolates (MacLean, 1988). Similarly, the *Xba I* sites from HSV-2 strain HG52 were deleted with variable isolation frequencies i.e. the 0.45 m.c. *Xba I* site was lost at a frequency of 2.0%; the 0.7 m.c. *Xba I* site was deleted at a frequency of 1.0%; the 0.91 m.c. *Xba I* site-deletion was obtained with an isolation frequency of 2.5%; and, the 0.94 m.c. *Xba I* site-deletion variants were isolated with a frequency of 20.0% (Harland, 1990). This data suggests that the isolation of restriction endonuclease site-deletion variants by selection enrichment technique depends upon the location of RE sites within the coding/non-coding sequences of the viral genome. In addition, the essential/non-essential nature of such coding sequences is also of crucial importance in this kind of variant isolation. For example, the 0.07 and 0.63 m.c. *Xba I* sites in HSV-1 strain 17+ are located within the UL2 and UL44 ORFs respectively. UL2 encodes the uracil-DNA glycosylase (Worrad and Caradonna, 1988) whereas UL44 encodes the glycoprotein C (Frink *et al.*, 1983; McGeoch *et al.*, 1988b). Both UL2 (Mullaney *et al.*, 1989) and UL44 (see reviews by Marsden, 1987; McGeoch, 1989) are non-essential in tissue culture. In contrast, the 0.29 and 0.45 m.c. *Xba I* sites are located within the essential ORFs UL22 (encodes gH; Gomples and Minson, 1986; McGeoch and Davison, 1986) and UL33 (whose gene product is required for capsid assembly; Al-Kobaisi *et al.*, 1991) and therefore required more *in vitro* manipulations to be deleted (MacLean, 1988). The 0.29 m.c. *Xba I* site was in fact deleted through a conservative single-base change using site-directed mutagenesis (MacLean and Brown, 1987a). Like the 0.07 and 0.63 m.c. *Xba I* site-deletion variants of HSV-1, the HSV-2 strain HG52 variants lacking *Xba I* sites were also isolated with relative ease indicating that possibly all four *Xba I* sites are located within the non-essential/non-coding sequences of the HSV-2 genome (Harland, 1990). The 0.91 m.c. *Xba I* site is located near the 3'-terminus of US4 within the coding region of the non-essential glycoprotein

gG2 (McGeoch *et al.*, 1985; Harland and Brown, 1988) whereas the 0.94 m.c. *Xba I* site is known to lie in an intergenic (non-coding) region (Whitton, 1984; Harland, 1990). However, the precise locations of the 0.45 and 0.7 m.c. *Xba I* sites are not known because of non-availability of the complete DNA sequence of HSV-2 strain HG52.

Apart from the difficulties encountered in obtaining the restriction endonuclease site-deletion variants by selection enrichment because of the presence of these sites within the essential coding regions of the HSV-1 genome (e.g. the *Xba I* site at 0.45 m.c.; see above), there are several other disadvantages of using this technique : (1) The changes leading to the RE site loss would frequently be non-conservative i.e. unlike the conservative single-base change which could be engineered by using site-directed mutagenesis (see later). (2) These restriction endonuclease site-specific alterations could arise as a result of small undetectable (using restriction endonuclease analysis) deletions and/or insertions which may impair the growth characteristics of the RE site-deletion variants at a later stage (although it was not observed in any of the *Xba I* site-deletion variants described above; MacLean, 1988; Harland, 1990; own observation). However, the alteration responsible for the 0.45 m.c. *Xba I* site in HSV-2 strain HG52 variants HG52X163X3X27 and HG52X163X2X53 may have also caused the temperature sensitivity of these variants (Harland, 1990). In addition, the RE site loss may be associated with sizeable deletions or insertions which would subsequently affect homology in intrastrain recombination studies. (3) The selection enrichment technique can only be used with restriction endonucleases whose total number of sites within the viral genome is limited e.g. 3 to 5. If a restriction endonuclease cleaves the viral genome more frequently, the chances of survival of viable genomes after DNA digestion are reduced to less than 1% (own observation). For instance, the *Hind III* restriction endonuclease makes 10 cuts in strain 17$^+$ DNA. When the variant 1721 lacking the 0.91 m.c. *Hind III* site was cleaved with *Hind III* for 1 hour at 37°C (as opposed to the normal 4 h digestion) and the digested DNA was transfected onto BHK21/C13 cell monolayers, no *cpe* was observed even at 5 or 7 days post infection. In addition, no *Hind III* site-deletion variant was isolated from the 300 plaques obtained and examined as a result of 15 min and 30 min digestion of 1721 DNA with *Hind III* and subsequent transfection (data not shown).

In the present study, the HSV-1 strain 17$^+$ variant 1702 (devoid of *Xba I* sites) was used as the parental virus to delete the *Hind III* restriction

endonuclease sites. As mentioned above, the 0.07, 0.45 and 0.63 m.c. *Xba I* sites were deleted by selection enrichment and the remaining *Xba I* site at 0.29 m.c. was deleted by site-directed mutagenesis. As the 0.63 m.c. *Xba I* site is located within the UL44 gene (encodes gC; see above) and the site loss was associated with a deletion of approximately 150 bp (Brown *et al.*, 1984), the *Xba I* site-deletion variants X2, 1701 and 1702 synthesize a truncated form of gC (MacLean, 1988). Moreover, the variants 1701 and 1702 are also *tk*$^-$ and it was speculated that the *tk*$^-$ phenotype was induced spontaneously during the isolation of 1701 lacking the 0.29 m.c. *Xba I* site beside those at 0.07 and 0.63 m.c. (MacLean, 1988). Therefore, all the restriction endonuclease site-deletion variants described herein are *tk*$^-$ and produce a truncated gC (data not shown).

The HSV-1 strain 17$^+$ genome (as well as the 1702 genome) contains 10 *Hind III* sites at 0.08, 0.1, two at 0.18, 0.26, 0.52, 0.58, 0.64, 0.88 and 0.91 m.c. Site-directed mutagenesis was used to delete the 0.08, 0.1, 0.18, 0.26, 0.64 and 0.91 m.c. *Hind III* sites (see Sections 3A:2 and 3B:1). Oligonucleotides spanning these *Hind III* sites with a conservative single base alteration (see Table 3) were synthesized and used in site-directed mutagenesis. The conservative change was produced within the *Hind III* recognition sequence (AAGCTT) in such a way that the third base (3' end) of the codon was replaced without affecting its coding potential. For example, the 18-mer oligonucleotide 5'-GGT CAT ***AAG CTA*** CAG CGC-3' (No. 11 in Table 3) was used to delete the *Hind III* site at 0.91 m.c. The third base (T) of the second codon (CTT) within the *Hind III* recognition sequence (indicated with bold & italicised nucleotides in the above cited sequence) was mutated (A) without altering the DNA sequence. As the 0.91 m.c. *Hind III* site is located within the promoter region of the essential glycoprotein D (gD; US6; Ligas and Johnson, 1988; McGeoch *et al.*, 1988b), disruption of its DNA sequence would have produced a non-viable virus in tissue culture [as was observed with *Lac Z* insertion mutant; see Section 3B:1 (a) (i)]. Thus, the above alteration had no effect on the synthesis of gD. Similar alterations were used to destroy the remaining (six in this study) *Hind III* sites (Table 3). The location of various *Hind III* sites on the HSV-1 strain 17$^+$ genome is given in Table 4. Reversion of the mutated sequence during DNA replication is possible albeit at a very low frequency. However, it can be avoided by introducing two conservative mutations instead of one. Furthermore, such a reversion was not observed during the course of the present study in which the *Hind III* site-deletion variants were used to isolate

further *Hind III* site-deletion mutants during which over 5,000 plaques were examined (see Section 3B:1).

The 0.91 m.c. *Hind III* site was deleted from the plasmid containing the HSV-1 *Bam HI j* fragment with relative ease using the site-directed mutagenesis procedure described by Oostra *et al.* (1983) and Liang *et al.* (1986). However, attempts to delete the remaining *Hind III* sites by the same procedure were unsuccessful despite the analysis of a total of 10,000 bacterial colonies (Section 3A:2), indicating that the efficiency of this method is lower than 0.1%. In contrast, the method described by Kunkel (1985) was found to be an efficient one and six *Hind III* sites were deleted using this method. Although the mutant isolation frequencies of greater than 50% (as claimed) were not obtained, the method gave an overall frequency of 2%. One of the possible reasons of such a big difference in mutant isolation frequencies could be the use of large (3 to 5 Kb) genomic fragments herein as a frequency of more than 25% was obtained by using smaller fragments (< 500 bp; Dr H Weir, personal communication). The pTZ range of vectors could be conveniently used for introducing site-specific mutations into any DNA sequence and were found very useful during the present study.

The plasmid lacking the 0.91 m.c. *Hind III* site was used in marker rescue experiments with 1702 and a desired variant (1721) was isolated from the 96 plaques analysed. The variant 1721 was further analysed with various other restriction endonucleases (beside *Hind III*) and contained no detectable deletion or insertion (less than 150 bp). This variant was then used in further marker rescue experiments to isolate the variants 1723 (lacking the 0.08 and 0.91 m.c. *Hind III* sites), 1724 (lacking the 0.1 and 0.91 m.c. *Hind III* sites) and 1725 (lacking the 0.64 and 0.91 m.c. *Hind III* sites). The variants 1723 and 1724 were recombined to isolate a variant (1727) lacking the *Hind III* sites at 0.08, 0.1 and 0.91 m.c. Similarly, 1727 was recombined with 1725 and the variant 1728 lacking the 0.08, 0.1, 0.64 and 0.91 m.c. *Hind III* sites was isolated (see detailed description in Section 3B:1).

Attempts to marker rescue the plasmids lacking the 0.18 and 0.26 m.c. *Hind III* sites with 1721 or 1727 were unsuccessful despite the analysis of over 3,400 plaques [see Sections 3B:1 (a) (iv) & (vi)]. Therefore, these plasmids were marker rescued into the 1702 genome and the variants 1722 (lacking the 0.26 m.c. *Hind III* site) and 1726 (lacking the two *Hind III* sites at 0.18 m.c.) were isolated. Recombination experiments between the variants 1725 and 1726 were carried out to generate a variant 1729 lacking

* The variant 1732 was actually isolated during a separate recombination experiment involving the variants 1722, 1726 and 1728 [see Section 3B : 1 (a) (vii); see also Figure 26.5].

the 0.18, 0.64 and 0.91 m.c. *Hind III* sites. Parallel recombination experiments between 1729 and 1722 as well as 1729 and 1728 have generated the variants 1730 lacking the *Hind III* sites at 0.18, 0.26, 0.64 & 0.91 m.c. and 1731 lacking the 0.08, 0.1, 0.18, 0.64 & 0.91 m.c. *Hind III* sites. Finally, the variants 1730 and 1731 were recombined to isolate the variants 1732* (lacking the 0.08, 0.1, 0.18, 0.26 and 0.91 m.c. *Hind III* sites) and 1733 devoid of the 0.08, 0.1, 0.18, 0.26, 0.64 and 0.91 m.c. *Hind III* sites. As the variants 1721 to 1733 were derived from 1702, these were also devoid of the four normally occuring *Xba I* sites at 0.07, 0.29, 0.45 and 0.63 m.c. Each of the above mentioned variant was further analysed with the commonly used restriction endonucleases (e.g. *Bam HI, Bgl II, Eco RI, Hpa I, Kpn I, Xba I,* etc.) and showed no other detectable deletion/insertion.

The infected cell polypeptide profiles of 1721 and 1733 were found identical to that of 1702 i.e. they are *tk*$^-$ and synthesize a truncated form of gC (data not shown). The growth characteristics of these viruses over both one-step and multiple-step (data not shown) experiments were similar to those of strain 17$^+$ and 1702. In addition, high titre virus stocks of 1721 to 1733 were obtained (Table 7).

A variant, 1734, lacking the 0.272 m.c. *Bgl II* site was fortuitously isolated. The genome structure of 1734 was identical to that of 1721 except that the 0.272 m.c. *Bgl II* site was missing. Restriction endonuclease analysis, hybridization experiments and immunoprecipitation experiments have shown no other alteration such as DNA deletions or insertions [Section 3B:1 (b)]. Therefore, it was assumed that the alteration causing the *Bgl II* site loss was semi-conservative. To increase the number of unselected markers (RE sites), the deleted *Bgl II* site was introduced into the genome of 1733. As a result of several recombination experiments [described in Section 3B:1 (c)], a variant (1738) lacking the 0.272 m.c. *Bgl II*, seven *Hind III* and four *Xba I* sites was generated.

The variants 1734 and 1738 showed similar growth characteristics to those of the parental viruses and high titre virus stocks were obtained for them. Moreover, the infected cell polypeptide profile of 1738 was essentially similar to those of 1702, 1721 and 1733.

Like 1734, another variant (1739) containing an additional *Hind III* site at 0.374 m.c. was spontaneously isolated (Section 3B:2) showing no other detectable (less than 150 bp) deletion or insertion. The variant 1739 was also devoid of the *Xba I* sites and was *tk*$^-$ (like the parental 1702 and 1721 viruses). A variant 1708 containing an additional *Xba I* site at 0.74 m.c.

has previously been isolated and characterized (MacLean and Brown, 1987c). In order to have an increased number of non-selected markers in intrastrain recombination studies, it was decided to introduce the additional *Hind III* site into the genome of 1708. Therefore, recombination experiments involving these two viruses were carried out and the variants 1740 to 1743 were generated [see Section 3B:2 (b)]. The variant 1743 contained 11 *Hind III* and 5 *Xba I* sites.

The general polypeptide profiles of 1739 and 1743 were found to be identical to those of 1702, 1721, 1733 and 1738. The growth properties of 1739 and 1743 were also very similar to those of the parental viruses. High titre virus stocks were obtained for the variants 1739 to 1743.

Thus, an HSV-1 strain 17$^+$ variant lacking 1 *Bgl II*, 7 *Hind III* and 4 *Xba I* sites as well as a variant containing the normally occuring *Bgl II* (12), 11 *Hind III* and 5 *Xba I* sites have now been obtained for use in intrastrain recombination experiments.

Apart from their use in recombination studies (Brown *et al.*, 1992; this thesis), the RE site-deletion variants have also been used in superinfection experiments rescuing viral DNA from latently infected cultures (Cook and Brown, 1987). In addition, such variants are useful in the development of eukaryotic vector systems which would allow the expression of foreign genes to high levels by creating unique restriction endonuclease sites into which appropriate DNA fragments could be inserted. The unique RE sites can easily be introduced within the intergenic regions which would not disrupt the functions of the neighbouring genes. Such a vector system has recently been developed and proved useful (Rixon and McLauchlan, 1990).

SECTION 4B : INTRASTRAIN RECOMBINATION STUDIES

Although recombination in HSV has long been established (Wildy, 1955; Subak-Sharpe, 1969) and both intratypic and intertypic (Timbury and Subak-Sharpe, 1973) recombination have been demonstrated, the molecular events leading to such exchange of genetic information including the functions of certain genes or sequences responsible for this are, as yet, undefined. Recombination studies using temperature-sensitive (*ts*) markers have led to the construction of linear genetic maps for both HSV-1 and HSV-2 (Brown *et al.*, 1973; Schaffer *et al.*, 1974; Timbury and Calder, 1976). However, correlation between order and distances of markers on the physical and genetic maps of *ts* mutations in HSV-1 revealed inconsistencies although

those of HSV-2 were found in good agreement (Stow and Wilkie, 1978; Stow *et al.*, 1978; Wilkie *et al.*, 1978, 1979; see Section 1F:3). Honess *et al.* (1980), using selected and unselected markers (present in both L and S components of the HSV-1 genome) such as *ts*, plaque morphology, TK phenotype, PAA resistance and sensitivity and polypeptide mobilities observed discrepancies between the linear physical and genetic maps and, therefore, suggested a circular genetic map. Because of such differences among the published genetic data, it was suggested that unselected markers should be included in recombination analysis to study the phenomenon more closely (Honess *et al.*, 1980). In addition, the use of a large number of such markers in both L and S components may reveal a clear linear or circular solution for linkage maps.

The successful use of restriction endonuclease (RE) sites as nonselected markers along with selected *ts* markers in the process of adenovirus recombination has been demonstrated (Young and Silverstein, 1980). Brown *et al.* (1984) suggested the use of unselected RE site markers in HSV recombination studies and as a first step towards such a study, reported the construction of *Xba I* site deletion variants. Umene (1985), following this suggestion, used two strains of HSV-1 differing in eight restriction endonuclease sites, thereby demonstrating the practical use of such unselected markers in HSV recombination. Although unselected markers were used by both Honess *et al.* (1980) and Umene (1985), they have also used two strains of HSV-1 as the parental input viruses. The presence of nonhomologous sequences between two strains of HSV-1 may influence the rate of recombination similar to that observed in recombination assays using viral or bacterial DNA being introduced into cultured mammalian cells (Roth and Wilson, 1985; Brouillette and Chartrand, 1987), conflicting observations have also been made (see Section 1F) indicating that nonhomology can also impose certain limitations on the recombining molecules. In addition, the possibility of growth advantage of one strain over the other will be diminished by using only one strain of HSV. Therefore, intrastrain recombination studies using identical HSV genomes apart from the alterations leading to the destruction of RE sites have been proposed (Brown *et al.*, 1984). The use of *Xba I* sites as unselected markers in intrastrain HSV-1 (strain 17+) as well as HSV-2 (strain HG52) recombination studies has recently been demonstrated (Brown *et al.*, 1992). However, since the total number of unselected markers in both HSV-1 and HSV-2 were small (five *Xba I* sites in each case), the

presence of any possible recombinational "hot spots" could not be determined. In addition, because of long distances between the markers used, the recombinogenicity of smaller regions of the HSV genomes could not be explored. Thus, implying that more frequent markers are required to further investigate the various factors involved in the process of HSV recombination.

As mentioned earlier, two HSV-1 (strain 17+) genomes (1738 and 1743) differing in 14 restriction endonuclease sites were generated over the course of this project. These viruses were used as the parental input to infect BHK21/C13 cells which were harvested at 24 h *pi*, titrated at the permissive temperature (PT; 31°C) and the unselected recombinant progeny examined for the presence or absence of 1 *Bgl II*, 8 *Hind III* and 5 *Xba I* sites (Section 3C). Of the 1008 progeny, 451 scorable recombinant genomes were isolated with an overall recombination frequency (RF) of 44.74%. In contrast, an overall frequency of 21.85% was observed during the intrastrain HSV-2 (strain HG52) recombination study (Brown *et al.*, 1992). However, in HSV-1 strain 17+ intrastrain recombination study, the overall recombinant isolation frequency was 43.75% although the number of total progeny screened was small, i.e. only 96 plaque isolates examined for the presence or absence of *Xba I* sites (Brown *et al.*, 1992). This data suggests that HSV-1 strain 17+ is more recombinogenic compared to HSV-2 strain HG52. Alternatively, the high frequency of recombinant isolation during the present study was because of the presence of larger number of unselected markers (14 compared to 5) although comparable number of recombinants were isolated using 5 unselected markers in the above cited HSV-1 study. The parental viruses used in that study did, however, contain selected *ts* markers and the above analysis was carried out at the PT as a control for the selected and unselected recombination analysis at the NPT.

Of the 451 scorable recombinants, 221 different genome structures were identified. However, the possibility of 8,192 identifiable genome structures using the 13 unselected markers is implicated on screening an extremely large number of progeny plaques. Amongst the 221 recombinant classes identified, the most abundant was the class involving a single crossover between the long (L) and short (S) components of the genome. Similarly, recombinant classes with multiple crossovers have shown the most abundant crossover between the L and S components, suggesting a possible occurrence of uneven number of crossovers between distant (50% of the genome length between the markers located in L & S components) markers.

The most abundant recombinant classes showing crossovers between adjacent markers within the L component of the genome had crossovers between the markers at 0.45 & 0.63 m.c. and 0.64 & 0.74 m.c. Again the distance between these markers is longer compared to the distances between other markers (see Table 15).

When the percentage of recombinants between pairs of markers was calculated (Table 14), it became apparent that the distance between the markers was indeed a main factor for the observed variation between different crossovers. This percentage was directly proportional to the distance apart between pairs of markers. The RF values as a function of genome length ranged from 0.24 to 1.89 per Kbp, giving an average frequency of 1.03 per Kbp (Table 15). These values are higher than those published for HSV-1 and HSV-2 (Honess *et al.*, 1980; Umene, 1985; Brown *et al.*, 1992). Mean two-factor recombination frequencies ranging from 2% to 40% were obtained for HSV-1 (Honess *et al.*, 1980) using a variety of selected and unselected markers. As the unselected markers used were different, i.e. polypeptide mobilities compared to the RE sites used herein, these RFs will not be discussed here for comparison purposes. However, an average recombination frequency of 0.7 per Kbp (using our method of calculation) was obtained by Umene (1985) using 8 RE sites as unselected markers whereas Brown *et al.* (1992) have reported an average RF of 0.43 per Kbp for HSV-2 and 0.45 per Kbp for HSV-1 using 5 *Xba I* sites as unselected markers in both HSV-1 and HSV-2.

The role of cellular factors in virus recombination is poorly understood. However, comparative studies using different host cells with adenovirus type 5 (Williams *et al.*, 1974) and HSV (Dasgupta and Summers, 1980) have demonstrated differences in recombination frequencies from host to host. These differences might be because of a defect in recombination pathways of some host cells or different cellular enzymes which promote the process of recombination. Both Williams *et al.* (1974) and Dasgupta and Summers (1980) found two-fold or less differences in RFs from host to host

and the latter group concluded that "each of the tested cell types [normal & mutant human skin fibroblasts, monkey kidney (Vero) cells and mouse L-cells (LMTK)] promoted HSV-1 recombination to an equivalent extent". However, results obtained by Brown *et al.* (1992) showed six to nine-fold differences in RFs among the HFL, CV-1, Hep 2, Vero and BHK21/C13 host cells. In addition, BHK21/C13 cells used in their (Brown *et al.*) studies as well as herein promoted HSV recombination 6-9 times more efficiently. Moreover, Vero cells (used by Umene, 1985) were found eight times less efficient than BHK21/C13 cells. Homology of parental genomes could be another factor in obtaining higher recombination frequencies herein compared to that obtained by Umene (1985). Different strains of HSV-1 were used by Umene whereas only one strain of HSV-1 (strain 17^+) was used in this study. Therefore, both cellular factors and homology of parental genomes might have contributed in promoting HSV recombination in this study.

The average RF of 1.03 per Kbp (Table 15) as a function of genome length was more than two times higher than that obtained by Brown *et al.* (1992). The highest recombination was observed between the markers at 0.07 & 0.08 (1.69 per Kbp), 0.26 & 0.27 (1.89 per Kbp) and 0.63 & 0.64 (1.89 per Kbp), giving an average RF of 1.82 per Kbp. The distance between each set of these markers was the smallest i.e. 1% genome length (GL) among the twelve sets of markers used in this study, indicating that HSV is more recombinogenic than that observed using distant markers. Recombination frequencies of 2-4% were observed using tightly linked (0.6% GL) genetic markers (HSV TK4 and HSV TK38), although the method of calculation (TK^+/total), assay conditions and markers were different (Dasgupta and Summers, 1980) compared to those used in the present study. Similarly, Brown *et al.* (1992) obtained higher recombination frequency (0.75 per Kbp) between the two markers whose distance apart was smaller i.e. 3% GL. An average RF of 1.365 per Kbp was calculated between markers at a distance of 2% GL (Table 15). This data suggests that more frequent markers throughout the genomes of HSV-1 and HSV-2 should be included to obtain true measurements of recombination frequencies. Furthermore, the presence of a large number of markers may also reveal "hot spots" of recombination.

The "hot spots" of recombination are specific elements reported to stimulate the process of recombination in both procaryotic and eucaryotic systems (see for example, Stahl *et al.*, 1975; Keil and Roeder, 1984; Kobori *et al.*, 1986). Specific sequences, such as, ***X*** (5'-GCTGGTGG-3') in phage λ

(Stahl *et al.*, 1975; Smith *et al.*, 1981) or a tetramer repeat $(AGGC)_n$ in the **I** region of the murine major histocompatibility complex (MHC) (Kobori *et al.*, 1986) and genes (for example, the **Int** and **Xis** genes in various phages; see review by Landy, 1989) containing the recombinase function have been shown to serve as "hot spots" of recombination. On the genome of herpes simplex virus, the sequences between 0.706 and 0.744 m.c. (Pogue-Geile *et al.*, 1985; Pogue-Geile and Spear, 1986; MacLean, 1988) and the *'a'* sequences (Mocarski *et al.*, 1980; Mocarski and Roizman, 1981) along with the repeat regions have been implicated to possess the property of recombinogenicity (Pogue-Geile and Spear, 1986; Umene, 1989, 1991). However, no direct evidence (including the present study) has yet been obtained to show conclusively that such sequences or genes are present in HSV. The possibility of the presence of "hot spots" of recombination in HSV cannot, however, be ruled out until a thorough investigation denying their presence is carried out.

The role of the four genomic isomers (P, I_L, I_S, I_{SL}) in HSV recombination has been a matter of controversy (Davison and Wilkie, 1983b; Morse *et al.*, 1977; Preston *et al.*, 1978). Umene (1985) has shown that intratypic recombinants of HSV-1 can be generated from all forms of DNA with the possibility of a preference for P or I_S over I_L or I_{SL} arrangements. Thus, confirming the results of Davison and Wilkie (1983b) on intertypic recombinants. However, the data from Brown *et al.* (1992) indicates that all the four isomers take part in intrastrain HSV-2 recombination. The results presented herein support and extend those of Brown *et al.* to HSV-1 recombination. The RF values obtained between any marker in the L component and the marker in the S component were almost identical (Table 16), indicating the participation of all the four isomers. If there was a preferential contribution of one arrangement over the others then because of the different distances between the twelve sets of markers (see Table 16), the RFs would have been significantly different. Unfortunately, only one marker (the 0.91 m.c. *Hind III* site) was available within the S component of the HSV-1 genome and therefore detailed analysis of the S region was not possible.

Ben-Porat *et al.* (1982) have demonstrated the involvement of only parental molecules in PRV recombination. However, time course experiments in HSV have shown an increase in the number of recombinants with time, indicating that both parental and progeny molecules are involved in the process (Ritchie *et al.*, 1977; Brown *et al.*, 1992). It would have been ideal

to further strengthen this conclusion by carrying out the time course of recombination experiments using the variants 1738 and 1743. However, it was not carried out because of the lack of time. The complexity of the recombinants analysed herein has nevertheless provided an indication that parental as well as progeny molecules were involved. Had there been parental contribution alone, the number of recombinants isolated and the high RFs observed would have been much lower. Similarly, the relationship between the processes of DNA replication and recombination was not investigated.

The intrastrain HSV-1 (strain 17+) recombination study presented confirms and expands the previous HSV studies (for example, the study carried out by Brown *et al.*, 1992) on: (1) HSV-1 is highly recombinogenic; (2) tightly linked markers improve the accuracy of measurement of recombination frequencies; (3) possibly all the four genomic isomers are involved in the process of HSV recombination; and (4) no "hot spot" of recombination was discovered.

To determine further the recombinogenic properties of HSV, it would be essential to have more unselected markers especially within the regions that could not be explored in this study. These regions include the S component of the genome and the regions with distant markers, such as, between the 0.45 & 0.63 m.c. and 0.64 & 0.74 m.c. within the L component. In addition, the presence of "hot spots" within the repeat regions of the genome can be investigated by introducing unique RE sites and their subsequent use in intrastrain recombination experiments.

SECTION 4C : ISOLATION OF HSV-1 (STRAIN 17±) DELETION/INSERTION VARIANTS

When HSV-1 strain 17+ genomes were analysed with *Hind III*, *Bgl II-Hind III* and/or *Bgl II-Xba I* restriction endonucleases to isolate the RE site deletion/insertion variants, a large number of variants showing genomic rearrangements were also detected. These include variants with sizeable DNA deletions ranging from 400 bp to more than 10 Kbp or insertions varying from 356 bp to >30 Kbp. Preliminary characterization of these variants was carried out as described in Section 3B:4.

The spontaneous isolation frequency of deletion/insertion variants of HSV-1 strain 17+ obtained during the present study was much

higher than that reported previously (MacLean and Brown, 1987b, c; MacLean, 1988). MacLean and Brown (1987b) reported the isolation of only one deletion variant (1703) from over 5000 plaque isolates examined, giving an overall frequency of approximately 0.02%. In contrast, a frequency of 24% was found on analysing the *wt* plaque isolates from an HSV-2 strain HG52 stock (Harland and Brown, 1985; Harland, 1990). It was suggested that these variants pre-existed in the HG52 stock and were not generated as a result of the *in vitro* manipulations such as transfection and/or extensive restriction endonuclease treatment. In addition, lower frequencies of such variants were detected in the elite stocks of other *wt* HSV-1 and HSV-2 strains as well as in the intertypic strain 17$^+$/strain HG52 recombinant R12-5, indicating that these frequencies vary from strain to strain (Brown *et al.*, 1984; Harland and Brown, 1985). Therefore, it was concluded that the HSV-1 strain 17$^+$ is considerably more stable than HSV-2 strain HG52 and that the deletion variant 1703 could have originated because of illegitimate recombination (MacLean and Brown, 1987b) influenced by the presence of three groups of tandemly reiterated sequences of high G+C content within the TR_L/IR_L repeat regions of the HSV-1 genome (Rixon *et al.*, 1984). The isolation frequency of approximately 0.62% (31 times higher than that found by MacLean and Brown, 1987b) observed herein still confirms the stability of strain 17$^+$ compared to strain HG52. However, the origin of these deletion/insertion variants is open to speculation.

DNA transfection (Calos *et al.*, 1983), restriction endonuclease treatment prior to transfection (Jones and Shenk, 1978; Brown *et al.*, 1984; Harland and Brown, 1985, 1988, 1989; Brown and Harland, 1987; MacLean and Brown, 1987a, b, c), genomic disruption (Longnecker and Roizman, 1986) and illegitimate recombination between small stretches of homologous sequences (Umene, 1986) have been shown to stimulate the isolation of deletion/insertion variants by making the non-essential/dispensible (as assessed in *in vitro* assays) regions of the viral genome unstable. The deletion variants described in this thesis [see Section 3B:4 (a) & (b)] along with many others (data not shown) were isolated during isolation of the RE site-deletion variants 1721 to 1738 using 1702 as the parental virus. As the variant 1702 was devoid of the *Xba I* sites and three out of four *Xba I* sites were deleted by the selection enrichment procedure, the HSV-1 (strain 17$^+$) DNA has already been used several times in transfection experiments and was subjected a number of times to an excess of *Xba I* digestion to generate the *Xba I* site-deletion variants B1/2, B9/6, X2 (Brown *et al.*, 1984), 1701 and

finally 1702 (MacLean and Brown, 1987a). In addition, it should be pointed out that no deletion/insertion variants were isolated during the isolation of 1721, 1723, 1724 and 1727 [see Section 3B:1 (a) (i), (ii) & (iii)]. Therefore, 1702 DNA was further used in transfection experiments to isolate these *Hind III* site-deletion variants before the variants with genomic alterations were detected. It is therefore possible that the selection enrichment procedure and subsequent transfection experiments have actually generated an undetectable number of molecules within the population of 1702, which when passaged several times (either through transfection experiments or latter rounds of plaque purification and virus growth) amplified to become detectable. This view is supported by the fact that most of the deletion/insertion variants of HSV-2 strain HG52 described by Harland and Brown (1985, 1988, 1989) & Brown and Harland (1987) were isolated during the isolation of *Xba I* site-deletion variants. Brown *et al.* (1984) have also reported the isolation of four deletion variants of the intertypic recombinant (R12-5) from 17 plaques screened following selection enrichment by *Xba I* of its DNA. Furthermore, the deleted genomes (as above) could have gone through several rounds of recombination events leading to the generation of different sizes of the deleted regions.

Most of the spontaneously isolated HSV variants reported by the above mentioned groups of workers as well as others involved one or more repeat regions (TR_L, IR_L, IR_S, TR_S) of the genomes, indicating that these regions are more prone to deletions and subsequent rearrangements. Similarly, most of the deletion variants isolated during the present study involved the repeat regions. For example, the variant 1714 had a deletion in both TR_L and IR_L; the variant 1721X193 contained a deletion involving IR_L and adjacent U_L sequences; the variant 1727X31 showed deleted U_S/TR_S; the variants with deletions ranging from 400 to 550 bp had deleted TR_L and IR_L. Because of the variation in the copy number of the tandemly reiterated short sequences found in R_L (Rixon *et al.*, 1984; Perry, 1986) and the *'a'* sequences (Locker and Frenkel, 1979; Davison and Wilkie, 1981; Mocarski and Roizman, 1981), variation in TR_L and IR_L has previously been identified (Lonsdale *et al.*, 1980). In addition, MacLean and Brown (1987b) have reported the isolation of 11 HSV-1 (strain 17^+) variants showing more extensive (up to several hundred bp) variation in TR_L/IR_L. However, the variation observed in the four variants plus the variant 1714 [see Section 3B:4(a)] was not because of such alterations but deletions ranging from approximately 400 bp to 759 bp. Sequence determination of the region

spanning the 1714 deletion revealed that the deletion has precisely removed an 18 bp DR_1 element of the *'a'* sequence located at the IR_L/IR_S joint region of the HSV-1 strain 17^+ genome (MacLean, A.R. *et al.*, 1991a). As the apparent size of the deletion in both TR_L and IR_L was the same (using RE analysis) in each of the deletion variant in which the deleted sequences involved both repeats of the L component, their genomes could have arisen as the result of the process of equalization. Equalization of the repeats could be mediated by a copying mechanism where one repeat acts as a template for the other (Roizman, 1979) or it could occur by intermolecular recombination followed by segregation (Ben-Porat *et al.*, 1984; Varmuza and Smiley, 1984). Therefore, a possible explanation of the origin of these variants is : (1) One repeat of the long component of the HSV-1 genome was deleted; (2) The viral genome with one deleted repeat has either pre-existed within the 1702 population or was created because that region became unstable due to continuous pressure exerted by the restriction endonuclease treatment and subsequent transfections; (3) Intermolecular recombination occurred during or soon after the formation of circular or concatemeric DNA molecules; and (4) By a process similar to that proposed in the double-strand break repair model (Szostak *et al.*, 1983; described fully in Section 1F:1), one repeat was copied by the other followed by cleavage of the concatemeric DNA to form unit length molecules containing equal repeats. In fact, evidence has recently been obtained about the equalization of repeats by a similar mechanism in PRV (Rall *et al.*, 1992).

The origin of other deletion variants such as 1721X193 and 1727X31 can also be explained in a similar manner. The variant 1721X193 showed a deletion of approximately 9.9 Kbp between 0.74 and 0.83 m.c., thus involving IR_L and the adjacent U_L sequences. It is known that the UL55, UL56, RL1 & RL2 genes as well as the latency associated transcripts (LATs) are dispensible in tissue culture (Stow and Stow, 1986; MacLean and Brown, 1987b, c; Stevens *et al.*, 1987; Spivack and Fraser, 1987; McGeoch *et al.*, 1988b; Chou *et al.*, 1990; MacLean, A.R. *et al.*, 1991a; McGeoch *et al.*, 1991). Although the precise end points of the deletion in 1721X193 are not known, restriction endonuclease analysis indicated that most of IR_L and adjacent terminal portion of U_L were deleted, suggesting that possibly all the genes mentioned above were deleted. The UL54 gene (IE2) is essential in tissue culture (Sacks *et al.*, 1985). As the variant 1721X193 is viable in tissue culture, the deletion seems to have no effect on UL54. However, determination of growth characteristics and analysis of immediate early

polypeptides of 1721X193 are necessary to find out whether the variant has any growth impairment compared to that of *wt* and produces the normal amounts of IE polypeptides (except that of Vmw110 because of possible deletion of one complete or partial copy of the RL2 gene). Sequence determination would be required to know the precise end points of the deletion. Since 1721X193 showed no variation in TR_L and the adjacent unique sequences, equalization of the repeats did not occur. However, it is possible that equalization has in fact occurred but because of the essential nature of the unique sequences (L) from the left end, the resultant virus was unable to replicate and therefore could not be detected. Hutchinson *et al.* (1992) have recently shown that the UL1 gene encodes a glycoprotein designated gL. In addition, they were unable to isolate a mutant virus containing a *Lac Z* insert within the UL1 ORF, indicating that gL is essential for HSV replication in tissue culture. As the extent of deletion in 1721X193 would have involved UL1 on equalization, such a variant was not isolated. Similarly, equalization was not observed in the case of the variant 1727X31. This variant had a deletion in U_S/TR_S. It has previously been shown that the IE3 (Vmw175) gene is diploid and the two copies are located entirely within the IR_S and TR_S respectively (McGeoch *et al.*, 1986a, 1988b). HSV variants containing only one copy of IE3 have been isolated (Longnecker and Roizman, 1986; Brown and Harland, 1987), suggesting that the other copy is dispensible *in vitro*. In addition, most of the US genes are non-essential in tissue culture (Post and Roizman, 1981; Sears *et al.*, 1985; Longnecker and Roizman, 1986, 1987; Umene, 1986; Brown and Harland, 1987). Therefore, the isolation of a variant (1727X31) showing deletion involving only one repeat of the short component of HSV-1 genome along with the adjacent unique sequences was not surprising.

The HSV-1 (strain 17^+) insertion variants isolated during the present study showed DNA inserts varying in size from 356 bp to greater than 30 Kbp. The nature and precise location of the insert in the variant 1740*In* was determined by dideoxynucleotide sequencing of the *Bam HI f'* fragment by Dr Amina Fu [see Section 3B:4 (c)]. Sequence determination has revealed that the 356 bp DNA insert in fact arose as the result of DNA duplication of sequences starting from 21 bp downstream of 3' terminus of the UL20 ORF. Therefore, no effect on the UL20 gene product or the growth pattern of 1740*In* compared to those of parental viruses was observed. Other insertion variants include the variant 1738X7 containing a DNA insert of approximately 6.6 Kbp; the variant 1743X138 with a DNA insert of more than 30.0 Kbp; the variant R-

582 showing an estimated DNA insert of 6.0 Kbp; the variant 1721X23 containing a DNA insert of approximately more than 9.0 Kbp; the variant 1721X71 with a DNA insertion of approximately 24.0 Kbp. These variants were only analysed by various restriction endonucleases and the analysis has not revealed the locations of the inserts. Hybridization experiments using the inserts as probes would be necessary to find out the approximate locations within the HSV-1 genome whereas sequence determination would be essential to know the precise end points. However, preliminary hybridization experiments with the variants 1721X23 and 1721X71 indicated that the inserts are possibly originated by duplication of HSV sequences (data not shown). Nothing is known about the nature of the inserts within the remaining variants.

HSV variants with deletions and insertions replacing the deleted sequences have previously been isolated (Poffenberger *et al.*, 1983; Jenkins *et al.*, 1985; Jenkins and Roizman, 1986; Umene, 1986; Brown and Harland, 1987; MacLean and Brown, 1987b; Harland and Brown, 1988, 1989). Deletions of upto 15 Kbp have been reported in HSV-1 (strain F) (Poffenberger *et al.*, 1983) and upto 13.5 Kbp in HSV-2 (strain HG52) (Harland and Brown, 1989). In both cases, the deleted sequences included the internal repeats (IR_L, IR_S) resulting in the generation of frozen L and S components in HSV-1 whereas frozen L and inefficiently inverting S component in HSV-2. The 15 Kbp deletion in HSV-1 was replaced by a 2 Kbp insert carrying the *tk* gene following insertional mutagenesis. While the 13.5 Kbp deletion in HSV-2 was spontaneously replaced by 1 to 14 copies of a 1 Kbp insert of bovine satellite DNA (Harland, 1990). Moreover, an HSV-1 strain 17^+ variant 1719 has recently been isolated and characterized (MacLean, A. R., 1992 : submitted for publication). This variant had a DNA duplication of approximately 7.5 Kbp located around Ori_L. The variant is stable with occasional *wt* virus being isolated possibly because of excision of the duplicated sequences during virus replication and homologous recombination. The variant 1719 showed *wt* growth characteristics *in vitro*. Apart from such variants, foreign DNA sequences and/or genes have been inserted using HSV-1 as an expression vector (see for example, Ho and Mocarski, 1988; Rixon and McLauchlan, 1990; Dormitzer *et al.*, 1992). However, the maximum limits of deletions and/or insertions within the HSV genomes are not known. The HSV-1 (strain F) variant 1358 with 15 Kbp deleted sequences was further characterized and its packaged DNA consisted of two submolar populations : the head-to-tail concatemers of 1358 DNA and defective genomes containing directly repeated sequence inserted in place of

the deleted L-S junction (Poffenberger and Roizman, 1985). The analysis of the HSV-2 strain HG52 variant, JH2614, revealed that a proportion of its molecules returned to the *wt* and another proportion was suggested to be defective and non-infectious (Harland and Brown, 1989). Such observations raise the question whether viral genomes with large deletions and/or insertions are really stable? In addition, is there any role of the so called non-essential/dispensible sequences in virus functions, e.g. in the growth impairment observed in some of these variants.

Apart from the variant 1740*In*, the insertion variants described herein were not plaque purified or characterized any further. Therefore, it is not known whether the inserts are stable or unstable. No deleted sequences were revealed by the restriction endonuclease analysis of these variants. It would be interesting to find out about the stability of large inserts particularly those within the genomes of 1721X71 and 1743X138. Moreover, analysis of these variants in terms of growth potential would give a better idea about the limitations of DNA inserts within the non-essential regions of the HSV genome.

The characterization of deletion/insertion variants has proved useful in elucidation of the essential/non-essential regions of both HSV-1 and HSV-2 genomes as well as the nature of viable genomes in relation to the size and necessity of both unique and repeat sequences (see Section 1E:1 for related references). For example, the isolation of HSV-1 strain 17$^+$ variant 1714 (described herein) and HSV-2 strain HG52 variant JH2604 (Harland and Brown, 1985; Taha *et al.*, 1989a, b) has provided an opportunity to define HSV genes involved in pathogenicity. It is now well established that the long repeats (R_L) of both strain 17$^+$ and strain HG52 contain an additional diploid gene termed RL1 or ICP34.5 (Taha *et al.*, 1989a, b, 1990; MacLean *et al.*, 1991a, b; McGeoch *et al.*, 1991; Dolan *et al.*, 1992). Detailed study of the variants 1716 (derived from the *wt* and 1714 viruses) and JH2604 is already underway and there are clear prospects for their use in HSV vaccines (Drs S M Brown, A R MacLean and J E Harland, personal communication).

SECTION 4D : FUTURE PROSPECTS

1. The removal of the remaining *Hind III* sites at 0.52, 0.58 and 0.88 m.c. from the variant 1738 would provide an HSV-1 (strain 17$^+$) genome with no *Hind III* sites. Such a genome would be useful in both intratypic and intertypic recombination studies because of the presence of additional

unselected markers particularly within the S component of the genome. The involvement of the four HSV isomers in recombination will be fully confirmed by having more markers such as the 0.88 m.c. *Hind III* site along with deletion of the *Bam HI* sites from the S component. However, analysis of the *Bam HI* fragments such as ***l'***, ***m'*** & ***n'*** would be difficult because of their small sizes (198, 156 and 90 bp respectively) and therefore the *Bam HI* sites surrounding these fragments would have to be used as single markers instead of two. The HSV-1 genome devoid of the *Hind III* sites can also be used in the development of an expression vector by inserting a unique *Hind III* site within an intergenic region and using that site for expression of any inserted foreign DNA.

2. The possible presence of "hot spots" of recombination can be investigated by generating HSV genomes with multiple unselected markers within particular regions of the genome and using such genomes in recombination studies. Deletion of closely spaced restriction endonuclease sites such as *Bam HI* would provide an opportunity to study the recombinogenicity of different regions of the HSV genomes in detail and therefore true recombination frequencies will be determined.

3. Recombination studies in the short genomic component as well as the repeats will particularly be of interest because of the presence of reiterated sequences within these regions. The use of only one strain of HSV in a recombination study provides isogenic material for good pairing and therefore increased chances of recombination between paired molecules. In addition, there is no "leakiness" due to the use of unselected markers. As the reiterated sequences have been suggested to mediate high recombination frequencies (Pogue-Geile *et al.*, 1985; Pogue-Geile and Spear, 1986; Umene, 1989, 1991), such a study will provide an insight in determining the recombinase function of these sequences.

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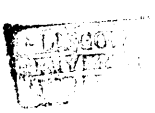

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