XBA I SITE LOSS MUTANTS AND DELETION/DUPLICATION VARIANTS OF HERPES SIMPLEX VIRUS TYPE 1 : ISOLATION, CHARACTERIZATION AND RECOMBINATION STUDIES

by

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SUMMARY

The aim of this project was to isolate a herpes simplex virus type 1 Glasgow strain 17 genome lacking all four Xba I restriction enzyme sites and to use the sites as non-selected markers to study intratypic HSV recombination. However, a large part of the work involved the analysis of variant genomes which were identified during the isolation of Xba I site negative viruses.

The parent virus used in these studies was the variant X2, from which the 0.07 map unit (m.u.) and 0.29 m.u. Xba I sites had been removed by selection enrichment (Brown *et al.*, 1984). The remaining two Xba I sites were deleted as follows : the Xba I site at 0.45 m.u., which lies within the gene (U_L33) encoding a predicted polypeptide of 14,000 molecular weight (mol. wt.), was removed by selection enrichment; while the Xba I site at 0.29 m.u., which lies within the gene (U_L22) encoding the essential glycoprotein H (Buckmaster *et al.*, 1984), was removed by site-directed mutagenesis. The variant devoid of Xba I sites (1702) shows normal growth characteristics *in vitro* and its polypeptide profile is indistinguishable from wild-type virus apart from the absence of the thymidine kinase (tk) polypeptide, a feature which is believed to be unrelated to the loss of the Xba I sites.

During *in vitro* latency experiments, Cook and Brown (1987) isolated a variant of X2 containing a novel Xba I site at 0.74 m.u. This virus was used in intratypic recombination experiments with wild-type strain 17 to generate a HSV-1 strain 17 variant (1708) containing a fifth

Xba I site, thus increasing the number of non-selectable markers between this virus (5) and 1702 (0). Different temperature-sensitive (ts) lesions were introduced into 1702 and 1708 as selectable markers.

A time-course of recombination was carried out at the permissive temperature and the appearance of non-ts recombinants assayed at the non-permissive temperature. Recombination was first detected at 4 h post infection, following the onset of DNA replication, and rapidly increased to 15% by 24 h post infection. The distribution of the non-selectable markers, ie. the Xba I sites, in ts^+ recombinant molecules was analyzed. From the experimental results a number of conclusions were drawn : (i) there was an increase in the complexity or number of crossovers in recombinants arising at later time-points, confirming the previous hypothesis of Ritchie et al. (1977) that both parental and progeny molecules take part in HSV recombination; (ii) in ts^+ recombinants, recombination was very high outwith the selected recombination region, suggesting that correct alignment of genomes plays an important role in determining the overall but not the relative rate of recombination; and (iii) due to the large distance between the Xba I sites, little can be determined about recombinational hotspots.

The HSV-1 genome consists of two unique sequences - the long unique (U_L) and the short unique (U_S) - flanked by inverted repeat elements known as the internal repeats (IR_L/IR_S) and the terminal repeats (TR_L/TR_S). During the isolation of 1702, a variant (1703) was isolated which has a deletion of approximately $5x10^6$ mol. wt. in the U_L and IR_L

regions of its genome, such that one copy of the immediate-early (IE) gene 1 and two unique open reading frames coding for predicted polypeptides of 20,000 mol. wt. and 22,000 mol. wt. (U_L55 and U_L56) are deleted. The variant 1703 synthesizes reduced levels of VmwIE110, the product of IE gene 1, and under immediate-early conditions apparently fails to synthesize VmwIE63, at both the polypeptide and RNA levels, despite there being no apparent deletion in the coding or controlling regions of the IE2 (VmwIE63) gene. 1703 also fails to synthesize the thymidine kinase polypeptide, although this is unrelated to either the deletion or the failure to synthesize VmwIE63. The variant 1703 exhibits normal growth characteristics in vitro.

During the analysis of eighty plaque isolates from one recombination experiment, fourteen variants with rearrangements around the long repeats were detected. Of these, eleven have extensive variation (up to 0.4×10^6 mol. wt.) in the size of the long repeats outwith the 'a' sequence. The remaining three variants have large scale deletion or duplication of both unique and repeat sequences. Variant 1704 has a deletion of 2.5×10^6 mol. wt. in IR_L/U_L and 0.8×10^6 mol. wt. in TR_L, such that sequences are deleted from both long repeats. The deletion in U_L removes the U_L55 and U_L56 open reading frames deleted in 1703 and terminates at least 500 bp downstream from the 3' end of the IE2 gene. Variant 1705 has a deletion of 3.5×10^6 mol. wt. in IR_L/U_L which again removes the U_L55 and U_L56 open reading frames and terminates around the 3' end of the IE2 gene. Variant 1706 has a similar deletion in U_L to 1705, but in this case the deleted sequences are replaced by sequences from the

left end of U_L, such that the long repeat is extended by $3x10^6$ mol. wt. and the overall genome size by $2x10^6$ mol. wt. In 1704, the synthesis of VmwIE63 is normal, whereas in 1705 and 1706 it is reduced to about 50% of the wild-type level. The three variants grow almost normally in vitro.

A variant (1709) which has a deletion of $0.5x10^6$ mol. wt. in the repeat elements of the short region (TR_S and IR_S) was isolated at the end of the project and thus no further characterization was carried out.

NON-STANDARD ABBREVIATIONS

It is assumed that the reader is familiar with the more standard abbreviations, such as DNA, RNA, h, min etc., and these are therefore not listed below.

APS	ammonium persulphate
BCdR	5-bromo-2′-deoxycytidine
BHI	brain heart infusion
BHK	baby hamster kidney
bp	base pairs
BSA	bovine serum albumin
BUdR	5-bromo-2′-deoxyuridine
CCV	channel catfish virus
Ci	Curies
CMV	cytomegalovirus
cpe	cytopathic effect
DMSO	dimethylsulphoxide
DNase	deoxyribonuclease
DR	direct repeat
DTT	dithiothreitol
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
EDTA	sodium ethylene diamine tetra-acetic acid
Fc	crystallisable fragment of immunoglobulin
HCMV	human cytomegalovirus
HFL	human foetal lung
HIV	human immunodeficiency virus
HSV	herpes simplex virus
HVS	herpes virus saimiri

ICP	infected cell polypeptide
IE	immediate-early
IgG	immunoglobulin G
IR	internal repeat
K	kilodaltons
kb	kilobase
M	molar
MDB	major DNA-binding protein
MI	mock infected
moi	multiplicity of infection
mol. wt.	molecular weight
m.u.	map units
NP40	Nonidet P40
NPT	non-permissive temperature
ori	origin of DNA replication
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming units
PRV	pseudorabies virus
PT	permissive temperature
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylenediamine
tk	thymidine kinase
TR	terminal repeat
ts	temperature sensitive
ts^+	wild type regarding temperature sensitivity

uv	ultra violet
Vmw	virus-specific polypeptide of apparent molecular weight
VZV	varicella-zoster virus
v/v	volume per volume
wt	wild-type
w/v	weight per volume

CONTENTS

INTRODUCTION

INTRODUCTION

I.1 OBJECTIVES

This project has involved the isolation of herpes simplex virus type 1 (HSV-1) genomes containing non-selectable markers and their use in recombination studies. HSV-1 variants with extensive genomic rearrangements have also been isolated and characterized.

The aim of the introduction is to provide a general overview of HSV with emphasis on those areas related to the project; in particular HSV evolution, recombination and genetics. Certain areas of interest to the author, such as DNA replication, the 'a' sequence and transcription have been dealt with in detail. However, other areas unrelated to the project, such as transformation, have been omitted. A section on general recombination is also included.

I.2 CLASSIFICATION OF HERPESVIRIDAE

The criteria for classification as a member of the family Herpesviridae are purely morphological (Fenner, 1976). The virion consists of four architectural elements : (i) a core, consisting of a fibrillar spool around which the double-stranded linear DNA is wrapped; (ii) an icosahedral capsid containing 12 pentameric and 150 hexameric capsomeres; (iii) variable amounts of an amorphous material asymmetrically arranged around the capsid and designated the tegument; and (iv) a membrane or envelope of 150-200nm surrounding the entire structure (Wildy et al., 1960;

Roizman and Furlong, 1974). Despite the fact that herpesviruses cannot be distinguished from each other by their morphology (Fenner, 1976), they are readily distinguishable by their biological properties, immunological cross-reactivity and the size, base composition and structure of their genomes (Roizman, 1982). More than eighty different members of the family have been identified by these criteria (Nahmias, 1972).

Herpesviruses have been classified on the basis of their biological properties (Roizman et al., 1978; Mathews, 1982; Roizman, 1982) and genome structure (Honess and Watson, 1977b; Roizman, 1982; Honess, 1984). These are outlined below.

I.2.a Classification on the basis of biological properties

On this basis, herpesviruses are subdivided into three subfamilies : Alpha-, Beta-, and Gammaherpesvirinae.

Alphaherpesvirinae usually cause acute, self-limiting disease in their natural host. The primary infection is often followed by the establishment of latency, typically in the dorsal root ganglia of the peripheral nervous system. A number of stimuli may cause reactivation of latent virus, leading to recurrent disease. The replicative cycle of these viruses is relatively rapid, susceptible cells often being lysed in less than 24 h. Prototypes of the group are herpes simplex virus types 1 and 2 (HSV-1 and HSV-2).

Betaherpesvirinae have a narrow host range. In vivo, they usually cause asymptomatic infections in immunocompetent individuals, but may cause severe generalized disease in immunosuppressed patients and

neonates. Latent virus has been demonstrated in secretory glands, lymphoreticular cells, kidneys and other tissues. In vitro, the replicative cycle is slow (greater than 24 h). Cytomegalovirus (CMV) is the prototype of the group.

Gammaherpesvirinae are the lymphoproliferative virus group. They have a narrow host range in vivo, replicating in either B or T lymphocytes, and latency is established in lymphoid tissues. In vitro, all known members of this subfamily will replicate in lymphocytes. Members include herpes virus saimiri (HVS) and Epstein-Barr virus (EBV), which replicate in T and B lymphocytes, respectively.

I.2.b Classification on the basis of genome structure

Herpesviruses differ considerably in their base composition (32-75% G+C), the size of their genome ($80\text{-}150 \times 10^6$ mol. wt.) and the arrangement of reiterated DNA sequences. A classification scheme based on this latter property has been proposed (Figure 1). While most of the identified herpesviruses have been classified on the basis of their biological properties, only a minority have been studied in sufficient detail to allow classification by genome structure.

(Honess and Watson, 1977b; Roizman, 1982; Honess, 1984)

Group A

Channel catfish virus (CCV) is a member of this group. Its genome is characterized by a single direct repeat at both termini (Chousterman et al., 1979). The DNA is present as only one isomer.

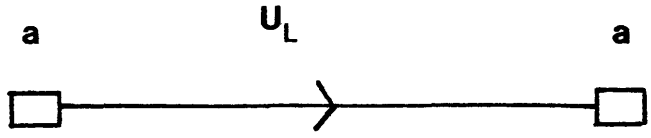
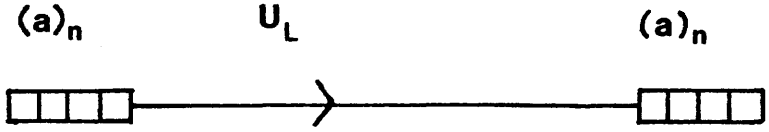
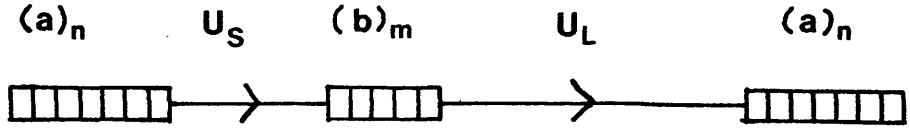
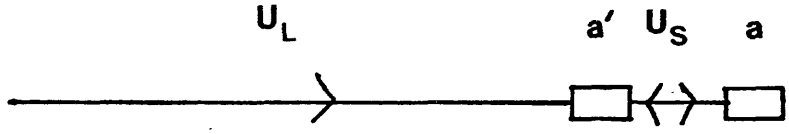
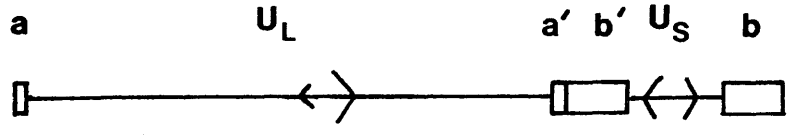
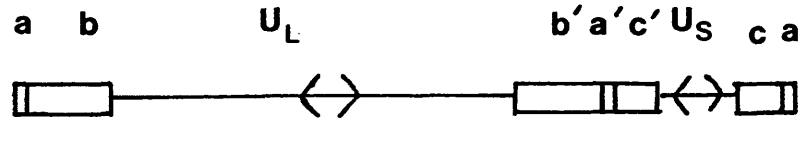
Group	Example	Genome Structure	Number of Isomers
A	CCV		1
B	HVS		1
C	EBV		1
D	PRV		2
E-I	VZV		2 major 2 minor
E-II	HSV-I		4

Figure 1. Genome structure of the herpesviruses.

The structures of the herpesvirus genomes are illustrated. Repeat sequences are represented as open boxes. U_L and U_S indicate long and short unique sequences and a, b and c indicate repeat sequences with a', b' and c' their complement. Arrows indicate the relative orientations of the unique sequences. An example of each group, A-EII, is illustrated and the number of isomers indicated. In VZV, U_S is in either orientation 50% of the time, while U_L is in one orientation 95% of the time.

CCV is channel catfish virus; HVS is herpesvirus saimiri; EBV is Epstein-Barr virus; PRV is pseudorabies virus; VZV is varicella-zoster virus; HSV-1 is herpes simplex virus type 1.

Group B

A member of this group is HVS, whose genome is characterized by multiple copies of a sequence present as a direct repeat at both termini. The number of repeats in each genome is roughly constant, although the number at each end varies extensively (Stamminger et al., 1987). Again, the DNA is present as only one isomer (Bornkamm et al., 1976).

Group C

A member of this group is EBV, whose genome contains multiple copies of a sequence present as a direct repeat at both termini and internal tandem reiterations of a second set of sequences. The DNA is present as only a single isomer (Raab-Traub et al., 1980).

Group D

Pseudorabies virus (PRV) is a member of this group. Its genome is characterized by the presence of two regions of unique sequence (the long and short unique, U_L and U_S), one of which (U_S) is flanked by inverted repeats (TR_S/IR_S), which allow inversion of U_S, leading to the presence of two isomers (Ben-Porat et al., 1979). Although the genome circularizes prior to replication, it is not terminally redundant (Harper et al., 1986).

Group E

This group has now been divided into two subgroups, EI and EII.

Group EI contains those viruses whose genomes contain two unique sequences (U_L and U_S) each flanked by inverted

repeats which share no homology. Varicella-zoster virus (VZV) is a member of the group. U_S is flanked by large inverted repeats through which it inverts, and is present in both orientations in equal amounts. U_L is flanked by short, 88 bp, inverted repeats, which allow apparently inefficient inversion, and U_L is therefore predominantly found in one orientation (95%). Thus, VZV DNA consists of 2 major and 2 minor isomers (Davison, 1984). PRV variants which have short inverted repeats flanking U_L are also included in group EI. Their genomes appear to have arisen as a result of duplication of sequences previously existing only at the terminus of U_L. In contrast to VZV, U_L inverts efficiently and the DNA population consists of 4 equimolar isomers (Lomnicizi et al., 1984, 1987).

Group EII is characterized by genomes with 2 unique regions (U_L and U_S) flanked by inverted repeats which share a short region of DNA directly repeated at the termini and indirectly repeated at the junction between the internal inverted repeats. Inversion of the unique DNA regions about the inverted repeats gives rise to four equimolar isomers in a population of DNA molecules. Exonuclease treatment of the ends leads to circularization, suggesting the presence of a terminal redundancy (Sheldrick and Berthelot, 1974; Wadsworth et al., 1975; Delius and Clements, 1976), a feature subsequently established by DNA sequencing (Davison and Wilkie, 1981; Mocarski and Roizman, 1981; Davison and Rixon, 1985). HSV-1 and HSV-2 are prototypes of the group.

I.3 MORPHOLOGICAL STRUCTURE OF HERPESVIRUSES

Herpesviruses consist of four morphologically distinct structures : core, capsid, tegument and envelope (Figure 2A). The double-stranded DNA genome is situated within the core (Ben-Porat and Kaplan, 1962; Epstein, 1962) and is toroidally wrapped around a central proteinaceous matrix (Chai, 1971; Furlong et al., 1972; Nazerian, 1974). VP21 appears to be a structural component of the core (Gibson and Roizman, 1972) and is probably analogous to Vmw43, which may be DNA-binding (Bayliss et al., 1975).

Surrounding the core is an icosahedral capsid approximately 100nm in diameter, consisting of 150 hexameric and 12 pentameric capsomeres with 5:3:2 axial symmetry (Wildy et al., 1960). The individual capsomeres are hollow elongated prisms and appear to be connected by intercapsomeric fibrils (Figure 2B) (Wildy et al., 1960; Vernon et al., 1981). The hexameric capsomeres are generally believed to have six-fold symmetry (Furlong, 1978) and it is probable that each capsomere is composed of six molecules of the major capsid protein, Vmw155 (Spear and Roizman, 1972; Marsden et al., 1976; Vernon et al., 1981; Steven et al., 1986). The virion protein p50 may form part of the pentameric capsomeres (Vernon et al., 1981). Vmw155 and p50 may be linked by disulphide bridges (Zweig et al., 1980).

An amorphous layer known as the tegument surrounds the capsid (Roizman and Furlong, 1974). Proteins believed to reside within the tegument and to intercalate between the capsid and the envelope include VP1-3 (>200K) (Gibson and Roizman, 1972) and the 65K virion transactivating factor

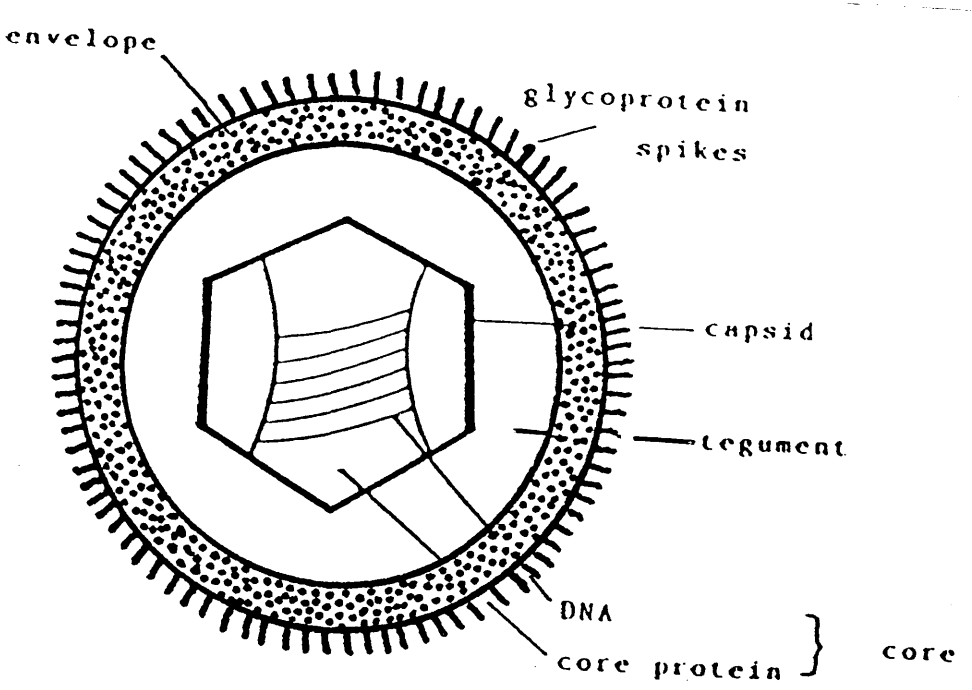
Figure 2. Herpes simplex virion structure.

A. Schematic diagram of HSV virions. The virions are composed of four main architectural elements - the core, capsid, tegument and envelope. Within the core, viral DNA is toroidally wound around a cylindrical protein mass. The virion envelope contains a number of glycoproteins, visible on electron microscopy as spikes protruding from the envelope.

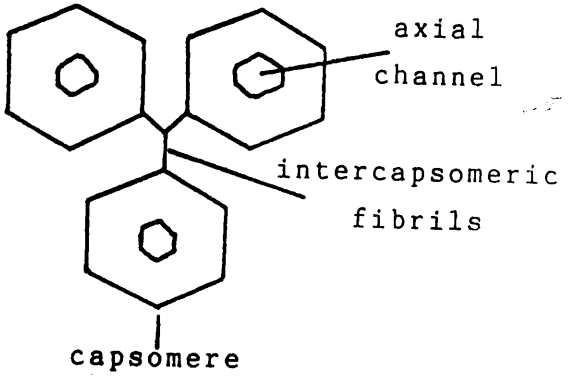
B. Intercapsomeric fibrils. The capsid is composed of 150 hexameric and 12 pentameric capsomeres. Adjacent capsomeres appear to be connected at their vertices by a matrix of intercapsomeric fibrils.

C. Six-fold symmetry of hexameric capsomeres. Hexameric capsomeres are believed to have six-fold symmetry, probably consisting of six molecules of the major capsid protein, Vmw155.

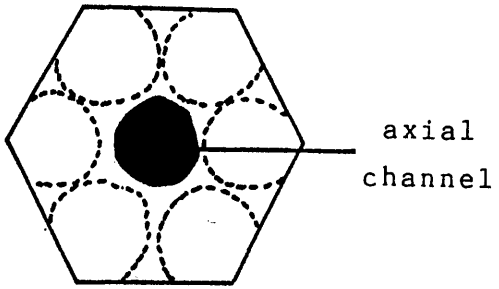
A.


envelope
glycoprotein
spikes
capsid
tegument
DNA
core protein
core

B.


axial
channel
intercapsomeric
fibrils
capsomere

C.


axial
channel

(Batterson and Roizman, 1983; Campbell et al., 1984). The tegument is surrounded by the virion envelope, which consists of a tri-laminar membrane with glycoprotein spikes, about 8-10nm long, projecting from the surface (Wildy et al., 1960). The virus envelope is believed to contain most of the virus glycoproteins (Spear and Roizman, 1972; Stannard et al., 1987), cell lipids (Asher et al., 1969), and spermidine (Gibson and Roizman, 1971).

I.4 HUMAN HERPESVIRUSES

Five herpesviruses have been shown to infect man as their primary host : HSV-1, HSV-2, VZV, EBV and HCMV. HSV-1 is commonly associated with mucocutaneous lesions of the face (Fiddian et al., 1983), but the virus can infect many (Hill et al., 1975)
organs, including the eye. Primary infection is often asymptomatic, but may lead to a range of clinical symptoms, including fever, sore throat, ulcerative and vesicular lesions, oedema, localized lymphadenopathy and general malaise (Whitley, 1985). Following primary infection, a latent state is normally established (see later, section I.5), the virus residing most commonly in the trigeminal ganglia (Bastian et al., 1972). The virus may periodically reactivate to cause recurrent herpetic lesions, localized to the dermatome supplied by the latently-infected dorsal root ganglion. (Wildy et al., 1982) Analysis of the restriction enzyme profile of virus DNA extracted from primary and recurrent lesions has indicated that : (i) most recurrent lesions are due to reactivation of the original latent virus, and (ii) except for very closely related cases, each individual has his own

*Papilloma virus possibly in conjunction with HSV-2 has also been associated with cervical carcinoma (zur Hausen, 1982).

unique strain of virus (Lonsdale et al., 1979). Around 90% of the adult population are seropositive for HSV (Whitley, 1985).

Herpes simplex virus type 2 has similar biological properties to HSV-1 and shares 50% homology with it at the DNA level (Kieff et al., 1972). This serotype is primarily associated with genital lesions (Dowdle et al., 1967; Kessler et al., 1977). The virus normally establishes latency in the sacral dorsal root ganglia, and periodically reactivates (Baringer, 1974). The distinction in location between HSV-1 and HSV-2 is not absolute : both viruses may be found either orally or genitally. Indeed, in one study it was found that in 50% of cases of genital herpes, HSV-1 was responsible (Chaney et al., 1983). An association between HSV-2 and cervical carcinoma has been suggested (Naib, 1966; Eglin et al., 1981; zur Hausen, 1982; Park et al., 1983).*

Varicella-zoster virus is the causative agent of chicken-pox (varicella) during its primary infection. This virus also becomes latent in dorsal root ganglia (Gilden et al., 1978). Reactivation, usually in adults, may lead to shingles (zoster) (Weller, 1976) : this is at least partly dependent on the immunological status of the individual (Rifkind, 1966; Galb, 1985).

Human cytomegalovirus infection in children or adults is usually asymptomatic or associated with a mild fever. However, infection of foetus via their mothers is believed to be a major cause of intrauterine death and congenital defects (Weller, 1971; Rapp, 1980). An association with the European form of Kaposi's sarcoma has also been suggested (Giraldo et al., 1975). Reactivation of the virus is usually

associated with immunosuppression, often as a result of malignant disease or chemotherapy, and primary infection with, as well as reactivation of, HCMV is also associated with renal transplant rejection (Weller, 1971; Plummer, 1973; Gold and Nankervis, 1976). It has been suggested that HCMV may be associated with cervical carcinoma (Alford and Britt, 1984; Fletcher et al., 1986).

Epstein-Barr virus is B-lymphotropic. Infection in childhood is often asymptomatic, while infection during adolescence or into adulthood may cause infectious mononucleosis (Evans et al., 1968; Henle et al., 1968; Evans and Hinderman, 1976). This virus is also associated with Burkitt's lymphoma (Epstein et al., 1964; de Thé et al., 1978) and nasopharyngeal carcinoma (zur Hausen et al., 1970; Nonayama and Pagano, 1973; Miller, 1985).

Recently, a novel herpesvirus, called human B-lymphotropic virus (HBLV), has been isolated from humans (Josephs et al., 1986; Salahuddin et al., 1986). It was isolated from six African patients with lymphoproliferative disease, two of whom were infected with human immunodeficiency virus and who had the clinical symptoms of AIDS. The electron microscopic structure and genome size of the virus allowed its classification as a herpesvirus. No serological cross-reactivity or genome cross-hybridization with other human or animal herpesviruses was detected. Although all the infected patients had antibodies to HBLV, antibodies were found in only four out of 220 sera from healthy controls. Thus, unlike the other human herpesviruses, HBLV would appear to be relatively rare in the human population, and may be associated with a range of

lymphoproliferative diseases. However, it remains to be determined whether this is a genuine human herpesvirus or if it is only the result of an opportunistic infection of immunosuppressed patients by an undiscovered animal herpesvirus.

I.5 HERPES SIMPLEX VIRUS LATENCY

It has long been believed that HSV undergoes a latent infection with periodic recurrences over many years (Goodpasture, 1929). The mechanism of latency and recurrence appears to be identical for HSV-1 and HSV-2. One individual may have primary and secondary infections with both serotypes (Wheeler, 1975). The site of recurrence is largely determined by the primary infection (Chang, 1971), with latency occurring in the sensory ganglion supplying the primary infected area (Bastian et al., 1972; Baringer and Swoveland, 1973; Baringer, 1974; McLennan and Darby, 1980).

Following primary infection, HSV travels up the neuronal axon (Cook and Stevens, 1973; Kristensson et al., 1974) to establish latency within the neuronal cell bodies in the dorsal (sensory) root ganglion (Baringer and Swoveland, 1973; McLennan and Darby, 1980; Cook et al., 1984). Latent HSV may reactivate from cultured ganglia, from human cadavers or experimentally infected animals, either spontaneously (Baringer and Swoveland, 1973; Baringer, 1974; Warren et al., 1977, 1978) or following superinfection with temperature-sensitive (ts) mutants at their non-permissive temperature (Brown et al., 1979; Wigdahl et al., 1982; Lewis et al., 1984). In vivo, HSV will remain latent within the

ganglia until triggered to reactivate (by mainly unknown stimuli, although uv light and stress may be important), potentially leading to the presence of detectable virus at the periphery (recurrence) or herpetic lesions (recrudescence) (Wildy et al., 1982).

The neurons themselves were shown to be the site of latent HSV, by studies involving the reactivation of ts mutants (McLennan and Darby, 1980) and by in situ hybridization (Puga et al., 1978). There is now some evidence that HSV may also become latent at the site of primary infection, but the cells involved are unknown (Scriba and Tatzer, 1981; Al-Saadi et al., 1983; Hill et al., 1983; Openshaw, 1983). More recently, HSV has also been isolated up to 11 days post-explantation from explanted human corneas, removed during the course of treatment for stromal keratitis (Shimeld et al., 1982; Tullo et al., 1985; Cook, 1988), and also from the corneas of latently-infected rabbits (Cook et al., 1987).

The state of latent virus DNA is unclear. Southern blotting analysis of ganglionic DNA suggests that latent HSV DNA lacks ends, while the joints are over-represented (see later, section I.6.a), suggesting that the DNA is not in an unintegrated linear form, but may be present as either long concatamers or in a circular form (Rock and Fraser, 1983, 1985). Recent buoyant density studies favour the latter state (Mellerick and Fraser, 1987). Whether HSV expression occurs during latency is also open to speculation. Puga et al. (1978) could find no evidence of viral transcription. In contrast, other workers have found limited transcription or antigen expression (Galloway et al., 1979, 1982; Green et

al., 1981; Kennedy et al., 1983) - but the presence of reactivating cells within the population cannot be excluded. Of much more interest is the finding, during latent infection, of transcripts from the long repeat regions of HSV which are not present in lytically-infected cells. These have been mapped to the opposite strand to the immediate-early 1 gene, starting downstream but with the 3' terminus overlapping IE1 (Stevens et al., 1987).

At present, the host and viral factors affecting latency and reactivation are extremely poorly, if at all, understood, and much work still remains to be done. Although animal models have been mainly used to study HSV latency, more recently in vitro systems, using cell cultures, have been developed (Wigdahl et al., 1983, 1984a,b; Russell and Preston, 1986; Cook and Brown, 1987). Such assays together with knowledge of the HSV DNA sequence and gene products should considerably help to address these points.

I.6 THE HERPES SIMPLEX VIRUS GENOME

I.6.a Structural features

The HSV-1 genome is a linear double-stranded DNA molecule of around 100×10^6 mol. wt. (155,000 bp in length) (Becker et al., 1968; Frenkel and Roizman, 1971; Kieff et al., 1971; Grafstrom et al., 1974; Clements et al., 1976; Davison, 1981). Partial denaturation studies suggested that HSV-1 has an overall G+C content of 67% (Kieff et al., 1971), although this varies in different areas of the genome, with the repeat regions having a higher G+C content than the unique regions : for example, the short repeat has

a G+C content of 79% (Davison and Wilkie, 1981; Murchie and McGeoch, 1982). Sequence analysis has shown that these estimates are not completely accurate. In HSV-1 strain 17, the overall G+C content is 68.3%; R_L is 71.6%; U_L is 66.9%; U_S is 64.9%; and R_S is 79.5% (Dr. D. J. McGeoch, personal communication). The genomes of HSV-1 and HSV-2 share 50% homology along their entire length (Kieff et al., 1972), are colinear and share the same structural features (Wilkie et al., 1979; Davison and Wilkie, 1983c); most of the detailed analysis having been carried out on HSV-1. This similarity at the DNA level also extends to the protein level : many analogous HSV-1 and HSV-2 proteins possess type-common as well as type-specific epitopes, and vary only slightly, if at all, in apparent mol. wt. (Honess and Watson, 1974; Halliburton et al., 1977a; Marsden et al., 1978). The only detailed sequence comparison between HSV-1 and HSV-2 has been in the short unique region (McGeoch et al., 1985, 1987), confirming earlier conclusions about colinearity drawn from hybridization (Kieff et al., 1972) and electron microscopy (Kudler et al., 1983) studies. The only large difference found by McGeoch et al. (1987) was in U_S4 (gG), the coding sequence in the HSV-2 gene being larger by approximately 1500 bp.

I.6.b Fragmentation of herpes simplex virus DNA in alkali

Herpes simplex virus DNA, like that of other herpesviruses, fragments upon denaturation with alkali (Kieff et al., 1971; Frenkel and Roizman, 1972; Wilkie, 1973). Following extraction from virions, usually only about 15% of the DNA is intact. Although HSV DNA does contain

stretches of short ribonucleotides (Gordini et al., 1973; Muller et al., 1979) it is unlikely that these significantly contribute to fragmentation upon alkali denaturation, since an equal amount of fragmentation was reported on neutral sucrose gradients following formamide denaturation (Roizman, 1979). It has been suggested that fragmentation is due to the presence of single-strand nicks or gaps in HSV DNA (Frenkel and Roizman, 1972; Wilkie, 1973; Wadsworth et al., 1976; Ecker and Hyman, 1981), probably randomly distributed on both strands (Wilkie, 1973; Ecker and Hyman, 1981).

I.6.c Organization of the herpes simplex virus genome

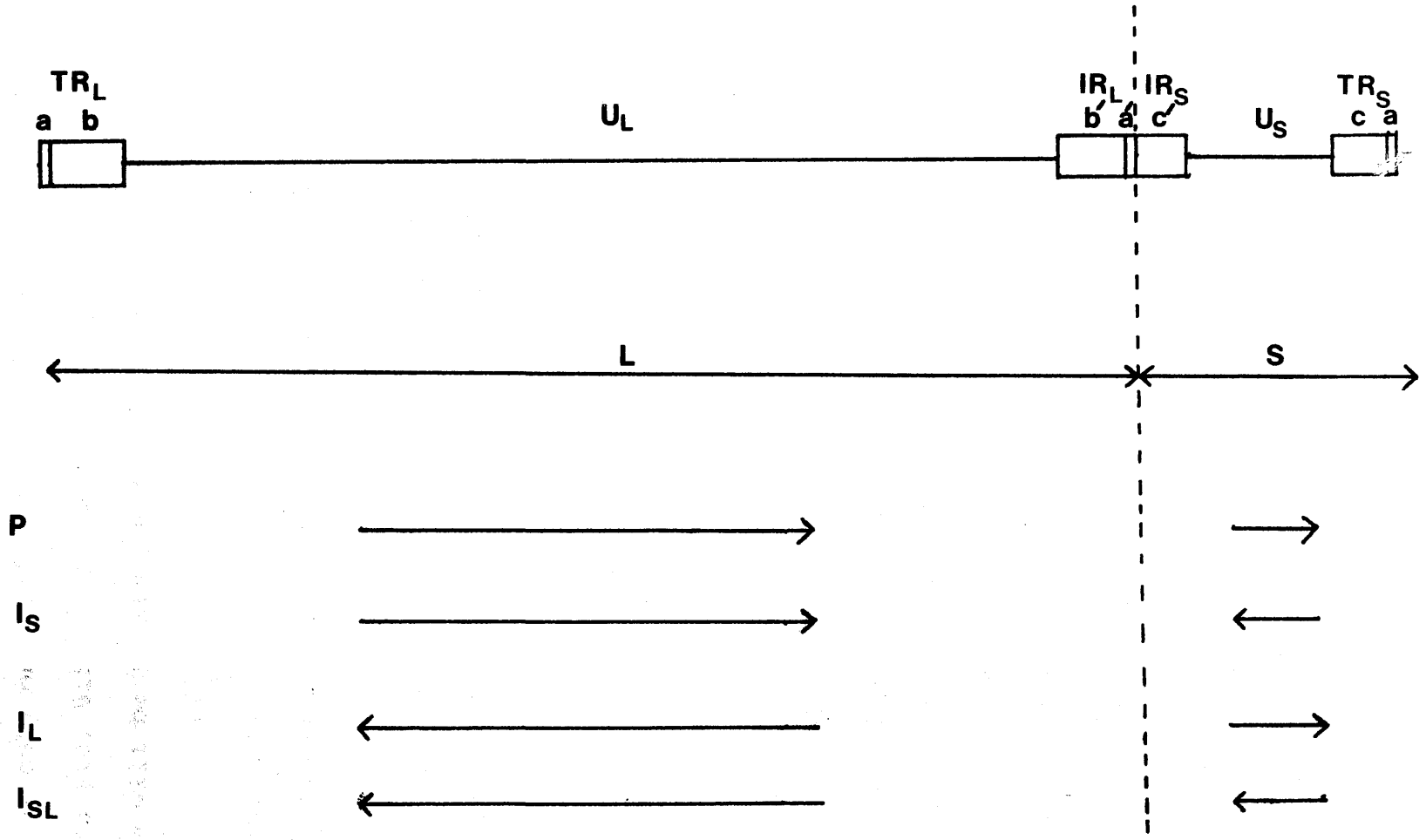
Herpes simplex virus DNA consists of 2 unique segments, the long unique (U_L) and the short unique (U_S), each bounded by a set of internal (IR) and terminal (TR) inverted repeats, IR_L/TR_L and IR_S/TR_S, respectively (Sheldrick and Berthelot, 1974; Delius and Clements, 1976; Figure 3). A short sequence, known as the 'a' sequence, is present as a direct repeat at the termini and in an inverted orientation at the L-S junction (Sheldrick and Berthelot, 1974; Delius and Clements, 1976; Wadsworth et al., 1976; Davison and Wilkie, 1981) and varies in size both between and within strains. (For a full description of the 'a' sequence, see later, section I.7).

Preparations of HSV DNA contain four equimolar isomers, which differ in the relative orientations of the two unique regions about the L-S junction (Figure 3); these are designated P (prototype), I_S (inversion of U_S), I_L (inversion of U_L) and I_{SL} (inversion of U_S and U_L) (Delius and Clements, 1976; Hayward et al., 1975; Morse et al.,

Figure 3. Structure of the HSV genome.

The genome consists of a long (L) and short (S) region, each comprising a unique sequence (U_L and U_S) flanked by inverted repeats, TR_L/IR_L and TR_S/IR_S respectively. The 'a' sequence is present as a direct repeat at the genomic termini and as an inverted repeat at the L-S junction. The remainder of R_L is known as b, b' and of R_S as c, c'.

Inversion of the L and S segments occurs between inverted copies of the 'a' sequence to generate four isomeric forms of viral DNA found in equimolar amounts. These are designated P (prototype), I_S (inversion of the short segment), I_L (inversion of the long segment) and I_{LS} (inversion of the long and short segments).


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

1977). Inversion is believed to be mainly due to site-specific recombination via the 'a' sequence (Mocarski et al., 1980; Mocarski and Roizman, 1981; Smiley et al., 1981). Provided that there are no cleavage sites within the repeat regions, one consequence of inversion is that, on a restriction enzyme profile, the terminal fragments will be present at only one-half the concentration of the internal fragments (ie. 0.5M), and those fragments spanning the L-S junction will be present at only one-quarter the concentration of the internal fragments (ie. 0.25M) (Wilkie, 1976). The four genome isomers would appear to be functionally equivalent (Davison and Wilkie, 1983 a,b; Preston et al., 1978; Poffenberger et al., 1983; Poffenberger and Roizman, 1985; Longnecker and Roizman, 1986), despite earlier claims to the contrary (Morse et al., 1977; Roizman, 1979).

I.6.d Presence of short reiterations in herpes simplex virus DNA

In addition to variation in the size of terminal fragments of HSV DNA, due to the presence of multiple copies of, and variation within, the 'a' sequence, several other fragments in the genome exhibit varied mobility (Locker and Frenkel, 1979; Davison and Wilkie, 1981; Lonsdale et al., 1981). Sequence analysis of several clones from regions exhibiting altered mobility has shown that such variation is due to the presence of short tandemly reiterated sequences which vary in length between 5 bp and 54 bp (Whitton and Clements, 1984b; Perry, 1986). Each family has a distinct sequence, generally having a high G+C content, homopolymer

runs and marked strand asymmetry with respect to purine versus pyrimidine content (Rixon et al., 1984). The locations of these repeats in HSV-1 around the short region and long repeat are shown in Figure 4, and have been numbered I-XII (McLauchlan, 1986). Reiteration I (Davison and Wilkie, 1981) is equivalent to the DR_2 repeat element of Mocarski and Roizman (1981), and lies within the 'a' sequence. Reiterations II-XII are situated within U_S genes 7, 10 and 11 (Rixon and McGeoch, 1984; McGeoch et al., 1985); in the introns of IE mRNAs 1, 4 and 5 (Murchie and McGeoch, 1982; Perry et al., 1986); downstream from the mRNA 3' termini of IE 1 and 3 (Davison and Wilkie, 1981; Rixon et al., 1984) and of U_S 8, 9, 10, 11 and 12 (McGeoch et al., 1985); and in an apparently untranscribed region in the long repeat (Perry, 1986). A thirteenth repeat is present in U_L, at around 0.5 map units (m.u.) (Dr. D. J. McGeoch, personal communication).

The copy number of repeated sequences varies between virus isolates and, similarly, serial passage and recloning of the one isolate leads to variation in the number of the repeats (Davison and Wilkie, 1981; Watson et al., 1981; Murchie and McGeoch, 1982; Rixon et al., 1984; Perry, 1986). Comparisons between HSV-1 and HSV-2 revealed that the DNA sequence of the repeat elements is either poorly conserved (reiteration IV, Whitton and Clements, 1984b) or is absent from the HSV-2 genome (reiterations I and VI, Davison and Wilkie, 1981; Whitton, 1984).

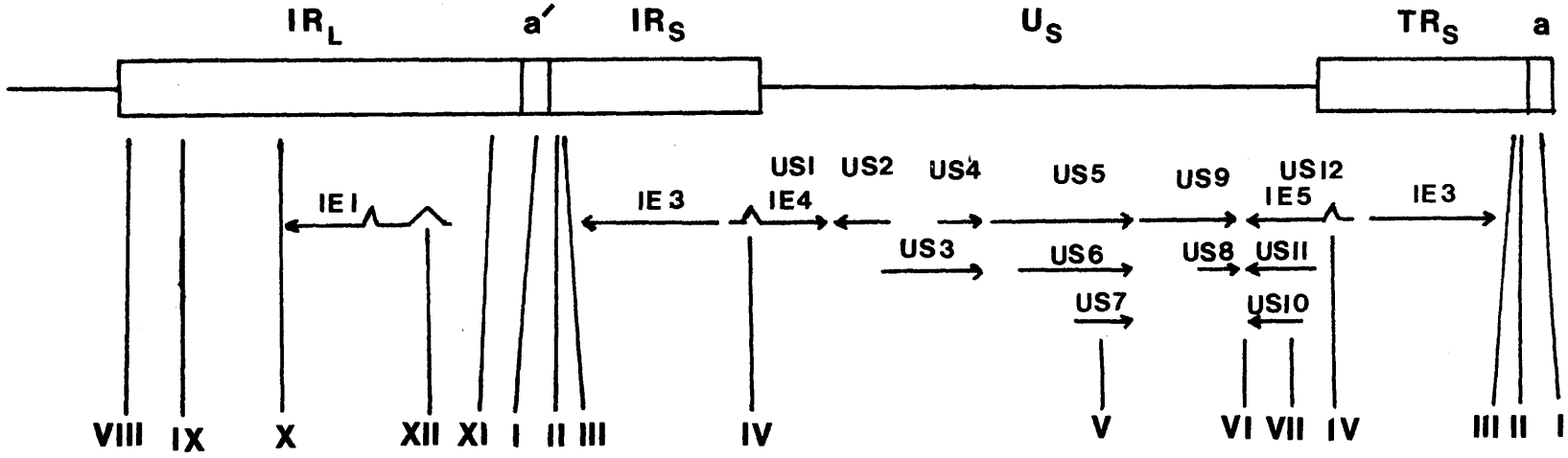
Immediate-early genes 1 and 3 are located entirely within the repeats (Clements et al., 1979; McGeoch et al., 1986; Perry et al., 1986) and are thus present in diploid

Figure 4. HSV-1 short tandem reiterations.

Locations of the short tandemly reiterated sequences within the S segment and R_L regions of HSV-1 strain 17 DNA. Positions of reiterated sequences (numbered I to XII) are shown relative to the locations of mRNAs mapping in this region of the HSV-1 genome.

A thirteenth reiteration is present in the middle of U_L.

Adapted from McLauchlan, 1986.


IR_L
a'
IR_S
U_S
TR_S
a
IE1
IE3
US1
IE4
US2
US4
US5
US9
US12
IE5
IE3
US3
US6
US8
US11
US7
US10
VIII
IX
X
XII
XI
I
II
III
IV
V
VI
VII
IV
III
II
I

amounts. Reiterations flanking the regions may serve to promote genetic exchange between the repeats, thus maintaining homology (Rixon et al., 1984; Umene, 1987). Such a process would lead to extensive variation in the copy number of the reiterations. This hypothesis depends on two assumptions : (i) Reiterations promote a high degree of recombination. In support of this assumption is the extensive variation found in the copy number of the repeat elements. In addition, reiteration II shares homology with an immunoglobulin class-switch sequence involved in high frequency recombination (Gomez-Marquez et al., 1985); and (ii) Reiterations are located in non-essential regions of the genome. Reiterations II and III, which lie between the 'a' sequence and 3' terminus of IE3, can be deleted without affecting virus viability or coding potential (Hubenthal-Voss and Roizman, 1985), as can reiterations VIII and IX, downstream from IE1 (MacLean and Brown, 1987b, c; this thesis).

I.7 HERPES SIMPLEX VIRUS 'a' SEQUENCE

The 'a' sequence of HSV is located at the L and S termini of the genome in a directly repeated orientation and at the L-S junction in inverted orientation (Wadsworth et al., 1975, 1976). Although only one 'a' sequence is found at the S terminus, there can be up to 10 'a' sequences at the L terminus and L-S junction, although a single 'a' sequence is the predominant form. Thus, restriction enzyme fragments from the L terminus and L-S junction vary in size and are often seen as more than one band on a gel (Wagner and

Summers, 1978; Locker and Frenkel, 1979; Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982b).

I.7.a Structure of the 'a' sequence

DNA sequencing of the 'a' sequence from several HSV-1 and HSV-2 strains has been reported (Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982b; Mocarski et al., 1985; Varmuza and Smiley, 1985). Its length, around 250-550 bp, varies, both within and between strains. The 'a' sequence can be divided into several structural regions (Mocarski and Roizman, 1981) (Figure 5). It consists of both unique (U) and directly repeated (DR) elements; variation in the copy number of these DR elements is mainly responsible for the difference in size between different 'a' sequences. The HSV-1 strain F L/S junction 'a' sequence (Mocarski and Roizman, 1981) can be represented as DR_1-U_b-$(DR_2)_{19}$-$(DR_4)_3$-U_c-DR_1 : DR_1 is a 20 bp sequence present at both ends; U_b and U_c are unique 58 bp and 65 bp sequences, respectively, and are named by virtue of their proximity to the b' and c' repeated regions, respectively; DR_2 is a 12 bp sequence present in 19-22 tandem copies; and DR_4 is a 37 bp sequence, present in 3 tandem copies.

The copy number of the DR_2 repeat varies extensively both between and within strains. For example, HSV-2 strain HG52 has only one copy of DR_2 (Davison and Wilkie, 1981), while HSV-1 strain F has 19-22 copies. Similarly, many strains, including HSV-1 strain 17 and HSV-2 strain HG52, contain only one copy of a DR_4 homology, which is therefore regarded as part of Uc (Davison and Wilkie, 1981). Hence, HSV-2 strain HG52 could be regarded as containing a single

Figure 5. Structure of the HSV-1 'a' sequence.

A. A HSV-1 genome in the prototype orientation.

B. An expansion of the 'a' sequence in the orientation found at the L-S junction, showing part of b' and c'. The 'a' sequence consists of unique (U) and directly repeated (DR) sequences.

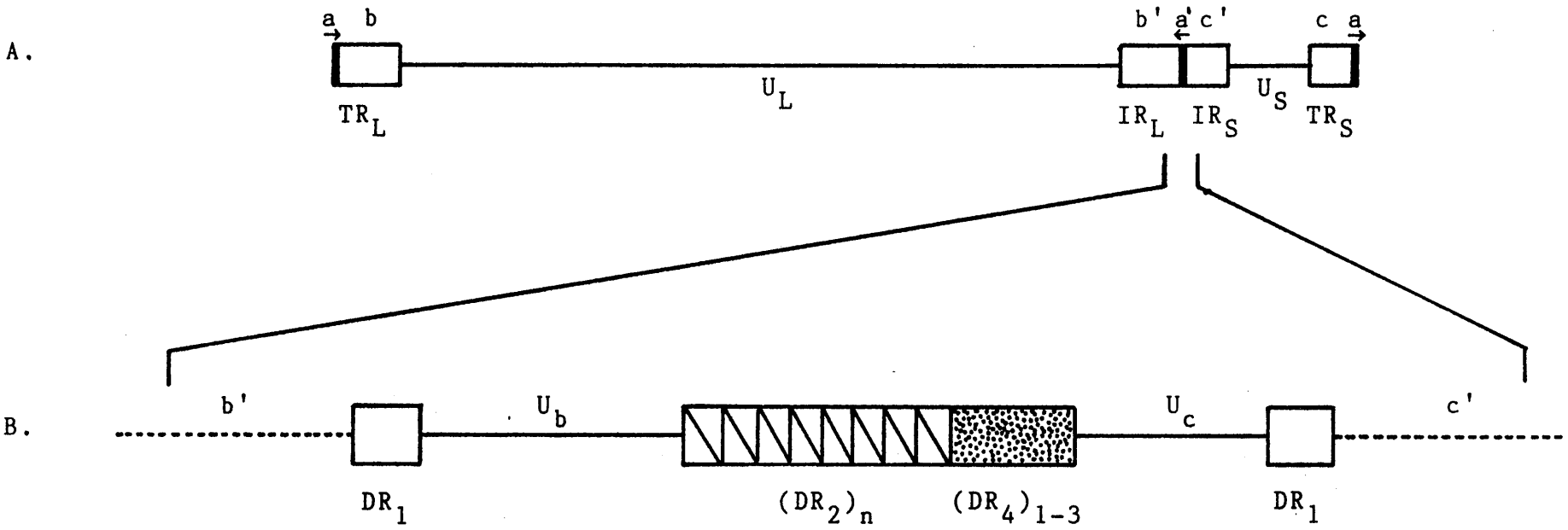
Ub : a unique sequence located towards the b' sequence.

Uc : a unique sequence located towards the c' sequence.

DR1 : a 17-21 bp element, present as a direct repeat at the ends of the 'a' sequence.

DR2 : a 12 bp element, present in 1 to at least 22 copies.

DR4 : a 37 bp element, present in 1 to 3 copies.


A.
a b
b' a' c'
c a
U_L
U_S
TR_L
IR_L
IR_S
TR_S
B.
b'
U_b
U_c
c'
DR_1
$(DR_2)_n$
$(DR_4)_{1-3}$
DR_1

unique sequence, bounded by a direct repeat (DR_1). HSV-1 strain Justin contains two copies of an extra direct repeat, called $DR_{3.5}$ (Mocarski and Roizman, 1981, 1982b; Mocarski et al., 1985).

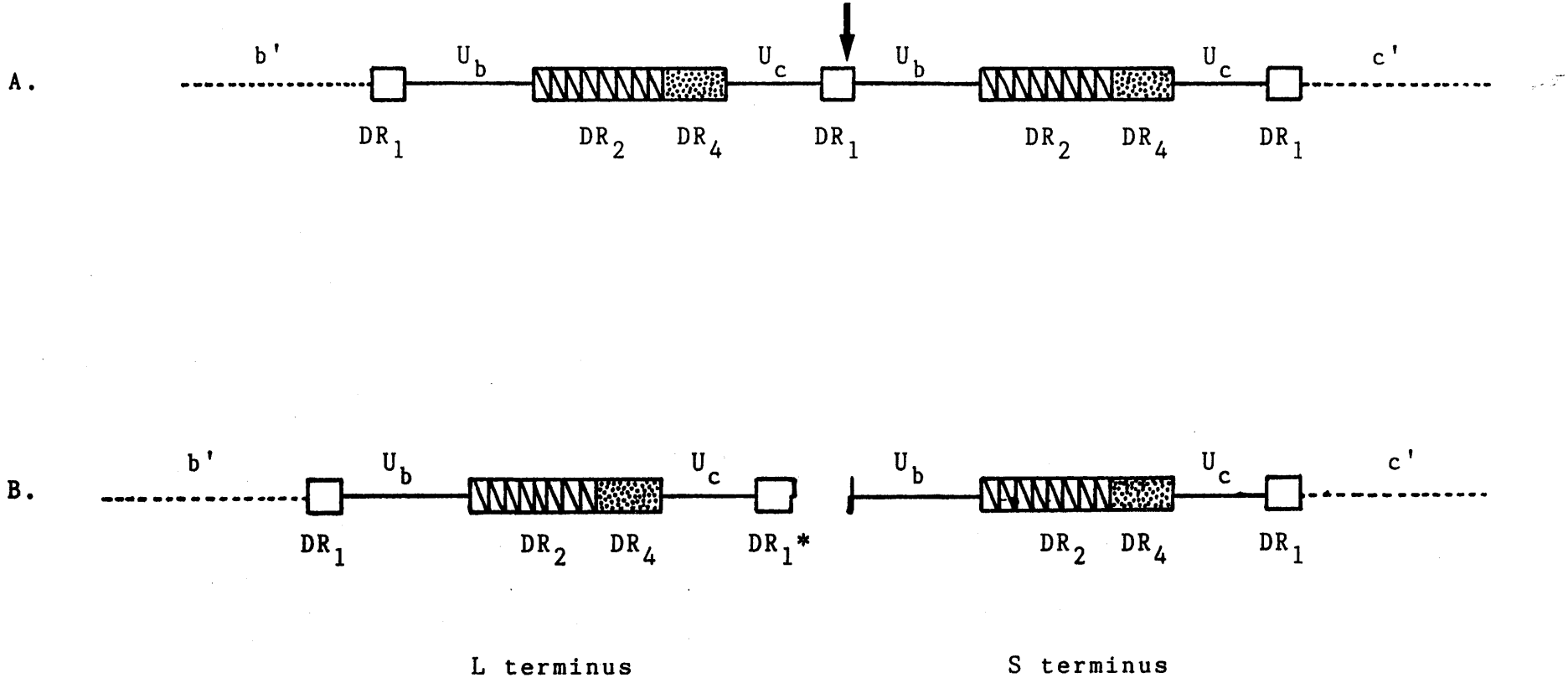
There is some conservation of DR_1, DR_2 and DR_4 between different virus strains (Mocarski and Roizman, 1981, 1982b; Mocarski et al., 1985; Varmuza and Smiley, 1985). Within U_b and U_c there are short, well-conserved sequences of about 20 bp located approximately 40 bp and 35 bp from the ends of the 'a' sequence, respectively (Davison and Wilkie, 1981; Deiss et al., 1986). These homologies in U_b and U_c are shared by the CMV 'a' sequence (Spaete and Mocarski, 1985) and homologous sequences are found at the termini of several other herpesviruses (Deiss et al., 1986). The functional role of these sequences is discussed in more detail below.

Tandemly reiterated 'a' sequences share the intervening DR_1 element (Figure 6). The S and L terminal 'a' sequences differ from a junctional 'a' sequence, in that they contain an incomplete copy of the terminal DR_1 : for HSV-1 strain F, DR_{1c} of the L-terminus 'a' sequence consists of 18 bp and a single nucleotide extended 3' (18.5 bp), while DR_{1b} of the S-terminus 'a' sequence consists of 1 bp and a single nucleotide extended 3' (1.5 bp) (Mocarski and Roizman, 1982b) (Figure 6); similarly, the HSV-1 strain 17 L-terminus 'a' sequence contains 20.5 bp of the 21 bp DR_{1c} element, with only 0.5 bp of the S-terminus DR_{1b} element (Davison and Rixon, 1985). Together the two partial sequences form a complete DR_1. Thus, cleavage of a double 'a' sequence at the shared DR_1 element could generate L and S terminal 'a' sequences (Figure 6). Likewise, circularization of the

Figure 6. Structure of double and L and S terminal 'a' sequences.

A. Tandem copies of the 'a' sequence share the intervening DR1 element. The 'a' sequences are shown in the orientation seen in Figure 5. Cleavage through the intervening DR1 element (↓) would generate the termini shown in B.

B. Structure of the L and S terminal 'a' sequences. These are shown in the same orientation as A. L and S terminal 'a' sequences possess only partial DR1 elements ($DR1^{*}$) with single nucleotide 3' extensions, such that annealing of an L and S terminus would form a complete DR1 element as in A.


A.
b'
U_b
U_c
U_b
U_c
c'
DR_1
DR_2
DR_4
DR_1
DR_2
DR_4
DR_1
B.
b'
U_b
U_c
U_b
U_c
c'
DR_1
DR_2
DR_4
DR_1*
DR_2
DR_4
DR_1
L terminus
S terminus

genome will form a double 'a' sequence sharing the intervening DR_1.

I.7.b Circularization

Following infection, the HSV genome rapidly circularizes : this is believed to be mediated by the 'a' sequence (Davison and Wilkie, 1983a; Poffenberger et al., 1983; Poffenberger and Roizman, 1985). Although it was originally suggested to occur by exposure of complementary cohesive termini following digestion with a processive exonuclease (Roizman, 1979), it is more likely that circularization takes place by ligation of the two termini, aided by the complementary single base 3' overhang (Mocarski and Roizman, 1982a,b; Davison and Rixon, 1985). Evidence in favour of the latter suggestion is the fact that HSV genomes containing heterotypic 'a' sequences circularize (Davison and Wilkie, 1983a) and that both PRV and VZV, although lacking a terminal redundancy, circularize following infection (Davison, 1984; Harper et al., 1986).

Circularization occurs in the absence of protein synthesis (Poffenberger and Roizman, 1985), although it is possible that proteins bound to the 'a' sequence may play a role.

I.7.c Promoter activity

Chou and Roizman (1986) have mapped the promoter of a gene (ICP34.5), whose coding region is in the long repeat (Ackerman et al., 1986), to the U_b/DR_2 region of HSV-1 strain F. The 5' termini of the transcripts map to DR_1 and to the long repeat outside the 'a' sequence. The promoter is

atypical of HSV genes in that there is no TATA consensus in the normal position (ie. around -25) - the best TATA homology (TTTAAA) being at -15. The function and essential nature of this protein are unknown. Interestingly, in the only other HSV strain sequenced in this region, HSV-1 strain 17, no open reading frame coding for ICP34.5 has been detected in the long repeat (Dr. D. J. McGeoch, personal communication). Thus, whether promoter activity is a general feature of most HSV 'a' sequences remains to be determined.

I.7.d Site-specific recombination

A HSV DNA population consists of 4 equimolar isomers differing in the relative orientation of U_L and U_S (Sheldrick and Berthelot, 1974; Hayward et al., 1975; Delius and Clements, 1976; section I.6.c, Figure 3). Inversion occurs about the L-S junction, and is mediated by site-specific recombination through the 'a' sequence (Mocarski et al., 1980; Mocarski and Roizman, 1981; Smiley et al., 1981). These workers showed that insertion of an additional 'a' sequence into the thymidine kinase (tk) locus leads to inversion of the region of DNA which lies between inverted repeats of the 'a' sequence. Sequences from other regions of the genome failed to induce inversion - although this conclusion has subsequently been modified (see below). Mocarski and Roizman (1982a) suggest that inversion is mediated by trans-activating viral products, since protein synthesis is required.

Davison and Wilkie (1983a) have shown that in HSV-1 strain 17/HSV-2 strain HG52 recombinants, where the L-S junction 'a' sequence differs from the terminal 'a'

sequence, isomerization does not occur. Therefore, lack of 'a' sequence homology may block site-specific recombination.

Chou and Roizman (1985) carried out a detailed deletion analysis of the 'a' sequence and localized the important sequences for inversion to DR_4 and DR_2. They suggest that DR_4 may be responsible for high frequency inversion, with recombination itself taking place between DR_2 elements, which are by themselves capable of inverting at a low frequency. Unequal recombination between 'a' sequences via DR_2 is believed to be at least partly responsible for the different copy number of DR_2 within the one strain (Mocarski and Roizman, 1982a,b). However, the constructs of Chou and Roizman (1985) did not exclude a role for DR_1 in inversion; indeed Varmuza and Smiley (1985) showed that deletion of both DR_1 elements led to extremely low frequency inversion.

I.7.e Other herpes simplex virus sequences capable of mediating inversion events

Other regions within the repeats are also capable of causing isomerization. A sequence within the long repeat, approximately 5 kbp from the 'a' sequence, appears capable of causing low frequency inversion of U_L (Longnecker and Roizman, 1986). Reiteration II within the short repeat appears capable of mediating high frequency inversion of U_S (Davison and Wilkie, 1981; Mocarski and Roizman, 1981; Varmuza and Smiley, 1984). As previously mentioned, this sequence is homologous to an immunoglobulin switch region (Gomez-Marquez *et al.*, 1985).

When sequences from other areas of the genome are inserted into the tk locus, in inverted orientation, they

generally fail to stimulate detectable inversion of the intervening sequences. However, when sequences from 0.706-0.744 m.u. (of HSV-1 strain F) are inserted into the tk locus, in inverted orientation, the intervening sequences are inverted and markers within these repeated sequences are converted to homology (Pogue-Geile et al., 1985; Pogue-Geile and Spear, 1986). This is similar to the picture seen with the repeats, where sequence differences (both point mutations and deletions) are, in most cases, rapidly converted to homology (Stow and Stow, 1986; Sacks and Schaffer, 1987; Umene, 1987). However, Harland and Brown (1985; and personal communication) failed to detect conversion to homology in variants containing deletions within one long repeat. One difference in the latter variants is the large size of the deletions and, in some cases, their proximity to the U_L/R_L boundary.

The significance of inversion, with respect to the virus replicative cycle, is unknown. Non-inverting variants in at least three orientations are viable, both in vivo and in vitro (Davison and Wilkie, 1983a; Poffenberger et al., 1983; Poffenberger and Roizman, 1985; Jenkins and Roizman, 1986; Longnecker and Roizman, 1986).

I.7.f Cleavage/packaging

Herpes simplex virus DNA replication involves the formation of head-to-tail concatamers which are cleaved into unit length genomes and packaged into nucleocapsids in the cell nucleus (Ben-Porat et al., 1976; Ben-Porat and Rixon, 1979; Jacob et al., 1979; Jongeneel and Bachenheimer, 1981) (see section I.8). So far, it has not been possible to

separate the two processes of cleavage and packaging (Ladin et al., 1982; Preston et al., 1983).

The HSV 'a' sequence has been shown to contain the cis-acting signals necessary for cleavage/packaging (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982; Vlazny et al., 1982; Stow et al., 1983; Deiss and Frenkel, 1986). Varmuza and Smiley (1985) and Deiss et al. (1986) have localized the sequences involved in cleavage/packaging. Sequences spanning the U_b and U_c homology regions would appear to be essential, and have been termed pac-1 and pac-2 (Deiss et al., 1986), cleavage appearing to occur in a sequence-independent manner at a set distance from the U_b and U_c cleavage/packaging signals. However, the constructs of neither group totally eliminated DR_4 or DR_1. Deiss et al. (1986) found that while $U_b^+U_c^-$ constructs did not result in the amplification of any of the input amplicons, $U_b^-U_c^+$ constructs were amplified and propagated (albeit inefficiently) - this required the insertion of a wild-type 'a' sequence from the helper virus. These results suggest that packaging begins at the L terminus, with cleavage occurring at U_c; the cut end can then ligate to a normal 'a' sequence; recombination could generate a normal U_b sequence, which would cause cleavage at U_b and termination of packaging at the S-terminus.

I.7.g Amplification of the 'a' sequence

If an amplicon containing a single 'a' sequence is used as a seed monomer, this will be packaged as tandem repeats of genome length containing an 'a' sequence at each end (Stow et al., 1983; Deiss and Frenkel, 1986). Three models have been proposed to account for the apparent amplification

of these single 'a' sequences (Varmuza and Smiley, 1985; Deiss et al., 1986).

I.7.g.i Directional cleavage-packaging model

This model was proposed by Deiss et al. (1986) as a modification of the 'theft' model of Varmuza and Smiley (1985). It is illustrated in Figure 7 for standard virus DNA head-to-tail concatamers, and involves the following steps : (i) A packaging complex encounters the HSV DNA and travels at random until an 'a' sequence containing a U_c signal is found; (ii) Double-stranded cleavage (producing a 3' single base extension) occurs at the DR_1 element proximal to the first U_c signal encountered (cleavage 1). If the complex has traversed into a ba_nc type junction starting from the L component (around 80% of the time, assuming random initial binding), the first cleavage will produce ba and ca_{n-1} termini. If the process started from the S component (around 20% of the time), the first cleavage will generate ba_n and c termini. In both cases, directional packaging will begin from the generated L terminus (ba or ba_n); (iii) Packaging will continue by scanning in the L-to-S direction, until a directly repeated junction is encountered; (iv) a second double-stranded cleavage (also yielding 3' single base extensions) will then occur at the DR_1 proximal to the U_b signal encountered within that junction. This cleavage (cleavage 2) will produce a ca terminus on the packaged molecule and a free ba_{n-1} terminus. Free termini possessing an 'a' sequence could subsequently be packaged into a separate capsid; in contrast, free termini lacking an 'a' sequence - which are not normally

Figure 7. Models for the cleavage and packaging of HSV DNA.

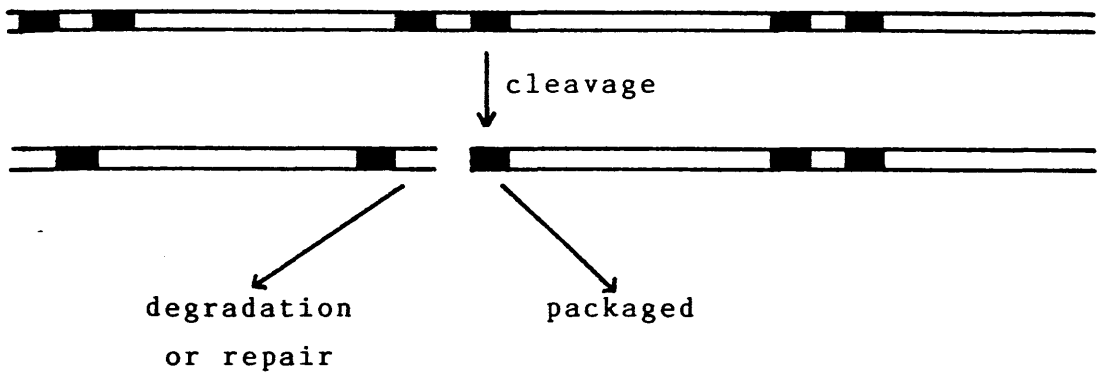
A. The wastage model (Varmuza and Smiley, 1985; Deiss et al., 1986) proposes that cleavage at a single 'a' sequence generates one functional terminus, containing an 'a' sequence, which is packaged and one non-functional terminus lacking an 'a' sequence which is rapidly degraded.

B. The single-strand nick/repair model (Varmuza and Smiley, 1985) proposes that the cleavage event generates two single-strand nicks at either end of a single 'a' sequence, the strands separate and are then repaired to generate two 'a' sequence-containing termini which could then be packaged.

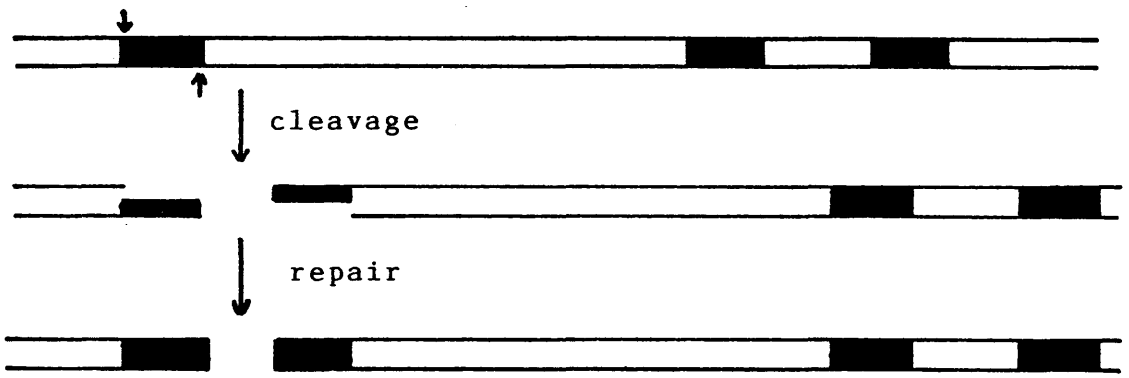
C. The double-strand/gap repair model (Deiss et al., 1986) is more complicated and is detailed in Figure 8. Simply, DNA is packaged prior to cleavage, the two directly repeated 'a' sequences then align and are amplified by a double-strand break/repair mechanism to generate two double 'a' sequence-containing junctions. Cleavage between the double 'a' sequences then generates four 'a' sequence-containing termini.

In A, B and C 'a' sequences are represented by ▬ and in C also by ▭ .

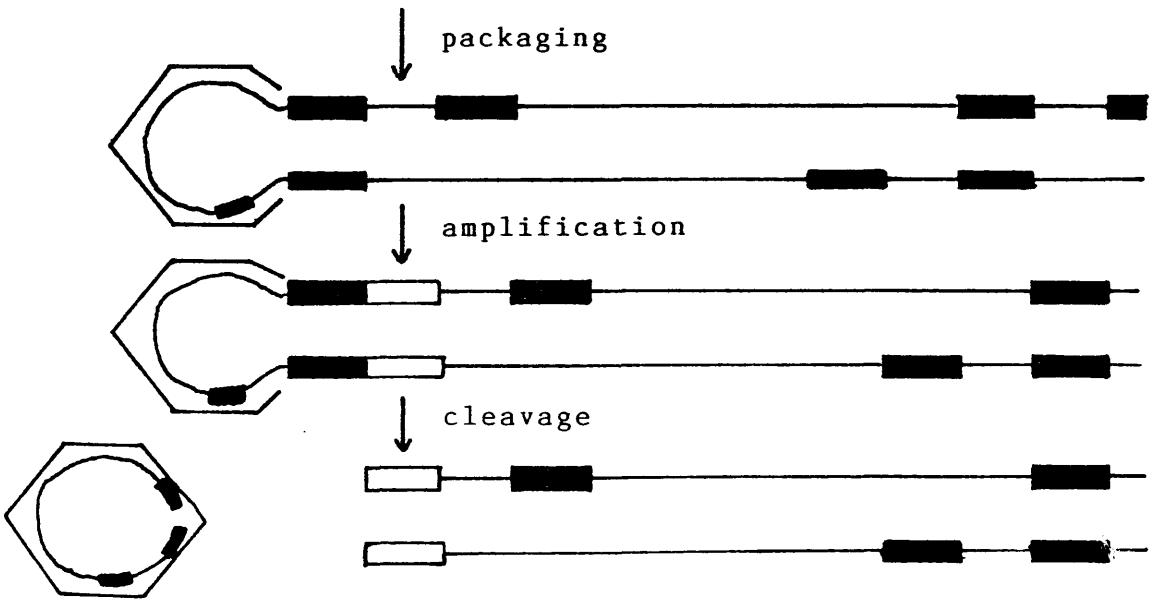
(a) Wastage model



(b) Single-strand nick/repair model



(c) Double-strand gap/repair model



detected - must either be rapidly degraded or repaired.

The model accounts for a number of features of HSV cleavage/packaging : (i) the generation of two terminal 'a' sequences from one junction 'a' sequence (Deiss and Frenkel, 1985) is explained by the loss of DNA between two 'a' sequences; (ii) the presence of only one 'a' sequence at the S terminus, but a variable number at the L terminus, with around 80% being single (Locker and Frenkel, 1979); (iii) it might also account for the apparent loss of 'a' sequences from multiple 'a' containing junctions - these could arise simply as a consequence of genome circularization following entry into the cell (Mocarski and Roizman, 1982a; Poffenberger and Roizman, 1985); and (iv) the presence of 3' single base extensions at the genome termini (Mocarski and Roizman, 1982b).

However, the model does not fully explain all the findings of Deiss et al. (1986). In addition, as termini lacking 'a' sequences have not been detected (Mocarski and Roizman, 1982a; Deiss and Frenkel, 1986), the degradation of such products must be very rapid.

I.7.g.ii Double-strand break and gap repair model

To explain some of the above anomalies, Deiss et al. (1986) have proposed a second model based on the double-strand break-repair model of Szostak et al. (1983). This model involves the interaction of two directly repeated junctions resulting in amplification of the 'a' sequence by a gene-conversion-like mechanism. The resultant junctions, containing double 'a' sequences, are then cleaved with 3' single base extensions to yield the genomic termini (Figures

7 and 8). Cleavage/packaging thus includes the following steps : (i) As in the previous model, the packaging complex travels at random until it reaches the first U_c signal (Junction 1). No cleavage occurs; (ii) Packaging occurs in the L-to-S direction until the packaging complex meets a directly repeated junction (Junction 2); (iii) The two junctions are juxtaposed (Figure 8a) and a U_c signal-directed double-stranded cleavage occurs within the DR_1 element of either 'a' sequence (Figure 8b); (iv) The resultant 3' terminus invades the homologous sequence in the other junction and is extended by copying the 'a' sequence in that junction while displacing the equivalent strand (Figure 8c,d); (v) The displaced strand serves as the template for repair synthesis of the second strand of the invading 'a' sequence (Figure 8e); (vi) The process terminates by resolution of the two Holliday structures (Holliday, 1964) and is expected to occur at or near DR_1 (Figure 8f); (vii) If the other junction also contained a single 'a' sequence, then the process would be repeated in the other direction (Figure 8g). The result is amplification of the 'a' sequence in both junctions to generate a double 'a' sequence in which DR_1 is flanked by both U_b and U_c packaging signals; (viii) Both double 'a' sequence junctions are cleaved (generating a 3' single base extension) between the newly inserted and original 'a' sequence. This cleavage could also be the result of staggered single-stranded cleavages generated by U_b and U_c of adjacent 'a' sequences (see section I.7.g.ii).

This model predicts that around 80% of the packaged genomes (initial binding in L) will carry ba and ca termini

Figure 8. Double-strand gap/repair model (Deiss et al., 1986).

A. DNA is packaged prior to cleavage and the directly-repeated 'a' sequences, which will form the L and S termini, then align (junction A and B, respectively).

B. Uc-directed double-strand cleavage occurs through the DR1 element ( ) of junction A. The cleaved ends align with the DR1 elements of junction B.

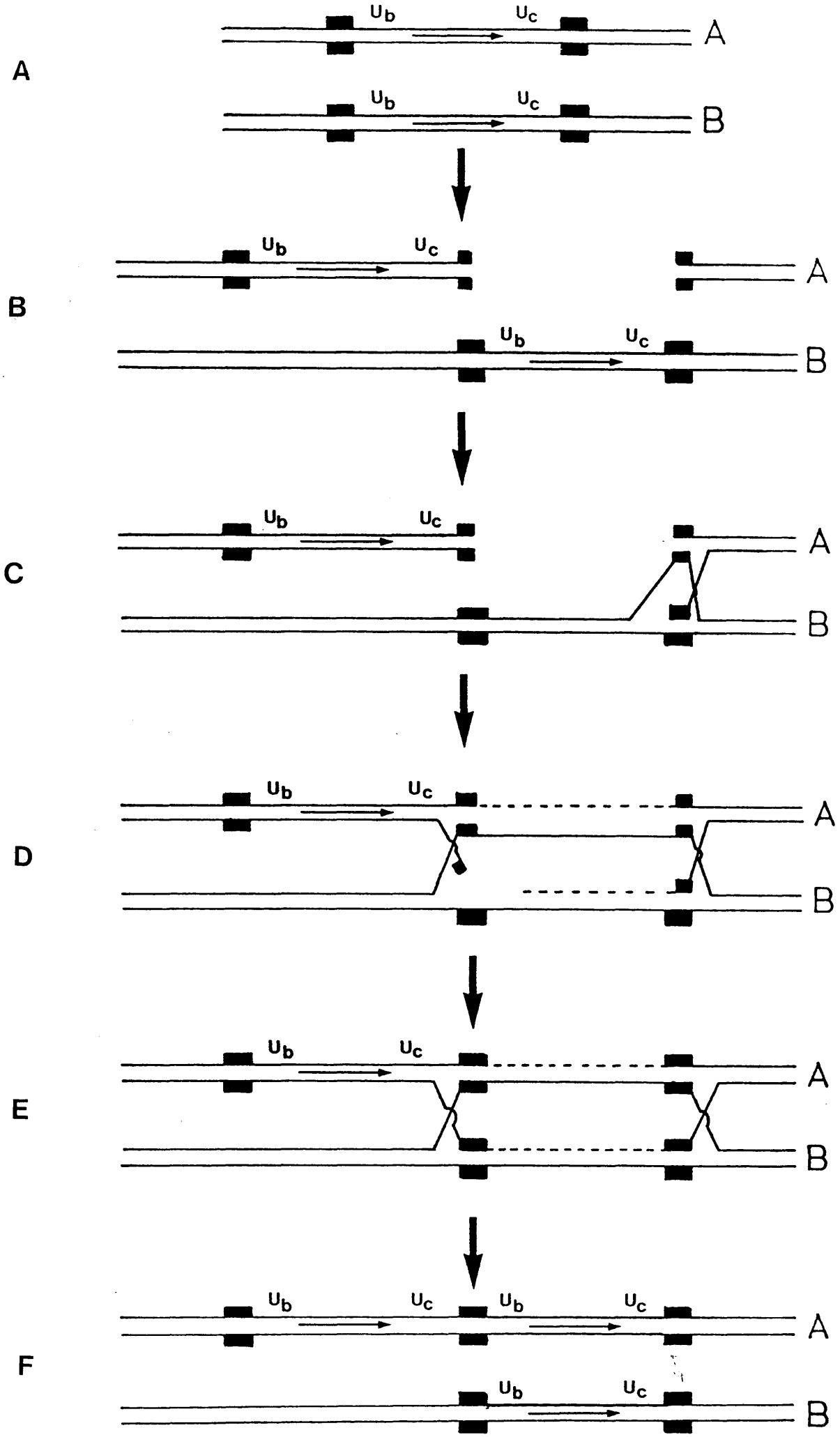
C. One strand from junction A then invades junction B, displacing the equivalent strand. (For simplicity this diagram is drawn with the top and bottom strands of junction B reversed).

D. The displaced strand from junction B aligns with partial DR1 elements of junction A and serves as a template for repair synthesis.

E. Similarly the non-displaced strand from junction B serves as a template for repair synthesis between the invading partial DR1 elements.

F. Resolution of the Holliday structure results in the presence of two directly repeated copies of the 'a' sequence at junction A.

G. A similar process is then envisaged to take place for junction B (not illustrated). Cleavage between the adjacent 'a' sequences then generates the L and S termini of the packaged DNA.


A
U_b
U_c
A
U_b
U_c
B
B
U_b
U_c
A
U_b
U_c
B
C
U_b
U_c
A
B
D
U_b
U_c
A
B
E
U_b
U_c
A
B
F
U_b
U_c
U_b
U_c
A
U_b
U_c
B

regardless of the number of 'a' sequences in the cleaved junctions, whereas the remaining packaged genomes (initial binding in S) will be terminated with ca and ba_n sequences. The termini of the free molecules generated by the cleavage would be of the type ba_n and ca_n or ca, and might be either degraded or serve as a target for new packaging cycles.

The model accounts for all the features of HSV cleavage/ packaging explained by the previous model, and also some of the other anomalies. (i) No termini devoid of 'a' sequences are produced. (ii) It explains the finding of multiple 'a' sequence-containing S termini seen in defective-containing stocks (Deiss and Frenkel, 1986). (iii) It would account for the structures found by Deiss et al. (1986). One problem is that the heterotypic 'a' sequences found in some recombinants (Davison and Wilkie, 1983a) could not participate in this form of recombination.

I.7.g.iii Single-strand repair synthesis model

Varmuza and Smiley (1985) have postulated that the genomic termini are generated by single-stranded nicks at DR_{1b} and DR_{1c}, directed by U_b and U_c signals from adjacent 'a' sequences, rather than double-stranded cleavages (Figure 7). Thus, one DNA strand is nicked at the future L terminus and the other at the future S terminus. Repair synthesis from both gaps then displaces the single strand to produce two blunt end termini, each bearing an 'a' sequence. As before, this model would predict that a double 'a' sequence would be cleaved by two single-stranded cleavages either on opposite sides of the same 'a' sequence to allow amplification and blunt-ended termini, or the U_b (S) signal

of one 'a' sequence and the U_c (L) signal of its neighbour could act in concert to generate a double-stranded, presumably staggered, cleavage producing the 3' single base extensions previously found and no amplification (Mocarski and Roizman, 1982a,b).

The model proposes that not all HSV molecules end in 3' overhangs and also has the problem of stability of large single-stranded regions during replacement synthesis.

All these models have arisen from the need to explain the apparent amplification of 'a' sequences from single 'a' sequence-containing amplicons (Deiss and Frenkel, 1986). However, in intact HSV, DNA circularization of the genome (Poffenberger and Roizman, 1985) would generate double 'a' sequences, occurring as direct repeats, at every second junction. Given this, and the finding that 80% of L termini and L-S junctions contain a single 'a' sequence (Locker and Frenkel, 1986), then in the majority of cases there is no need to postulate amplification of the 'a' sequence - cleavage of the double 'a' sequences being adequate. Possibly, a variant of one or all of the above mechanisms occurs in a minority of infections with intact virus, but the latter mechanisms are the only way to generate unit length DNA molecules containing terminal 'a' sequences from defective amplicons. It may explain some of the unusual defective amplicons seen in these studies (Deiss and Frenkel, 1986; Deiss et al., 1986).

The cleavage/packaging function of the 'a' sequence is essential for viral packaging and propagation, as illustrated by studies on defective amplicons (Spaete and

Frenkel, 1982; Stow et al., 1983).

I.7.h Protein interactions at the herpes simplex virus 'a' sequence

The 'a' sequence serves as the cis-acting sequence necessary for a number of functions, including promoter activity, circularization, site-specific recombination and cleavage/packaging of viral DNA. As such, it would be expected that many proteins, both HSV encoded and cellular, would interact in a sequence-specific manner to mediate these functions. At present, little is known about such DNA-protein interactions. A small polypeptide has been shown by electron microscopy to be bound to both the termini and L-S junction of DNA extracted from virions (Wu et al., 1979). Its function and identity is unknown. Kudler and Hyman (1979), on the basis of protection of the 3' ends of virus DNA from exonuclease digestion, suggested that a polypeptide may be bound at the ends of HSV DNA. Dalziel and Marsden (1984) have shown that two late, HSV-1-induced polypeptides of apparent mol. wt. 21,000 and 22,000 (21K/22K) appear to interact specifically with the 'a' sequence of HSV-1 in vitro. These are almost certainly the true late 21K/22K polypeptides encoded by gene U_S11 (Rixon and McGeoch, 1984; Johnson et al., 1986), which have been shown to be strong DNA-binding proteins (Bayliss et al., 1975; MacLean et al., 1987). The function of the interaction with the 'a' sequence is unclear, since genomes lacking U_S11 are viable in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987) and isomerize and package DNA in an apparently normal fashion. Interestingly,

the products of gene U_S11 were shown, by immune electron microscopy, to localize strongly to nucleoli of infected cells (MacLean et al., 1987; Puvion-Dutilleul, 1987) - viral DNA, in contrast, localizes to the nucleoplasm and is apparently excluded from the nucleolus (Rixon et al., 1983).

Using a DNase I footprinting assay, MacLean (1987) has demonstrated a HSV-induced DNA-protein interaction within DR_2 and the U_b homology region. The U_b interaction is specific for infected cells, being induced by both HSV-1 and HSV-2. The DR_2 interaction is also induced by both HSV-1 and HSV-2, although a different pattern is observed with mock extracts. Interestingly, the DR_2 repeat element shares homology with an Sp1 binding site (Jones and Tjian, 1985; Kadonaga et al., 1985). The significance of these interactions, and which, if any, viral polypeptides are involved, remains to be determined, although the products of gene U_S11 are not involved (MacLean, 1987).

I.8 HERPES SIMPLEX VIRUS DNA REPLICATION

Little is known about the detailed mechanism of DNA replication of HSV, although the cis-, and at least some of the trans-, acting functions have now been determined. Immediately after infection, HSV DNA accumulates in the nucleus where it rapidly circularizes, apparently by ligation of the terminal 'a' sequences. DNA replication at 37°C is first detected approximately 3 hours post infection, peaking by about 9-11 hours and being virtually complete by 16 hours post infection (Wilkie, 1973).

The nature of the replicating DNA is unclear, although replicative forks and bubbles have been seen on electron microscopy (Friedmann et al., 1977). Analysis is complicated by the size and fragility of the replicative complex, and Ben-Porat and Rixon (1979) have described replicating HSV DNA as "large tangled masses".

Newly replicated HSV DNA is endless, ie., it lacks detectable terminal restriction enzyme fragments, indicating circular or concatameric forms. The latter are likely to be predominant, since junction fragments are not over-represented (Jacob et al., 1979; Jongeneel and Bachenheimer, 1981).

The circular template monomer is probably first amplified by theta replication (Ben-Porat and Tokazenski, 1977; Jean et al., 1977; McGeoch, 1987). Subsequently, the DNA is replicated by a rolling circle mechanism, to form long head-to-tail concatamers (Jacob et al., 1979), which are processed, cleaved and packaged into nascent nucleocapsids in the cell nucleus (Vlazny et al., 1982).

I.8.a Herpes simplex virus origins of DNA replication

Herpes simplex virus has been shown to contain three origins of DNA replication. Originally, information came from electron microscopy studies of replicating DNA (Friedmann et al., 1977) and studies on defective particles within an HSV stock. In order to be replicated and propagated, defective molecules must contain an origin of replication, as well as a packaging signal. Two classes of

defective molecules were found. Both contained sequences from the end of the short region (the 'a' sequence for cleavage/packaging (section I.7.f)). Class I defective molecules also contained sequences from either end of the S segment, while Class II defective molecules contained additional sequences from the middle of the L segment (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982).

I.8.a.i ori$_S$

Using a plasmid-based system, the origins of DNA replication in the short region, ori$_S$, have been localized to a 90 bp fragment in R_S situated between the 5' ends of the divergent transcripts for IE3 and IE4/5 (Stow, 1982; Stow and McMonagle, 1983). Thus, ori$_S$ is present as two identical copies in the intact genome, one in IR_S and one in TR_S (see Figures 9 and 10). In HSV-1 strain 17, ori$_S$ contains an imperfect palindrome of 42 bases with an $(AT)_6$ sequence at the centre (Figure 11). Both this central $(AT)_6$ region and sequences surrounding the palindrome are essential for viral DNA replication (Stow and McMonagle, 1983; Stow, 1985). Recently, Hubenthal-Voss et al. (1987) have found evidence for a transcript spanning ori$_S$. This transcript is 3' co-terminal with IE3.

In HSV-2 strain HG52, a similar sequence has been identified in R_S, although here it is present in duplicate as part of a 137 bp imperfect direct repeat (Whitton and Clements, 1984a). In VZV, Stow and Davison (1986) have identified a sequence with ori function in R_S. This sequence will allow replication of a plasmid containing it following superinfection with either VZV or HSV-1. There is some

Figure 9. Open reading frames in HSV-1 strain 17 (1).

Open reading frames in the left hand 75 kb of HSV-1 strain 17 (Dr. D. J. McGeoch - personal communication). The open boxes are repeated sequences and a is the 'a' sequence. Open reading frames in U_L are numbered U_L1- 36, with 15/1 and 15/2 referring to the two coding exons of gene U_L15. Some of the open reading frames whose polypeptide products are known are labelled. MCP is the major capsid protein. 'Challberg' indicates those genes, neccessary for plasmid-based HSV DNA replication, whose products are as yet unknown. The origin of DNA replication in U_L (ori$_L$) is also indicated. Dotted lines indicate that the open reading frame is continued onto the following line.

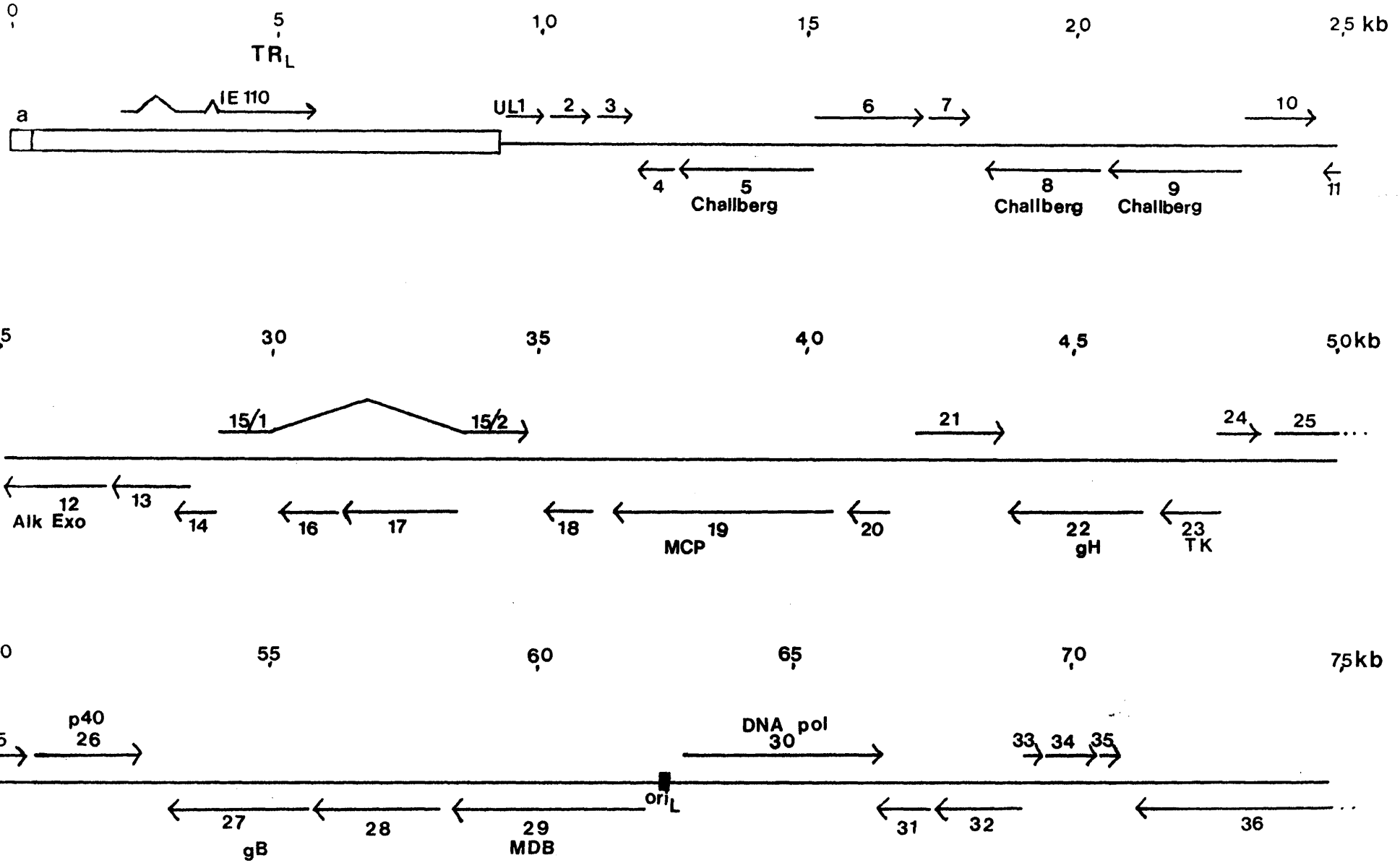

0
5
1,0
1,5
2,0
2,5 kb
TR$_L$
a
IE 110
UL1
2
3
6
7
10
4
5
Challberg
8
Challberg
9
Challberg
11
2,5
3,0
3,5
4,0
4,5
5,0kb
15/1
15/2
21
24
25
12
Alk Exo
13
14
16
17
18
19
MCP
20
22
gH
23
TK
5,0
5,5
6,0
6,5
7,0
7,5kb
25
p40
26
DNA pol
30
33
34
35
ori$_L$
27
gB
28
29
MDB
31
32
36

Figure 10. Open reading frames in HSV-1 strain 17 (2).

Open reading frames in the right hand half of HSV-1 strain 17 (Dr. D. J. McGeoch - personal communication). Similar labelling to Figure 9 is used. Open reading frames in U_L are numbered U_L36-56 and those in U_S, US1-12. RR1 and RR2 are the large and small subunits of ribonucleotide reductase. $65K_{DBP}$ is the 65K DNA-binding protein which is distinct from $65K_{TIF}$, the tegument transactivating protein (Marsden et al., 1987). U_S5 is marked g* to indicate that it encodes a potential glycoprotein, which has not yet been identified. The origin of DNA replication in the short region (ori_S) is also indicated.

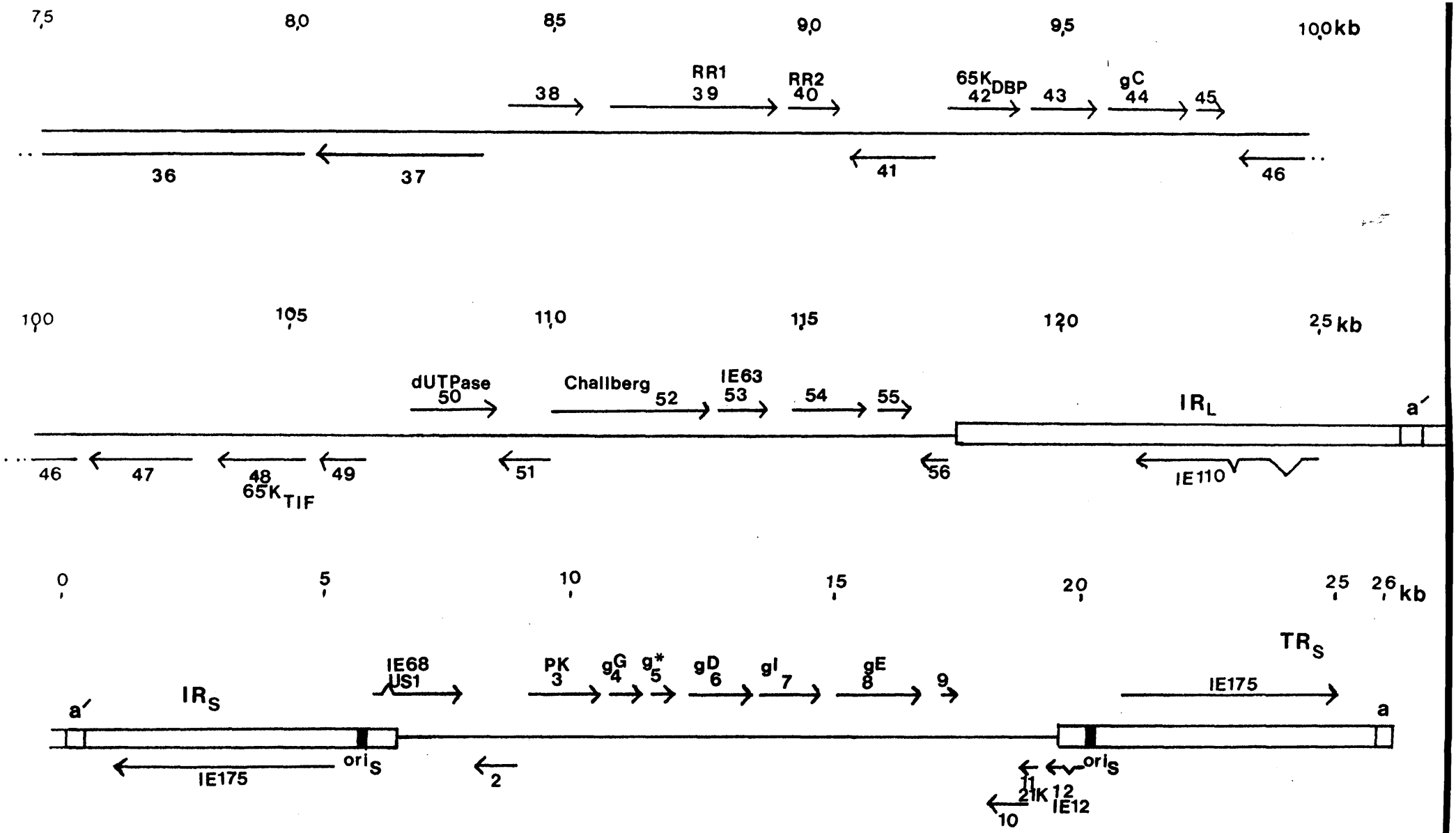

7,5
8,0
8,5
9,0
9,5
10,0kb
38
RR1
39
RR2
40
65K
42
DBP
43
gC
44
45
36
37
41
46
100
105
110
115
120
25kb
dUTPase
50
Challberg
52
IE63
53
54
55
IR_L
a´
46
47
48
65K
TIF
49
51
56
IE110
0
5
10
15
20
25
26kb
TR_S
IE68
US1
PK
3
g_4^G
g*_5
gD
6
gI
7
gE
8
9
IE175
IR_S
a´
a
IE175
ori_S
2
ori_S
11
21K
12
IE12
10

Figure 11. ori_S.

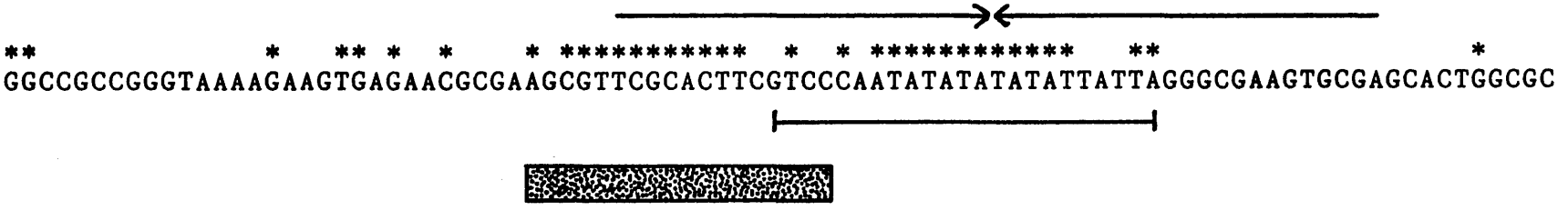
The 90 bp minimal ori_S sequence of HSV-1 strain 17. The sequence of the 90 bp fragment containing the *cis*-acting sequences required for ori_S function (Stow and McMonagle, 1983) is shown. This represents the upper strand in the 5'-3' direction of the ori_S sequence within the short internal repeat.

→← represents the 45 bp imperfect palindrome within this region.

* represents bases conserved between the VZV and HSV-1 ori_S fragments.

|—| represents the sequence deleted by Stow (1985), resulting in a plasmid which fails to replicate.

▒▒▒ represents the bases on this strand protected by the ori_S-binding protein (Elias *et al.*, 1986).


** * ** * * * ********** * * ********** ** *
GGCCGCCGGGTAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGCGC

homology between ori_S of HSV-1 and VZV. The VZV ori_S also contains a palindrome, although apart from the central $(AT)_n$ residues, it is not conserved compared to HSV-1. However, there is a block of 11 conserved residues which in HSV-1 lies across the boundary of the palindrome, but is outwith the palindrome in VZV. This 11 bp conserved sequence forms part of the binding site in the HSV-1 ori_S for an HSV-1-induced nuclear protein (Figure 11) (Elias et al., 1986).

I.8.a.ii ori_L

Analysis of defective genomes indicated that the origin in the long segment, ori_L, was situated between 0.360 and 0.419 m.u. (Spaete and Frenkel, 1982). However, when fragments spanning these sequences were cloned in standard vectors, between 100-650 bp were deleted. Deleted fragments possessed no origin activity, although following serial passage in the presence of wild-type helper virus the deleted sequences were restored and the plasmids were then capable of replicating. Sequences were presumably restored by recombination with the helper virus (Spaete and Frenkel, 1982; Weller et al., 1985).

Weller et al. (1985), using a yeast plasmid vector, succeeded in cloning ori_L in an undeleted form : this fragment will replicate in a plasmid-based system following superinfection with HSV. Sequence analysis of ori_L from HSV-1 KOS (Weller et al., 1985), HSV-1 Ang (Grey and Kaerner, 1984) and HSV-1 strain 17 (Quinn and McGeoch, 1985) has shown that ori_L contains a perfect 144 bp palindrome, which is presumably responsible for the deletion events. The

palindrome constitutes the minimum requirement of ori_L (McGeoch, 1987) and shows a high degree of homology, including a central (A+T) rich region, to ori_S. Sequences at one side of ori_S, including the binding site of Elias et al. (1986), are contained on both arms of ori_L, suggesting that ori_L may be bidirectional but ori_S only unidirectional (Weller et al., 1985; McGeoch, 1987).

Lockshon and Galloway (1986) have identified and sequenced ori_L in HSV-2. Again, it is a perfect palindrome, showing a strong degree of homology to the HSV-1 sequence. Stow and Davison (1986) found no evidence for ori_L in VZV. No sequence homology or functional origin was detected. In HSV-1, ori_L is situated between the divergent transcripts for the DNA polymerase and the major DNA-binding protein (MDB) (Quinn and McGeoch, 1985; Figure 9) , two of the proteins essential for DNA replication (Challberg, 1986; see below). The significance of the location of both ori_L and ori_S between divergent transcripts encoding polypeptides with a major role in viral replication and transcription is unknown. It is interesting to note that ori_L is situated almost exactly in the middle of U_L (Dr. D. J. McGeoch, personal communication) at 0·41 m.u.

The significance of the three origins of replication in HSV is unknown. The duplication of ori_S may be a consequence of its location in R_S and have no functional significance; viruses lacking one copy of ori_S (Longnecker and Roizman, 1986; Brown and Harland, 1987) grow normally in vitro, thus two copies of ori_S do not appear to be required in vitro at least. The role and relative contribution to DNA replication of ori_L and ori_S are at present unknown. Recently, a HSV-1

variant lacking ori_L has been isolated; this virus would appear to grow normally *in vitro* and also to establish latency in experimental animals (Polvino-Bodnar *et al.*, 1987). No viruses lacking both copies of ori_S have yet been isolated, although this may be due to the presence of a potential gene spanning ori_S (Hubenthal-Voss *et al.*, 1987).

I.8.b Proteins involved in DNA replication

Plasmid-based assay systems have allowed the *cis*-acting origins of replication to be identified and characterized. Until recently, information concerning proteins involved in replication has come from genetic studies. From these, it appears that the DNA polymerase (Hay and Subak-Sharpe, 1976; Chartrand *et al.*, 1979, 1980; Coen *et al.*, 1982; Honess *et al.*, 1984), the MDP (Bayliss *et al.*, 1975; Powell and Purifoy, 1976; Powell *et al.*, 1981; Weller *et al.*, 1983), ribonucleotide reductase (Dutia, 1983; Preston V. *et al.*, 1984) and the alkaline exonuclease (Moss, 1986) are required for DNA synthesis. Several other complementation groups of *ts* mutants are also DNA negative (McGeoch, 1987; Schaffer *et al.*, 1987).

Recently, a plasmid-based assay system has been developed to identify those genes required for efficient replication of plasmid molecules containing an HSV origin of replication (Challberg, 1986; McGeoch *et al.*, 1987; Wu *et al.*, 1988). Seven genes have been identified as essential for DNA replication (Figures 9 and 10); all have homologues in VZV (McGeoch *et al.*, 1988; Wu *et al.*, 1988). Of these, two are the DNA polymerase and the MDB. A third is the 65K DNA-binding protein ($65K_{DBP}$), previously identified by

Bayliss et al. (1975) and Marsden et al. (1987). This polypeptide is also known as ICSP34,35 (Vaughan et al., 1984, 1985). The function of the latter two polypeptides, and the other four open reading frames, has yet to be determined. All these essential genes fall within a DNA negative complementation group (McGeoch, 1987; Schaffer et al., 1987).

It is possible that other HSV genes involved in DNA replication might not be recognized in this assay. These could include any genes whose function could be substituted by host cell proteins or are involved in cleavage/packaging of HSV DNA. Interestingly, the apparently essential ribonucleotide reductase (Dutia, 1983; Preston V. et al., 1984) was only required for optimal activity in this assay (Wu et al., 1988). However, the construction of a variant with a deletion in the ribonucleotide reductase has recently shown that this enzyme is non-essential in dividing tissue culture cells (Goldstein and Weller, 1988). Another apparently essential gene, that for the alkaline exonuclease (Moss, 1986), was also not required in this plasmid-based assay of DNA replication.

I.8.b.i DNA polymerase

Herpes simplex virus encodes its own DNA polymerase (Keir and Gold, 1963; Keir et al., 1966; Hay et al., 1971) which has been shown to be essential for DNA replication (Hay and Subak-Sharpe, 1976; Chartrand et al., 1979, 1980). Unlike the cellular enzyme, the HSV DNA polymerase is sensitive to phosphonoacetic acid (PAA) (Hay and Subak-Sharpe, 1976), a pyrophosphate analogue. Unlike other

eukaryotic DNA polymerases, it also contains a 3'-5' exonuclease activity, which may serve to increase the fidelity of DNA replication (Knopf, 1979).

I.8.b.ii Major DNA-binding protein

The MDB (Bayliss et al., 1975) has been shown to be essential for HSV DNA replication (Powell and Purifoy, 1976; Powell et al., 1981; Weller et al., 1983; Quinlan and Knipe, 1985) and to bind to single-stranded DNA preferentially (Bayliss et al., 1975; Knipe et al., 1982). Interestingly, given the presence of an (A+T) rich region at the centre of the HSV origins of replication, it has the property of destabilizing poly A/T helices in vitro (Powell et al., 1981). It also has the ability to stabilize single-stranded DNA (Ruyechan, 1983). Using ts mutants with lesions within the gene for MDB, Littler et al. (1983) demonstrated at the permissive temperature only an interaction between the MDB, DNA polymerase and alkaline exonuclease. The MDB has also been shown to have a role in the regulation of gene expression (Godowski and Knipe, 1983; see section I.10.d.ii).

I.8.b.iii 65K DNA-binding protein

The $65K_{DBP}$ is essential for viral replication (McGeoch, 1987; Schaffer et al., 1987; Dr. V. Preston, personal communication), is strongly DNA-binding (Bayliss et al., 1975; Powell and Purifoy, 1976), and is distinct from the 65K virion polypeptide (Marsden et al., 1987) responsible for transactivating immediate-early genes (Campbell et al., 1984). It has been mapped to gene U_L42 (Figures 9 and 10;

McGeoch, 1987; Parris et al., 1987). The polypeptide is also known as ICSP34,35, and has been shown to be strongly associated with the DNA polymerase (Vaughan et al., 1984, 1985; see section I.8.b.i). The function of the $65K_{DBP}$ is unknown, although it has been shown to copurify with a topoisomerase I activity present in HSV-1 virions (Muller et al., 1985). It is not believed to be a topoisomerase (Dr. H. S. Marsden, personal communication).

I.8.b.iv Alkaline exonuclease

Following infection with HSV there is an increase in the deoxyribonuclease activity within the cells. The activity has an alkaline pH optimum (Keir and Gold, 1963; Keir, 1968; Morrison and Keir, 1968; Hay et al., 1971) and is virus encoded (Francke et al., 1978), mapping to 0.145-0.185 m.u. (Moss et al., 1979; Preston and Cordingley, 1982). The latter workers showed that the major product of in vitro translation from this region was an 85K polypeptide, subsequently shown to be encoded by a 2.3 kb mRNA (Banks et al., 1983; Costa et al., 1983). Moss (1986), by further characterization of a previously described ts lesion in this gene (Francke et al., 1978; Moss et al., 1979), showed that the alkaline exonuclease activity was essential for DNA synthesis and infectious virus production.

I.8.b.v Ribonucleotide reductase

A HSV-induced ribonucleotide reductase activity which differs biochemically from the host cell enzyme (Ponce de Leon et al., 1977; Langelier et al., 1978; Huszar and Bacchetti, 1981) has been demonstrated in both HSV-1 (Cohen,

1972) and HSV-2 (Cohen et al., 1974) infected cells. Ribonucleotide reductase catalyzes the reduction of all four ribonucleotides to the corresponding deoxyribonucleotides. The HSV-induced enzyme has been shown to be virally encoded (Dutia, 1983), with the HSV-1 polypeptide Vmw136, which maps between 0.580-0.585 m.u., being an essential component (Preston V. et al., 1984). The sensitivity of the virus enzyme to hydroxyurea suggested that it consists of two subunits (Langelier and Buttin, 1981). Using antibodies against Vmw136, it has been shown that a polypeptide, Vmw38, coprecipitates with Vmw136. These two polypeptides also copurify (Huszar and Bacchetti, 1983; Bacchetti et al., 1984; Preston V. et al., 1984). Vmw38 is coded to the right of Vmw136 (Figures 9 and 10), the two messages being 3' coterminal (McLauchlan and Clements, 1982).

Using a HSV-1 ts mutant in the Vmw136 component of ribonucleotide reductase, Frame et al. (1985) could precipitate at the permissive temperature Vmw136 and Vmw38 together, using antibodies against either polypeptide. However, at the non-permissive temperature only the polypeptide against which the antibody was directed was precipitated, suggesting that Vmw136 and Vmw38 form a complex at the permissive temperature but not at the non-permissive temperature. The complex may be essential for ribonucleotide reductase activity (Frame et al., 1985; Bacchetti et al., 1986); a view supported by the recent finding that synthetic oligopeptides corresponding to the carboxy-terminus of Vmw38 inhibit ribonucleotide reductase activity in vitro, and are thought to do so by interfering with complex formation (Cohen et al., 1986; Dutia et al.,

1986).

Herpes simplex virus also encodes a number of other enzymes which are involved in DNA metabolism.

I.8.b.vi Deoxynucleoside pyrimidine kinase (thymidine kinase)

Herpes simplex virus encodes a thymidine kinase (tk) (Kit and Dubbs, 1963) which phosphorylates both thymidine and deoxycytidine using the same active site (Jamieson and Subak-Sharpe, 1974). This enzyme has been mapped to 0.300-0.309 m.u. and subsequently sequenced (McKnight, 1980; Wagner et al., 1981). The enzyme is non-essential in growing cells but is essential for virus growth in serum-starved cells (Jamieson and Subak-Sharpe, 1974). Viruses lacking tk would appear to have reduced potential for latency and pathogenicity (Field and Wildy, 1978).

I.8.b.vii Deoxyuridine triphosphate nucleotidylhydrolase (dUTPase)

The dUTPase catalyzes the hydrolysis of dUTP to dUMP and, as a consequence, minimizes the incorporation of dUTP into DNA. Herpes simplex virus has been shown to encode a dUTPase (Wohlrab et al., 1982; Preston and Fisher, 1984) which maps to 0.69-0.70 m.u. (Figures 9 and 10). The dUTPase activity has been shown to be non-essential in vitro (Fisher and Preston, 1986).

I.8.b.viii Uracil DNA glycosylase

Herpes simplex virus encodes a uracil DNA glycosylase

*This enzyme, which is responsible for removing uracil from DNA, is encoded by gene UL2 (Dr. D.J. McGeoch, personal communication).

(Caradonna and Cheng, 1981) which maps to 0.065-0.08 m.u. and has a molecular weight of 39,000 kd following *in vitro* translation (Caradonna *et al.*, 1987).*

Varicella-zoster virus and HVS have been shown to encode a thymidylate synthetase, although HSV apparently lacks this enzyme (Honess *et al.*, 1986; Thompson *et al.*, 1987).

I.8.b.ix Protein kinase

Although this enzyme is not necessarily involved in DNA replication, it is dealt with in this section.

McGeoch and Davison (1986a) have identified a gene homologous to the protein kinase of eukaryotic cells and oncogenic retroviruses in the short region of HSV-1 (McGeoch *et al.*, 1985), HSV-2 (McGeoch *et al.*, 1987) and VZV (Davison and Scott, 1986), three alphaherpesviruses. No homologue has been found in EBV, a gammaherpesvirus. The role of this enzyme during HSV infection has not been determined, although it is not essential for growth in tissue culture (Longnecker and Roizman, 1987). As the polypeptide is synthesized at early times, it may act to modify host proteins or phosphorylate virus proteins. The product of the above gene is responsible for the novel protein kinase activity observed following infection with HSV (Blue and Stobbs, 1981; Katan *et al.*, 1985; Purves *et al.*, 1986, 1987).

I.9 HERPES SIMPLEX VIRUS POLYPEPTIDES

Approximately fifty HSV-induced polypeptides have been recognized by one-dimensional SDS-PAGE (Honess and Roizman, 1973, 1974; Powell and Courtney, 1975; Marsden *et al.*, 1976). Sequence analysis of the HSV-1 genome (Dr. D. J. McGeoch, personal communication) has indicated that the genome encodes 70 distinct polypeptides. Some polypeptides are not recognizable following one-dimensional SDS-PAGE of total cell extracts, requiring immunoprecipitation with specific antisera for recognition (see, for example, Buckmaster *et al.*, 1984; Frame *et al.*, 1986). It is possible that not all the open reading frames encoding polypeptides have been identified; thus, the total number of HSV-1 specified polypeptides may be somewhat higher than 70 (Dr. D. J. McGeoch, personal communication). On two-dimensional SDS-PAGE, at least 230 distinct polypeptide species have been identified (Haarr and Marsden, 1981), although many of these are related families in which one primary translation product has undergone several stages of post-translational modification.

I.9.a Post-translational modification of herpes simplex virus polypeptides

The difference in the apparent mobilities on SDS-PAGE of some *in vitro* translated HSV polypeptides compared to those from infected cell extracts indicates that many HSV polypeptides undergo some form of post-translational modification (Preston, 1977). There are four main types of post-translational modification, namely glycosylation,

phosphorylation, sulphation and cleavage, although a limited amount of polyribosylation (Preston and Notarianni, 1983) and fatty acyl glycosylation of glycoproteins (Johnson and Spear, 1982) also occurs.

I.9.a.i Proteolytic cleavage

Proteolytic cleavage appears to be involved in the maturation of HSV glycoproteins. In gD, the amino-terminal 25 amino acids, presumably constituting the signal peptide, are removed from the mature polypeptide (Eisenberg et al., 1984). Balachandran and Hutt-Fletcher (1985) also suggest that in the processing of gG-2 the 108K final product arises by proteolytic cleavage of a 120K glycosylated precursor. gE also appears to undergo proteolytic cleavage. Mature gE is found on the surface of infected cells and virions (Baucke and Spear, 1979). In the presence of serum, three gE-related cleavage products of 32/34/35K are found in the medium (Hope et al., 1982; Cross et al., 1987). The significance of these secreted products is not clear.

I.9.a.ii Sulphation

All the major HSV glycoproteins (gB, gC, gD and gE) are sulphated, with gE being the major sulphated glycoprotein (Hope et al., 1982; Hope and Marsden, 1983).

I.9.a.iii Phosphorylation

Sixteen HSV-1 and eighteen HSV-2 polypeptides are phosphorylated (Pereira et al., 1977; Marsden et al., 1978). Wilcox et al. (1980) demonstrated that phosphate cycles on and off several HSV polypeptides and suggested that these

proteins act as phosphate donors. Many phosphoproteins are DNA-binding proteins, with their DNA-binding properties differing according to their state of phosphorylation. For example, in its phosphorylated form, ICP29 (54,000 mol. wt.) was strongly DNA-binding, whereas Vmw136 (the large subunit of ribonucleotide reductase) bound DNA more strongly in its non-phosphorylated form (Wilcox et al., 1980).

I.9.b DNA-binding proteins

Approximately sixteen HSV-1-induced and twelve HSV-2-induced polypeptides have been shown to possess DNA-binding properties (Bayliss et al., 1975; Powell and Purifoy, 1976; Hay and Hay, 1980; Wilcox et al., 1980). It is unknown how many of these bind DNA directly. The MDB (Powell et al., 1981) and $65K_{DBP}$ (Vaughan et al., 1985) have been sufficiently purified to suggest that they interact directly with DNA. Purification procedures used to obtain apparently pure preparations of both the alkaline exonuclease and DNA polymerase involved as a final step DNA cellulose chromatography (Powell and Purifoy, 1977; Banks et al., 1983), suggesting that these two enzymes also interact directly with DNA. The situation with VmwIE175 is unclear (see section I.10.d.i). Several of these virus proteins, for example, the DNA polymerase (Vmw145) and alkaline exonuclease (Vmw87), have enzymatic activity (see section I.8.b.i and iii). Vmw21 has been shown to bind to the 'a' sequence (Dalziel and Marsden, 1984; see section I.7.h). The functions of most of the other DNA-binding proteins are still unknown.

I.9.c Glycoproteins

Seven HSV glycoproteins have been identified (see Table 1 for a summary of their functions). These are gB, gC, gD (Spear, 1976), gE (Baucke and Spear, 1979), gG (Marsden et al., 1978, 1984; Roizman et al., 1984; Frame et al., 1986; McGeoch et al., 1987), gH (Buckmaster et al., 1984) and gI (Longnecker et al., 1987). An unidentified open reading frame in U_S, U_S5, also appears to have the potential to encode a glycoprotein (McGeoch et al., 1985), although no other potential glycoproteins have been identified in U_L (Dr. D. J. McGeoch, personal communication; see Figure 12). No HSV-2 homologue of gH has yet been identified. There is a large size difference between gG-1 and gG-2, with gG-2 containing a 1460 bp insert. It is interesting to observe that there is a clustering of glycoproteins in U_S with U_S genes 4-8 all coding for actual or potential glycoproteins. There may possibly have been gene duplication at one point (McGeoch et al., 1987).

Of the identified glycoproteins, several have been shown to be non-essential in tissue culture - gC (Heine et al., 1974), gG, gE, gI and U_S5 (Longnecker and Roizman, 1986, 1987; Longnecker et al., 1987; Harland and Brown, 1988). Ts lesions have been identified in gB (Ruyechan et al., 1979; Little et al., 1981) and gH (Weller et al., 1983; Buckmaster et al., 1984; Gompels and Minson, 1986), indicating that these glycoproteins are essential. The requirement for gD is at present unknown.

Pulse-chase experiments and two-dimensional SDS-PAGE have demonstrated that HSV glycoproteins are processed in several discrete steps (Haarr and Marsden, 1981; Palfreyman

Table 1 : Properties of the HSV-encoded glycoproteins

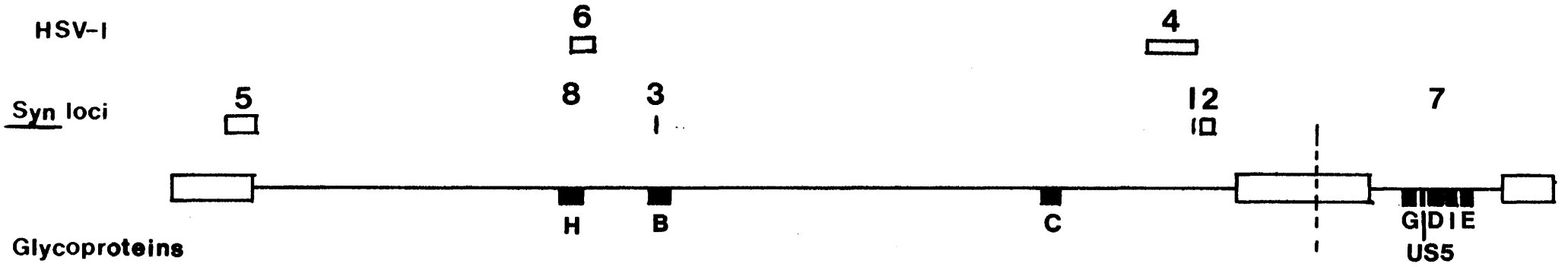
GLYCOPROTEIN	POSSIBLE FUNCTIONS
gB **UL27**	Essential for replication in tissue culture. Implicated in adsorption, penetration and cell fusion.
gC **UL44**	Not essential for replication in tissue culture. Implicated in adsorption, penetration and cell fusion. gC-1 can act as a C3b-receptor.
gD **US6**	No ts mutant yet described. Implicated in adsorption, and in cell fusion.
gE **US8**	Not essential for replication in tissue culture. Implicated in adsorption. Interacts with gI to form an Fc-receptor.
gG **US4**	Not essential for replication in tissue culture.
gH **UL22**	Essential for replication in tissue culture. Implicated in cell fusion and in cell-to-cell spread of infectious virus.
gI **US7**	Not essential for replication in tissue culture. Interacts with gE to form an Fc-receptor.

Figure 12. HSV-1 glycoproteins and syn loci.

The HSV-1 genome is represented in the prototype orientation together with the approximate map locations of the genes encoding the viral glycoproteins (below the line). The glycoprotein predicted to be encoded by gene US5 has not yet been identified.

Above the line is indicated the approximate map positions of the identified *syn* loci (▭).

(Adapted from Marsden, 1987).


HSV-I
6
4
Syn loci
5
8
3
12
7
H
B
C
G
D
I
E
US5
Glycoproteins

et al., 1983), involving the addition of both N-linked and O-linked oligosaccharides as indicated by inhibition by tunicamycin and monensin, respectively (Pizer et al., 1980; Johnson and Spear, 1982; Hope and Marsden, 1983). Several functions have been suggested for HSV glycoproteins (see Table 1; reviewed in Marsden, 1987). Neutralizing antibodies against all the glycoproteins (except gI) have been identified : gB (Powell et al., 1974; Showalter et al., 1981), gC (Zweig et al., 1980; Balachandran and Hutt-Fletcher, 1985), gD (Powell et al., 1974; Showalter et al., 1981), gE (Para et al., 1983), gG (Balachandran and Hutt-Fletcher, 1985) and gH (Showalter et al., 1981; Buckmaster et al., 1984).

It has been shown that gB and gD (Johnson et al., 1984) and, to a lesser extent, gC and gE (Fuller and Spear, 1985) appear to be involved in virus adsorption to cells. Little is known about the cellular receptors to which HSV binds, except that they are serotype-specific (Vahlne et al., 1979, 1980; Addison et al., 1984). In addition, gB and, to a lesser extent, gC appear to play a role in penetration (Littler et al., 1981; Bzik et al., 1984; DeLuca et al., 1984; Epstein et al., 1984).

I.9.c.i Cell fusion

Some HSV-1 variants induce cell fusion, or syncytium (syn) formation (Hoggan and Roizman, 1959). Seven or eight viral loci involved in cell fusion have been identified (reviewed in Spear, 1985; Marsden, 1987; Figure 12). Syn loci 1-3 were mapped by Ruyechan et al. (1979). The syn 1 locus has now been completely sequenced and a point mutation

identified in 2 *syn* mutants mapping here (Bond and Person, 1984; Debroy *et al.*, 1985; Pogue-Geile *et al.*, 1985; Pogue-Geile and Spear, 1986). An open reading frame for a potential transmembrane polypeptide has been identified.

The position of the *syn* 2 locus is about 0.745 m.u. (Machuca *et al.*, 1986). Its relationship to gC is uncertain (Manservigi *et al.*, 1977; Honess *et al.*, 1980; Machuca *et al.*, 1986), but there appears to be a strong but not absolute correlation. The *syn* 3 locus is contained within gB (Manservigi *et al.*, 1977; DeLuca *et al.*, 1982; Bzik *et al.*, 1984). The *syn* 4 locus has been identified by isolation of a *ts* mutant in cell fusion (Little and Schaffer, 1981), as has the *syn* 5 locus (Little and Schaffer, 1981). The *syn* 6 locus maps to the 5' end of the tk gene (Sanders *et al.*, 1982) and may be the same as the *syn* 8 locus involving gH (Gompels and Minson, 1986), which is also situated 5' of tk (Sharp *et al.*, 1983; McGeoch and Davison, 1986b). The *syn* 7 locus is situated within gD (Noble *et al.*, 1983; Gompels and Minson, 1986; Minson *et al.*, 1986).

I.9.c.ii Egress

The HSV virion acquires its envelope primarily at the nuclear membrane (Morgan *et al.*, 1969; Marsden, 1987) and is transported from the perinuclear space to the outside of the cell, probably via transport vesicles operating between the rough endoplasmic reticulum, the Golgi apparatus and the cell surface (Johnson and Spear, 1982; Spear, 1985). Buckmaster *et al.* (1984) have suggested that gH may play a role in egress and cell-to-cell spread of HSV, since a monoclonal antibody against gH inhibits plaque formation.

I.9.c.iii Fc-receptor

A receptor which binds to the Fc region of IgG (Fc-receptor) has been identified on the surface of HSV infected cells and virions (Baucke and Spear, 1979; Para et al., 1980, 1982; Johansson et al., 1984). The Fc-receptor molecule was originally believed to be gE, but more recently it has been shown to consist of a complex between gE and gI, neither of which on its own has receptor activity (Johnson et al., 1988).

I.9.c.iv C3b-receptor

Human endothelial cells infected with HSV-1 express receptors for the C3b component of complement (Cines et al., 1982). This activity is not present following HSV-2 infection. Friedman et al. (1984) demonstrated that gC-1, but not gC-2, functions as a C3b-receptor. Sequence analysis has shown that gC-1 relative to gC-2 has a 28 amino acid insert (Frink et al., 1983; Swain et al., 1985), which may explain the different function of these two polypeptides.

The physiological significance of the presence of Fc- and C3b-receptors on HSV infected cells is unclear, but it might be expected to modulate the immune response to HSV. Neither activity is essential in tissue culture.

I.9.d Other herpes simplex virus-induced polypeptides

Herpes simplex virus encoded enzymes and proteins involved in replication are discussed in section I.8.b; HSV immediate-early polypeptides are dealt with in section I.10.d.i. A detailed map of open reading frames, identified

by DNA sequence analysis, is shown in Figures 9 and 10, and the identity of known genes is indicated (Dr. D. J. McGeoch, personal communication).

I.10 HERPES SIMPLEX VIRUS GENE EXPRESSION AND mRNA SYNTHESIS

I.10.a General properties of herpes simplex virus transcription

Herpes simplex virus DNA is transported to the nucleus following infection (Hummeler et al., 1969) and transcribed throughout infection by the host RNA polymerase II (Alwine et al., 1974; Ben-Zeev et al., 1976; Costanzo et al., 1977). There is no evidence for a novel RNA polymerase activity in HSV infected cells (Lowe, 1978).

I.10.b Herpes simplex virus RNA processing

Like other eukaryotic mRNAs, HSV transcripts are capped at their 5' ends (Bartoski and Roizman, 1976; Moss et al., 1977). Some mRNAs are also internally methylated at A residues (Moss et al., 1977), although this is not the case with mRNA synthesized at late times after infection (Bartoski and Roizman, 1976).

Herpes simplex virus mRNAs are also polyadenylated (Bachenheimer and Roizman, 1972; Stringer et al., 1977) using the same polyadenylation signal as the host (Proudfoot and Brownlee, 1976). Approximately 30 bp downstream from the polyA signal is situated a consensus 'GT box' sequence, YGTGTTYY (Gil and Proudfoot, 1984; McLauchlan et al., 1985), believed to be important in mRNA termination.

Few HSV transcripts are spliced. The 5' ends of the mRNA for IE genes 4 and 5 are situated within the short repeat region, with the coding sequence contained at opposite ends of U_S. These transcripts are spliced, with the intron being contained entirely within the repeat, and hence being the same for both genes (Whitton and Clements, 1984b; see Figure 13). Gene U_L15 is also spliced, with the intron being positioned between two coding exons (Dr. D. J. McGeoch, personal communication, Figures 9 and 10). The mRNA from this gene is a 2.7 kb late product with a 4 kb splice (Costa et al., 1985a). IE1 mRNA is also spliced, containing two introns of 765 bp and 135 bp, situated between coding exons (Perry et al., 1986; Figure 13). Minor spliced species of gC have been found in infected cells, although most of these would not encode a full length polypeptide (Frink et al., 1981, 1983).

Regions of the HSV-1 genome have been extensively transcript mapped (Wagner, 1985), although the only complete transcript analysis is for the short region of HSV-1 (Rixon and McGeoch, 1984, 1985; McGeoch et al., 1985; Figure 14). One feature of U_S is the presence of four 3' co-terminal mRNA families, with each message containing a different 5' end and coding for the most 5' gene. Also, the coding regions of genes U_S10 and U_S11 are partly overlapping, being translated in different reading frames on the same strand. Throughout the HSV genome there are 15 known 3' co-terminal families of mRNA. There appear to be few areas which are transcribed from, or are coding on, both strands (Dr. D. J. McGeoch, personal communication).

Gene UL38 is encoded by two 5' co-terminal mRNAs which both appear to encode the same polypeptide product (McGeoch et al., 1988, Figure 10).

Figure 13. HSV-1 immediate-early genes.

The HSV-1 genome is represented in the prototype orientation and the map locations of the five immediate-early genes are illustrated as 1 to 5 indicating IE1 to 5.

IE1 encodes VmwIE110; IE2 encodes VmwIE63; IE3 encodes VmwIE175; IE4 encodes VmwIE68; and IE5 encodes VmwIE12.

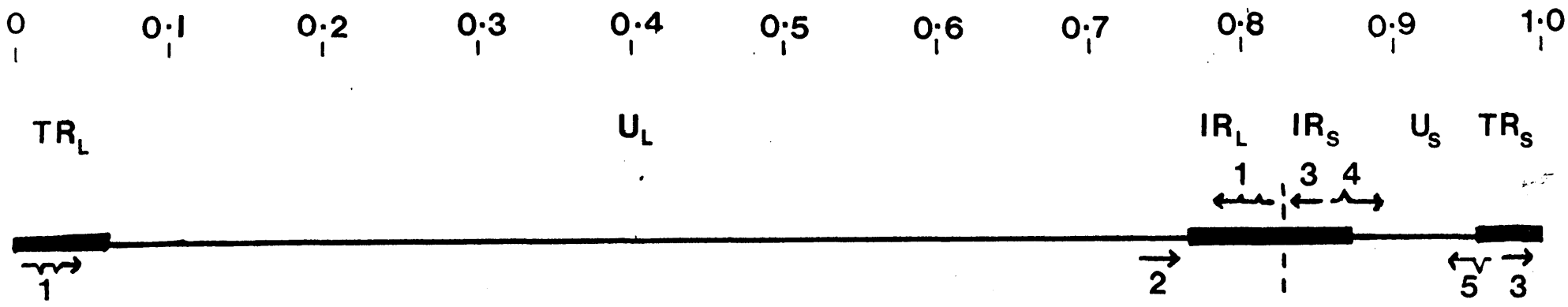
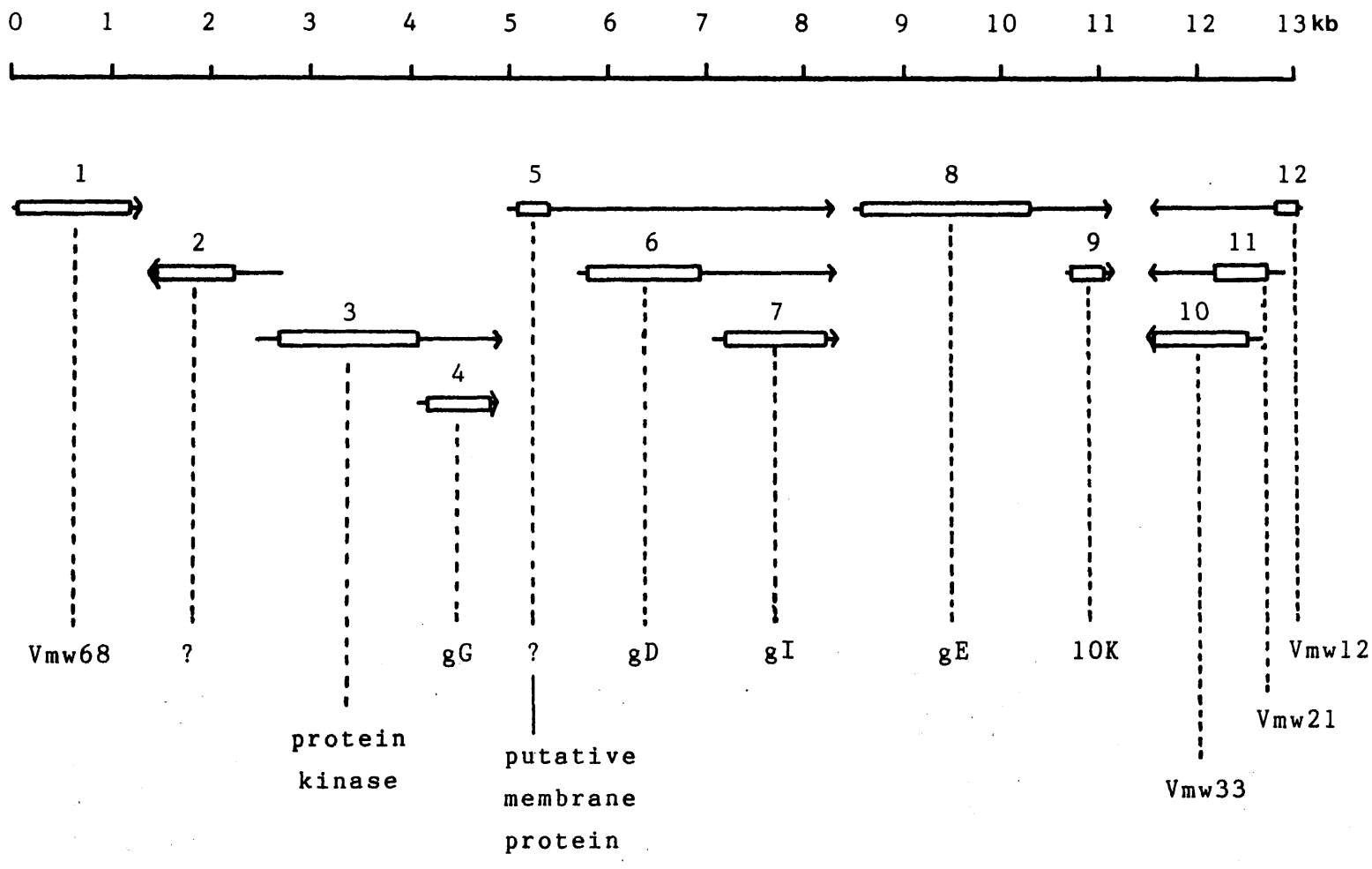

0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
TR_L
U_L
IR_L
IR_S
U_S
TR_S
1
3
4
1
2
5
3

Figure 14. Genome organization within the short unique region.

The short unique region, approximately 13,000 bp, is shown. The transcripts mapped to this region and their predicted coding regions (▭) are indicated. Where known, the polypeptides encoded by these genes are given. There are four 3' co-terminal families consisting of U_S3 and 4; U_S5, 6 and 7; U_S8 and 9; and U_S10, 11 and 12. Where a message spans more than one open reading frame in these families, only the most 5' open reading frame is translated.


0 1 2 3 4 5 6 7 8 9 10 11 12 13kb
1
2
3
4
5
6
7
8
9
10
11
12
Vmw68
?
protein kinase
gG
?
putative membrane protein
gD
gI
gE
10K
Vmw33
Vmw21
Vmw12

I.10.c Herpes simplex virus temporal regulation

Infection with HSV induces the synthesis of about 50 recognizable polypeptides (Honess and Roizman, 1973, 1974, 1975; Powell and Courtney, 1975; Marsden et al., 1976) which can be broadly divided into three temporal classes, immediate-early (IE or alpha), early (E or beta) and late (L or gamma), based on their detection in the presence and absence of metabolic inhibitors of protein and DNA synthesis (Honess and Roizman, 1974; Swanstrom and Wagner, 1974; Clements et al., 1977; Jones and Roizman, 1979). There is no apparent clustering of viral genes according to their temporal class, with the possible exception of the IE genes (Clements et al., 1977; Wagner, 1985).

Regulation of viral gene expression would appear to be predominantly at the level of transcription, although there is evidence that gD is controlled at least partly at the translational level. At late times, gD mRNA continues to accumulate while the level of gD polypeptide synthesis is reduced (Johnson and Spear, 1984). Only transcriptional control will be discussed here.

Herpes simplex virus gene expression has been analyzed using ts mutants, metabolic inhibitors of protein and DNA synthesis, deletion mutants, plasmid-based transient expression systems and biochemically-transformed cell lines containing HSV gene products.

I.10.c.i Immediate-early gene expression

There are five major IE genes, IE1, IE2, IE3, IE4 and IE5, which code for polypeptide products VmwIE110, VmwIE63, VmwIE175, VmwIE68 and VmwIE12, respectively (Clements et

al., 1979; Preston, 1979a; Easton and Clements, 1980; Figure 13). The properties of these polypeptides are outlined in section I.10.d.i. IE1 and IE3 map to the repeat regions and are therefore diploid, while IE4 and IE5 share a common promoter and 5' untranslated leader. IE2 maps to the right end of U_L.

During infection at 37°C, IE RNA can be detected by 1 h post infection (later classes by 2-3 h post infection). The rate of synthesis is maximal at around 2 h post infection, with maximal levels of mRNA being present approximately 3 h post infection, although IE RNA is still detectable at late times in infection (Harris-Hamilton and Bachenheimer, 1983; Godowski and Knipe, 1986).

Immediate-early 3 RNA declines very sharply, reflecting its transcriptional shut-off by 4 h post infection (Godowski and Knipe, 1986; Yager and Bachenheimer, 1987). On the basis of experiments using metabolic inhibitors, it was first suggested that early gene products negatively regulate IE gene expression (Honess and Roizman, 1974, 1975). Godowski and Knipe (1986) have suggested that the MDB, an early protein, may play a role in the down-regulation of IE3 (section I.10.d.iii). There is also evidence from ts mutants (Preston, 1979a) and plasmid expression studies (Gelman and Silverstein, 1986, 1987) that VmwIE175 represses its own and possibly other IE gene synthesis.

Immediate-early RNA is transcribed in the absence of de novo protein synthesis (Kozak and Roizman, 1974; Clements et al., 1977; Costanzo et al., 1977), although synthesis is stimulated 5-10 fold by the major structural component of the virion tegument, Vmw65 (Post et al., 1981; Mackem and

Roizman, 1982; Batterson and Roizman, 1983; Cordingley et al., 1983; Campbell et al., 1984; Preston C. et al., 1984).

I.10.c.ii Early gene expression

Early gene transcription begins after the appearance of functional IE products, peaking at about 4-6 h post infection. The criteria defining early genes are not as clear-cut as those defining IE genes. They have been subdivided into $beta_1$ and $beta_2$ depending on their requirement for functional IE polypeptides (Pereira et al., 1977; Roizman and Batterson, 1985). An example of a $beta_1$ gene is the large subunit of ribonucleotide reductase which may be expressed under IE conditions and by some ts mutants of VmwIE175 that do not otherwise express early gene products (DeLuca et al., 1985).

Some polypeptides, although expressed at early times in infection, require DNA synthesis for maximal expression, and hence are sensitive to PAA. gD is an example of such a protein (Gibson and Spear, 1983; Johnson et al., 1986). VP5, although described as a late gene, is expressed to a similar extent as gD in the absence of DNA replication, but is more typical of late genes in its response to IE gene products (Costa et al., 1985b; DeLuca and Schaffer, 1985; Harris-Hamilton and Bachenheimer, 1985; Everett, 1986). These genes have variously been described as early-late, leaky-late (beta-gamma), and $gamma_1$ genes, depending on the research group and the degree of sensitivity to inhibition of DNA replication (Harris-Hamilton and Bachenheimer, 1985; Roizman and Batterson, 1985; Wagner, 1985).

I.10.c.iii Late gene expression

Late gene products can be first detected by 2-3 h post infection, and reach maximum levels of accumulation by 10-16 h post infection. This parallels DNA synthesis which is initiated about 2 h post infection and peaks by about 8 h post infection (Wilkie, 1973; Roizman, 1979). In the presence of metabolic inhibitors of virus DNA synthesis, or following infection with DNA-negative ts mutants, there is a reduction in the amount of the genome transcribed and in the number of virus-induced polypeptides seen (Honess and Roizman, 1974, 1975; Swanstrom and Wagner, 1974; Powell et al., 1975; Marsden et al., 1976; Jones and Roizman, 1979; Holland et al., 1980; Pederson et al., 1981).

Late genes have been subdivided into two classes, $gamma_1$ (leaky late) and $gamma_2$ (true-late), depending on their requirement for DNA replication (Roizman and Batterson, 1985; Wagner, 1985). The expression of $gamma_1$ genes is reduced in the absence of DNA synthesis, but is still readily detectable. In contrast, the expression of $gamma_2$ genes is reduced to barely detectable levels in the absence of DNA synthesis (Powell et al., 1975; Godowski and Knipe, 1985; Johnson et al., 1986).

VP5 (see section I.10.c.ii) is an example of an early-late gene, while U_S11, which encodes Vmw21 (Rixon and McGeoch, 1984; McGeoch et al., 1985; Johnson et al., 1986; MacLean et al., 1987), and gC (Hall et al., 1982; Yager and Bachenheimer, 1987) are examples of true-late genes.

I.10.d Polypeptides involved in gene regulation

A number of HSV polypeptides have been implicated in

the control of gene expression, both by analysis of ts mutants and plasmid-based transient expression systems. The major class of these is the IE polypeptides.

I.10.d.i Herpes simplex virus immediate-early polypeptides

Herpes simplex virus type 1 encodes five IE polypeptides named according to their mobility on SDS-PAGE in terms of their apparent mol. wt. (Vmw) (Marsden et al., 1976; Preston et al., 1978; Watson et al., 1979) or infected cell polypeptide (ICP) number (Honess and Roizman, 1974; Morse et al., 1978). Equivalent polypeptides are found in HSV-2 (Easton and Clements, 1980).

Gene	Apparent mol. wt. (HSV-1)	ICP number
IE1	VmwIE110	ICP0
IE2	VmwIE63	ICP27
IE3	VmwIE175	ICP4
IE4	VmwIE68	ICP22
IE5	VmwIE12	ICP47

With the exception of VmwIE12, all the IE polypeptides are phosphorylated (Pereira et al., 1977; Marsden et al., 1978; Fenwick and Walker, 1979; Ackermann et al., 1984; Sears et al., 1985) and are located both in the cytoplasm and in the nucleus where they are tightly associated with chromatin (Hay and Hay, 1980, 1981).

VmwIE110

No ts mutants have been isolated in IE1. Only one copy is required for normal virus growth and gene expression in

tissue culture (Brown et al., 1984; Harland and Brown, 1985; MacLean and Brown, 1987b). Stow and Stow (1986) have constructed a virus which contains a large deletion in both copies of IE1. Although this deleted gene, when substituted for wild-type IE1, was inactive in stimulating early gene expression (see below) in a transient expression assay (Perry et al., 1986), the virus was capable of growing on a number of cell lines; however the virus yield was reduced 20-100 fold compared to wild-type HSV-1. Its ability to plaque was more significantly impaired than its yield, indicating that initiation of infection was poor. Polypeptide synthesis in the mutant was normal compared to wild-type virus. Sacks and Schaffer (1987) have also isolated two deletion mutants in IE1, which have very similar properties to the mutant of Stow and Stow (1986).

Sandri-Goldin et al. (1987) have constructed cell lines expressing an antisense IE1 message. Expression of VmwIE110 was reduced to less than 10% of wild-type levels, with little effect on the expression of HSV early and late genes or virus yield.

Thus, it appears that while VmwIE110 may play a role in stimulating gene expression and initiating infection, it is not an absolutely essential polypeptide in the HSV-1 lytic cycle in vitro. In vivo the mutant of Stow and Stow (1986) becomes latent and reactivates in the mouse footpad model at a frequency comparable to its wild-type parent (Dr. G. B. Clements, personal communication).

In contrast the mutant of Stow and Stow (1986) is unable to reactivate latent virus from an in vitro latency model (Russell et al., 1987).

VmwIE63

A number of ts mutants have been isolated in IE2,

suggesting that this gene is essential (Sacks et al., 1985). MacLean and Brown (1987b, and this thesis) have isolated a variant of HSV-1 which, although not containing a detectable deletion in IE2, fails to synthesize detectable VmwIE63 under immediate-early conditions. Interestingly, the ts mutants of Sacks et al. (1983) at the non-permissive temperature overproduce VmwIE175 and VmwIE63, synthesize normal levels of early proteins and some early-late proteins, but despite producing normal levels of DNA, severely underproduce late polypeptides. Everett (1986) has shown that VmwIE63 on its own has no effect on early and late promoters, but in conjunction with VmwIE110 and VmwIE175 causes increased transcription from a late promoter (VP5). Thus, it is possible that VmwIE63 is not required to be produced at immediate-early times, as long as it is synthesized at early times to stimulate late gene expression. This explanation would be compatible with the findings of MacLean and Brown (1987b).

VmwIE175

Only one copy of IE3 is required for normal virus growth and gene expression in tissue culture (Longnecker and Roizman, 1986; Brown and Harland, 1987). However, IE3 has been shown to be essential in the lifecycle of HSV-1, having an important role to play in the transcriptional programme. Many ts mutants in VmwIE175 have been isolated (Marsden et al., 1976; Watson and Clements, 1978, 1980; Preston, 1979a; Dixon and Schaffer, 1980; DeLuca et al., 1984). Deletion mutants in both copies of IE3 have also been isolated and, while capable of growing on cell lines expressing VmwIE175,

they are incapable of growing on normal (non-permissive) cells, which fail to complement this gene (DeLuca et al., 1985).

Most ts mutants in VmwIE175 overexpress IE polypeptides and fail to express early and late polypeptides (Marsden et al., 1976; Watson and Clements, 1978, 1980; Preston, 1979a; Dixon and Schaffer, 1980). In temperature shift-up experiments, these mutants resume IE gene expression and discontinue early gene expression, suggesting that functional VmwIE175 is required continuously throughout infection for early gene expression and IE gene repression (Dixon and Schaffer, 1980; Watson and Clements, 1980). Ts mutants in IE3 have also been isolated in which early, but not late, genes are expressed. In some cases, no DNA is synthesized, while in others DNA replication is apparently normal, implying that VmwIE175 plays a role both in DNA replication and late gene expression (DeLuca et al., 1984). These findings suggest that VmwIE175 may have several functional domains : the position of the ts lesion within the polypeptide determining the effect on gene expression. The difference in behaviour of deleted IE3 genes compared to ts IE3 genes in a plasmid-based transient expression assay may support this suggestion (DeLuca and Schaffer, 1987).*

VmwIE175 has been isolated as a homodimeric complex (Metzler and Wilcox, 1985). It is a phosphoprotein, there being three phosphorylated forms, a, b and c, which can be separated on the basis of their mobility on SDS-PAGE (Pereira et al., 1977). VmwIE175 from tsK, an HSV-1 strain 17 mutant with a ts lesion in IE3, contains only a single amino-acid substitution compared to wild-type VmwIE175

(Davison et al., 1984), and at the non-permissive temperature this polypeptide lacks the phosphorylated form c (Preston, 1979a,b) and fails to be poly-ADPribosylated (Preston and Notarianni, 1983), suggesting that such modifications may play a role in some of the functions of VmwIE175.

VmwIE175 has been shown to be a DNA-binding protein, although it is unknown if this is a direct or indirect interaction (Bayliss et al., 1975). Hay and Hay (1980) reported that VmwIE175 from crude extracts of cells infected under IE conditions bound to DNA, and Faber and Wilcox (1986) showed that partially-purified VmwIE175 could also bind to DNA. In contrast, Freeman and Powell (1982) showed that purified VmwIE175 had lost the ability to bind to DNA; this ability was restored by the addition of uninfected cell extracts. These authors concluded that VmwIE175 did not bind directly to DNA, but bound via a cellular protein. However, it is equally possible that VmwIE175 had been destabilized during purification and lost its DNA-binding activity; addition of proteins may restabilize VmwIE175, restoring its DNA-binding properties.

Recently, DNA sequences have been identified which bind protein complexes containing VmwIE175. Using a DNA-binding immunoassay, Faber and Wilcox (1986) have shown that partially-purified VmwIE175 binds to one site in the gD promoter and two sites in the tetracycline gene of pBR322. These three sites yielded a good consensus (ATCGTCNNNNYCGRC). As the site in gD lies outwith the essential gD promoter region, its significance is unknown (Everett, 1983; Faber and Wilcox, 1986).

Kristie and Roizman (1986a,b) have also demonstrated interactions between protein complexes containing VmwIE175 and specific DNA sequences using a gel retardation assay and monoclonal antibodies against VmwIE175. They detected binding to the regulatory sequences of IE genes 1, 2 and 3 and a late gene. However, little significant binding was detected at the tk promoter, an early gene responsive to stimulation by VmwIE175 (O'Hare and Hayward, 1985a). Analysis of the binding site in IE3 revealed that it contained sequences from -135 to -194 (Kristie and Roizman, 1986b). Binding did not require the presence of Spl binding sites and the consensus sequence of Faber and Wilcox (1986) was not present. In contrast, Muller (1987) was unable to detect binding in this region of the IE3 gene promoter using infected cell extracts, but could detect binding around the transcriptional start site of the gene : DNase I footprinting experiments demonstrated that the binding site contained a sequence compatible with the consensus binding site of Faber and Wilcox (1986), and is contained within the region believed to be important for autoregulation by VmwIE175 (O'Hare and Hayward, 1987).

The anomalies between these results, and the significance and effects of these DNA-protein interactions, are at present unclear. It is possible that binding specificities depend on the processing of VmwIE175 and on the presence of other proteins at different times in infection. Muller (1987) did not detect his complex until 2 h post infection, the time at which down-regulation of IE3 starts (Godowski and Knipe, 1986).

Pizer *et al.* (1986), using an *in vitro* transcription

assay, have demonstrated that the interaction between VmwIE175 and the IE3 promoter can lead to down-regulation, while interaction with an early or late promoter can lead to stimulation.

VmwIE68

A deletion mutant in IE4 exhibits host-range dependence, growing normally in some cell lines but not in others (Post and Roizman, 1981; Ackermann et al., 1985; Sears et al., 1985). These workers suggest that in the permissive cells a cellular factor substitutes for VmwIE68. They further speculate that VmwIE68 may have a role to play in late gene expression, on the basis of the pattern of gene expression in non-permissive cell lines (Sears et al., 1985).

VmwIE12

Immediate-early gene 5 has been shown to be non-essential for growth in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987). It is the only non-nuclear and unphosphorylated immediate-early polypeptide (Pereira et al., 1977; Hay and Hay, 1980, 1981). Little is known about its function but O'Hare and Hayward (1985a) suggest that it may have a role to play in regulation of early genes.

I.10.d.ii Vmw65

Stimulation of IE gene expression involves the interaction of the major tegument polypeptide, Vmw65, (UL42, Figure 10) with upstream regulatory regions of the IE genes (Post et al.,

1981; Mackem and Roizman, 1982b; Batterson and Roizman, 1983; Cordingley et al., 1983; Campbell et al., 1984; Preston C. et al., 1984; see section I.10.f.i).

The DNA sequence of Vmw65 has now been determined (Dalrymple et al., 1985; Pellet et al., 1985). It has been shown to be a different polypeptide from the $65K_{DBP}$ previously identified (Bayliss et al., 1975; Marsden et al., 1987; section I.8.b.v), and does not bind to DNA alone (Marsden et al., 1987; Muller, 1987). It is possible that Vmw65 may alter the specificity of a cellular DNA-binding protein, or interact indirectly with specific DNA sequences via a cellular or viral protein : interactions between cellular proteins and the HSV TAATGARATTC motif (Gaffney et al., 1985) have been identified (Kristie and Roizman, 1987; R.Thompson, personal communication), and such interactions may be altered following viral infection, with Vmw65 being found in the DNA-protein complex (Preston et al., 1988).

I.10.d.iii Major DNA-binding protein

The MDB has been shown to affect gene transcription. Defects in the MDB lead to increased expression of all classes of HSV genes (Godowski and Knipe, 1983, 1986). In the absence of DNA replication, functional MDB is required for the switch-off of IE3 transcription and to decrease the levels of at least some genes of all temporal classes (Godowski and Knipe, 1985, 1986). These results could either be interpreted as a general repression of HSV transcription by the MDB or, alternatively, since the MDB down-regulates IE3 synthesis at early time-points, lack of this function could lead to increased VmwIE175 levels and, as a

consequence, higher levels of HSV early and late gene expression.

I.10.e Transactivation of herpes simplex virus promoters in plasmid-based transient expression systems

I.10.e.i Immediate-early promoters

Immediate-early gene products have been shown to affect the level of expression from IE promoters. VmwIE110 stimulates expression from all IE promoters (O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986, 1987). In contrast, VmwIE175 at high molar ratios inhibits expression from its own promoter, although some stimulatory effects are found at low molar ratios (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985b; Gelman and Silverstein, 1986, 1987). VmwIE175 has also been shown to have a similar effect on all the other IE promoters, except that of IE gene 2, where a general stimulatory response was found (Gelman and Silverstein, 1987). When VmwIE110 and VmwIE175 are added together, VmwIE175 inhibits the stimulatory effect of VmwIE110 (Gelman and Silverstein, 1986, 1987). In contrast, when a cloned ts VmwIE175 gene is used, this may stimulate the expression from IE promoters and give a synergistic effect with VmwIE110 (DeLuca and Schaffer, 1985; Gelman and Silverstein, 1986, 1987). Vmw65 also stimulates IE promoter activity (Gelman and Silverstein, 1986, 1987). Interestingly, Gelman and Silverstein (1987) illustrated that the cell type used affected the response of these promoters to transactivation.

I.10.e.ii Early promoters

Short-term transfection assays have been used to determine the role of IE polypeptides in early gene expression. Although the results of several groups are not completely compatible, they give the same overall profile. Both VmwIE110 and VmwIE175 can stimulate expression from early promoters when used in isolation (Everett, 1984a; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985; Gelman and Silverstein, 1986). However, when used in conjunction, VmwIE110 and VmwIE175 have a synergistic effect (Everett, 1984a, 1986; Quinlan and Knipe, 1985; Gelman and Silverstein, 1986). Both early promoters such as tk and early-late promoters such as gD have been used with similar results. O'Hare and Hayward (1985a) also suggested that, in combination with VmwIE110 and VmwIE175, VmwIE12 may increase the response of at least some early promoters. This remains to be confirmed.

Everett (1986) has shown that VmwIE63 on its own has no effect on early and late promoters, but in conjunction with VmwIE110 and VmwIE175 causes increased transcription from the VP5 (an early-late) promoter. No effect was seen on either the tk or gD promoters.

I.10.e.iii Late promoters

True-late promoters also appear to be transactivated by IE gene products. The U_S11 promoter is stimulated to a small extent by VmwIE175 and VmwIE110 alone, but to a much greater extent when both are present together (Johnson, 1987). VmwIE63 was shown to have no effect on U_S11 expression (Johnson, 1987). Shapira et al. (1987) have reported similar

findings with the gC promoter, as have DeLuca and Schaffer (1985) using the L42 promoter. In contrast, using the L42 promoter, Mavroma-Nazos et al. (1986) found that VmwIE175 alone caused significant stimulation of transcription. It would appear that transactivation of late promoters is probably similar to that of early promoters.

Johnson and Everett (1986a), using a plasmid system, have demonstrated that DNA replication is required for the abundant expression of the true-late gene, U_S11, although in the absence of DNA replication some limited transcription does take place. As such, this mimics the system in the intact virus (Powell et al., 1975; Swanstrom et al., 1975; Johnson et al., 1986). Johnson and Everett (1986a) have shown that this is not only a case of an increase in template copy number leading to a higher level of transcripts, but that DNA replication per se plays a role, possibly leading to either a structural change at the promoter, or to different transactivation factors being present, thereby leading to an increase in the promoter activity. They speculate that replication may increase the availability of the promoter for transcription factors (Johnson and Everett, 1986a).

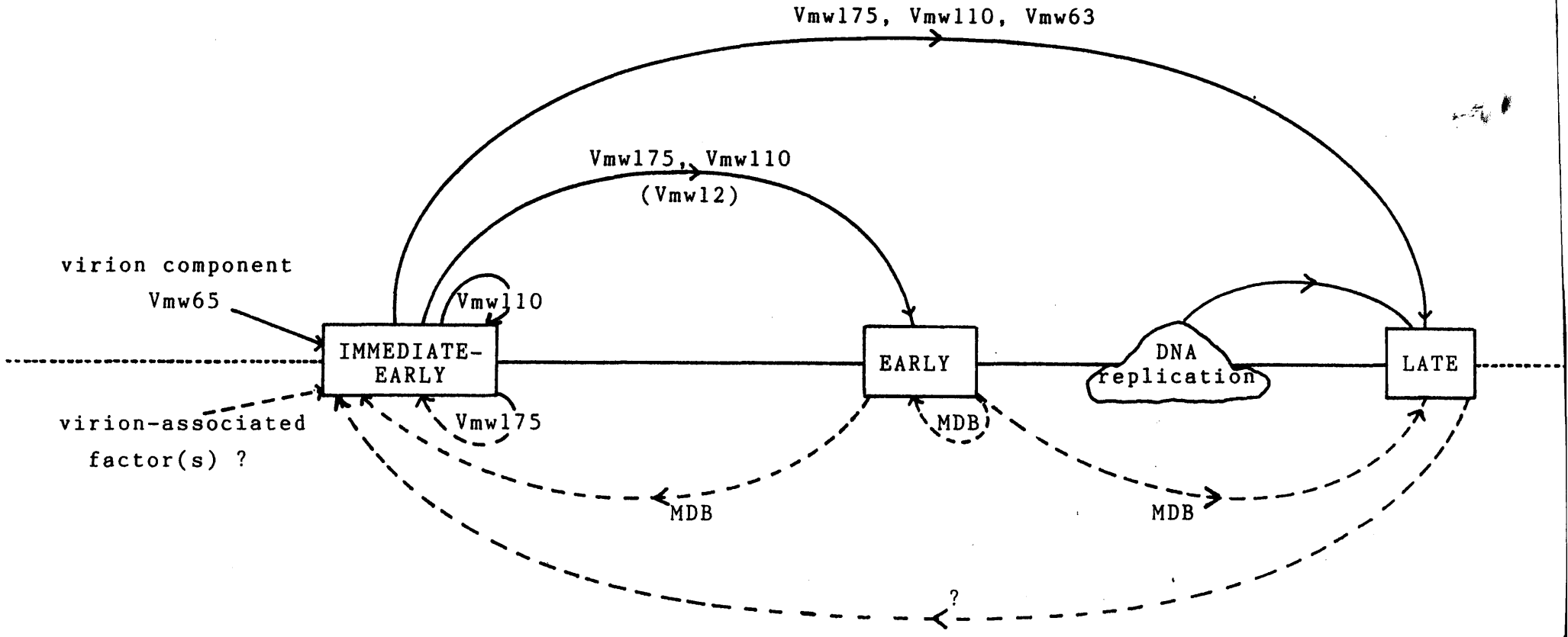
Figure 15 summarizes the role of HSV-encoded factors in the regulation of transcription of different classes of HSV genes.

Figure 15. Regulation of HSV transcription.

A summary of the viral polypeptides believed to influence viral gene expression. These are discussed fully in the text. Factors shown above the central line and indicated by a solid arrow (⟶) have a positive influence in the direction shown, while those shown below the central line and indicated by a dotted arrow (⇢) have a negative influence in the direction shown. The role of VmwIE12 in early gene expression remains to be established. ? indicates that the factor responsible has not been fully characterized.

MDB is the major DNA-binding protein.

Adapted from Johnson, 1987.


Vmw175, Vmw110, Vmw63
Vmw175, Vmw110
(Vmw12)
virion component
Vmw65
Vmw110
IMMEDIATE-
EARLY
EARLY
DNA
replication
LATE
virion-associated
factor(s) ?
Vmw175
MDB
MDB
MDB
?

I.10.f Sequence requirements for herpes simplex virus gene expression

I.10.f.i Immediate-early promoters

Immediate-early regulatory regions can be subdivided into (i) a minimal transcription unit that controls basal level expression and (ii) upstream sequences that are required for correct temporal regulation (Post et al., 1981; Mackem and Roizman, 1982a,b; Cordingley et al., 1983; Kristie and Roizman, 1984; Preston C. et al., 1984; Bzik and Preston, 1986). The minimal transcription unit includes the CAP site, TATA box and GC-rich elements which may include Sp1-binding sites (Jones and Tjian, 1985). The upstream regulatory sequences all contain an element responsive to Vmw65, the virion component (Mackem and Roizman, 1982a,b; Cordingley et al., 1983; Preston C. et al., 1984). The responsive sequence has a consensus of TAATGARATTC (Mackem and Roizman, 1982a,b; Murchie and McGeoch, 1982; Whitton et al., 1983; Whitton and Clements, 1984a; Gaffney et al., 1985), with at least one copy being present upstream of all the HSV-1 and HSV-2 IE genes.

The upstream regulatory region of IE3 has been extensively analyzed (Lang et al., 1984; Preston and Tannahill, 1984; Bzik and Preston, 1986). An enhancer-like element was discovered which worked in a distance and orientation independent manner but, unlike the typical SV40 enhancer (Banerji et al., 1981), did not act when situated at the 3' end of the gene, and was responsive to Vmw65 (Lang et al., 1984; Preston and Tannahill, 1984). The sequences responsible for enhancer activity and Vmw65 responsiveness

could be separated - the enhancer activity was assigned to three separate elements, two Spl-binding sites (GGGCGG) (Jones and Tjian, 1985) and one GA-rich box with homology to the SV40 enhancer core (Weiher et al., 1983). The most important element for response to Vmw65 was the TAATGARATTC sequence, but the GA-rich element augmented this effect.

I.10.f.ii Early promoters

The tk promoter has been analyzed in great detail using deletion and 'linker-scanning' mutagenesis (Ace et al., 1988) and assaying in either microinjected frog oocytes or transfected cells superinfected with HSV. The tk promoter is active constitutively in oocytes in the absence of HSV gene products (McKnight and Kingsbury, 1982; McKnight et al., 1984; Eisenberg et al., 1985; El Kareh et al., 1985; Jones et al., 1985). Four regions required for efficient transcription of the tk promoter have been identified. These include the TATA box at -25, two GC hexanucleotides at -55 and -100, and a CAAT box at -85. The GC hexanucleotides are recognized by Spl (Jones and Tjian, 1985) and the CAAT box by CAAT box-binding protein (Jones et al., 1985). Some of the deletions constructed by the above workers have been inserted into intact virus. These four regions were again found to be important, as was an additional region, just 3' to the RNA CAP site, ie. within the untranslated leader sequence (Coen et al., 1986).

The gD promoter has been analyzed in detail by Everett (1983, 1984b). Deletion analysis has shown that sequences from -83 to +17 are sufficient for fully regulated expression of gD. Four important regions were identified.

(i) The CAP site region (-4 to +11); (ii) the TATA box (-25 to -18); (iii) a GC-rich region at -53 to -41, and (iv) a second GC-rich region at -73 to -63. The latter two regions have good homology to the Sp1 consensus binding site (Jones and Tjian, 1985; Jones et al., 1985; Karadonna et al., 1986). Both the gD and tk promoters have similar sequence requirements, with the exception that the gD promoter does not have an obvious CAAT box.

The early-late VP5 promoter has been shown to require sequences up to between -73 to -125 for full activity (Costa et al., 1985b). Thus, it probably also requires upstream regulatory sequences, resembling tk and gD.

No virus-specific sequences have been detected in either the gD (Everett, 1983, 1984b) or tk (Eisenberg et al., 1985; Coen et al., 1986) promoters when comparing sequences required for transactivation in the presence or absence of viral superinfection. This suggests that virus transactivating factors may modify the action of the existing cellular transcription factors, or may themselves use existing transcription factor binding sites. HSV-1 infection has also been shown to activate at least some cellular promoters (Everett, 1983, 1984b). This effect was mediated by IE polypeptides (Everett, 1984a).

I.10.f.iii Late promoters

Johnson and Everett (1986b) have investigated the sequence requirements of the true-late gene, Us11 (Johnson et al., 1986). They have shown that only sequences from -31 to +39 are required for efficient transcription. This includes the TATA box which they conclude may be the only

sequence requirement for late gene expression. They converted the gD promoter into a late promoter by deleting sequences up to the TATA box and linking it to an origin of DNA replication.

Similar sequence requirements have been found for the true-late gene, gC (Hall et al., 1982; Yager and Bachenheimer, 1987) with the TATA box appearing to be essential (Homa et al., 1986; Shapira et al., 1987).

The sequence requirements of the three temporal classes of HSV genes are illustrated in Figure 16. As infection proceeds, the sequence requirements appear to decrease. For late genes, it is possible that the effect of DNA replication may be able to compensate for the lack of upstream promoter sequences (Johnson and Everett, 1986b), which are responsive to cellular transcription factors such as Spl (Jones and Tjian, 1985).

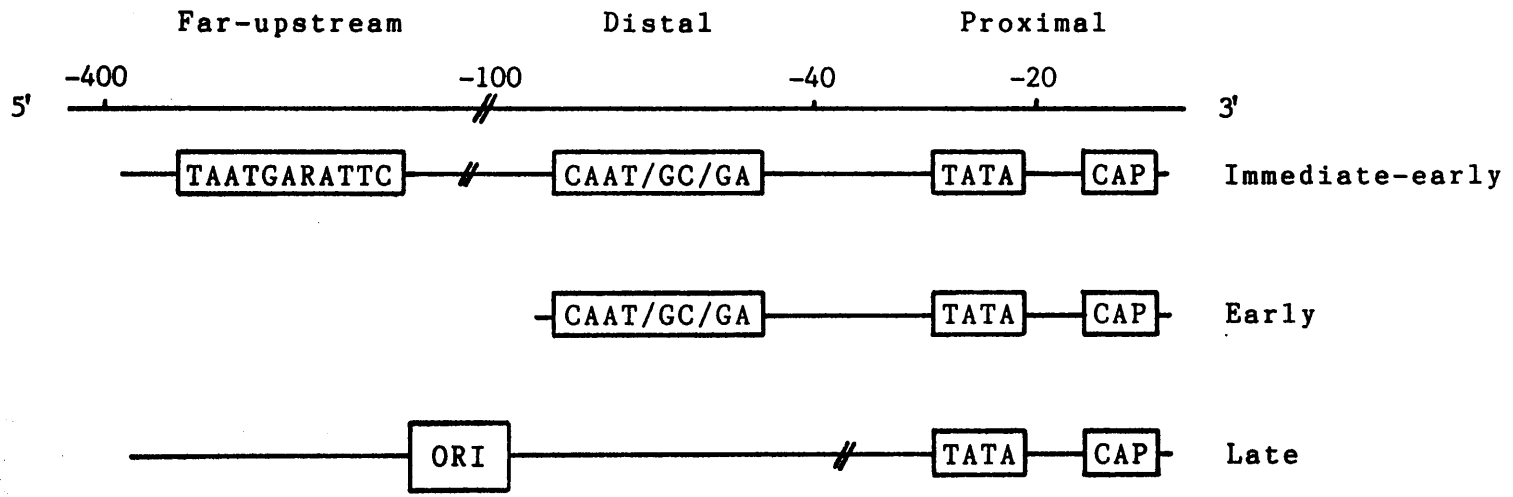
I.11 EFFECTS OF HERPES SIMPLEX VIRUS INFECTION ON HOST CELL METABOLISM

Infection of permissive cells by HSV leads to alterations in host cell functions and macromolecular synthesis. Mitosis ceases (Wildy et al., 1961), DNA synthesis is inhibited (Roizman and Roane, 1964), and a rapid shut-off of most host polypeptide synthesis occurs (Sydiskis and Roizman, 1966, 1967) accompanied by a degradation of cellular mRNAs (Schek and Bachenheimer, 1985). Host polysomes are disaggregated following infection (Sydiskis and Roizman, 1966, 1967), and newly formed

Figure 16. HSV gene 'promoter' signals.

Cis-acting signals required for the regulation of HSV transcription. Adapted from Johnson and Everett (1986a). Promoter element requirements seem to decrease the later the temporal class of the gene. Thus, late promoters consist of only a 'TATA-box' and cap-site region (CAP), although they require an active origin of DNA replication (ORI) in cis for efficient expression; early genes also require a distal promoter element, containing at least one of either a 'CAAT' box, GC-rich motifs or GA-rich motifs; while immediate-early genes require, in addition, a far upstream element, including the consensus sequence TAATGARATTC, as well as flanking modulatory sequences for response to the virion transactivating factor, Vmw65.

This diagram is not drawn to scale.


Far-upstream
Distal
Proximal
-400
-100
-40
-20
5'
3'
TAATGARATTC
CAAT/GC/GA
TATA
CAP
Immediate-early
CAAT/GC/GA
TATA
CAP
Early
ORI
TATA
CAP
Late

polyribosomes contain predominantly virus-encoded mRNA (Stringer et al., 1977).

Infection with HSV-2 usually results in a more rapid inhibition of host protein synthesis compared to HSV-1 (Powell and Courtney, 1975; Pereira et al., 1977; Fenwick et al., 1979; Schek and Bachenheimer, 1985), although HSV-2 strain HG52 is an exception, causing poor host shut-off (Marsden et al., 1978).

The factors involved in host shut-off are unclear, with at least two factors being involved. One is a virion component (Sydiskis and Roizman, 1967; Fenwick and Walker, 1978; Fenwick et al., 1979; Schek and Bachenheimer, 1985), although this function is not essential in tissue culture (Read and Frenkel, 1983). A second factor, synthesized later in infection, and which can act in the absence of the virion factor, is required for complete host shut-off (Honess and Roizman, 1974; Marsden et al., 1976). The virion-associated factor has been mapped to between 0.52-0.59 m.u. (Morse et al., 1978), as has the factor responsible for inhibition of DNA synthesis (Fenwick et al., 1979).

Some cellular genes, however, are up-regulated, especially the heat shock genes (Notarianni and Preston, 1982; LaThangue et al., 1984) and some genes which are also up-regulated in transformed cells (MacNab et al., 1985). Everett (1985) has demonstrated that HSV infection can activate stably-integrated cellular genes.

I.12 THE EVOLUTION OF HERPESVIRUSES

Herpesvirus evolution can be subdivided into three

areas : the relatedness of polypeptides in different viruses; G+C content; and the structure of the DNA repeat elements. There is overlap between these areas. Such analysis has been considerably aided by the increasing availability of DNA sequence information (for example, Baer et al., 1984; McGeoch et al., 1985, 1987; Davison and Scott, 1986).

I.12.a Polypeptide relatedness

Prior to large scale DNA sequencing, it was clear that many alphaherpesviruses did share a common ancestry as determined by antigenic cross-reactivity and DNA cross-hybridization (Davison and Wilkie, 1983c; McGeoch, 1987). There was no evidence for relatedness between sub-families. In HSV-1 and VZV, two alphaherpesviruses, a high degree of homology has been found between the proteins encoded by the genome (Davison and McGeoch, 1986; Davison and Scott, 1986; McGeoch, 1987). Gene organization in the short region is quite different, at least partly due to movement of the repeats between the two viruses (see section I.12.d). However, all the VZV genes in the short segment have counterparts in the HSV-1 short segment, although six HSV-1 genes have no counterpart in VZV (Davison and McGeoch, 1986). There is much less difference between the long segments, where only three or four genes in both HSV-1 and VZV have no counterpart (McGeoch, 1987) and the genes are arranged, in the main, colinearly. VZV has only an 88 bp long repeat and the IE1 gene equivalent lies within U_L (Davison and Scott, 1986; McGeoch, 1987). All seven essential HSV-1 replication genes have counterparts in VZV

(McGeoch et al., 1988; Wu et al., 1988); in some cases the sequence homology is among the highest seen when comparing these genomes, possibly reflecting their importance in the viral lifecycle (McGeoch, 1987). At least some of the genes in HSV-1 and VZV are functionally equivalent, as VZV can complement ts mutants in VmwIE175 and VmwIE63 (Felser et al., 1987).

The genomes of alpha- (HSV-1 and VZV) and gamma- (EBV) herpesviruses have also been compared. Davison and Taylor (1987) have carried out an extensive analysis of VZV and EBV. At the level of gene homology, they found twenty-nine pairs of genes which display some homology, ranging from strong to weak. They also identified fourteen pairs of genes which, although containing no amino acid homology, are believed to be homologous by the nature of their position or properties, such as distribution of hydrophobic residues. All of these related genes are in the U_L regions of both genomes : the repeat elements and short regions appear unrelated. Within U_L, conserved genes are located in three large blocks, whose relative locations differ in the two genomes. Within each block, the gene arrangement is generally conserved, although there are some minor rearrangements.

From the analysis of HSV-1, VZV and EBV, it would appear that the L segment represents the core of the conserved herpesvirus gene complement. In contrast, the S segment varies both between and within subgroups (McGeoch, 1987).

Herpesvirus genes have also been compared with non-herpesvirus genes. Thus, homologies have been found with

genes of prokaryotic and eukaryotic cells and viruses. Both subunits of ribonucleotide reductase are homologous to eukaryotic and prokaryotic enzymes (Nikas et al., 1986). Thymidylate synthetase (TS) of VZV and HVS is also homologous to prokaryotic and eukaryotic TS (Davison and Scott, 1986; Honess et al., 1986; Thompson et al., 1987). A protein kinase has been identified in HSV and VZV which has homology to eukaryotic protein kinases (McGeoch and Davison, 1986a). The DNA polymerase of HSV and vaccinia, a pox virus, are also homologous and may have evolved from the cellular DNA polymerase alpha (Earl et al., 1986; McGeoch, 1987). These homologies between viral and cellular genes may help to identify the origin of the genetic material which gave rise to the ancestral herpesvirus genome from which it is believed present day herpesviruses have arisen (Honess, 1984; McGeoch, 1987).

I.12.b G+C content

The three genomes discussed above, and for which the complete DNA sequence is available, have G+C contents of 68.3% (HSV-1; Dr. D. J. McGeoch; personal communication), 46% (VZV; Davison and Scott, 1986) and 60% (EBV; Baer et al., 1984). Thus, although HSV-1 and VZV are very closely related, their G+C content differs by 22%. For herpesviruses as a whole, G+C content varies from 32-75% (Honess, 1984). In general, the G+C content increases towards the repeats (for example, Davison and Scott, 1986; McGeoch et al., 1986, 1987; Perry, 1986). Herpesviruses are densely packed with genes. This is true both of viruses with a high G+C content, such as HSV (McGeoch, 1984; McGeoch et al., 1985; Dr. D. J.

McGeoch, personal communication), and those with a low G+C content, such as VZV. Therefore, the difference in G+C content must be largly due to the use of degenerate codons as well as differences in the amino acid composition of related polypeptides. The G+C content spans the whole range acceptable for polypeptide coding sequences (Woese and Bleyman, 1972).

As a generalization, alphaherpesviruses have a high, betaherpesviruses an intermediate, and gammaherpesviruses a low G+C content, although there are obvious exceptions (for example, VZV). Thus, viruses with similar biological properties tend to have a similar G+C content (Honess and Watson, 1977b; Honess, 1984).

The reason for the variation in G+C content is not clear. There are two views : selectionists believe that the differences in G+C content arise as a consequence of the cellular environment; non-selectionists believe that genome composition is the product of some difference in, or intrinsic property of, the genetic system, subject to, but not directed by, external selection (Honess, 1984).

The selectionist view has generally been favoured : that the variations in base composition are due to adaptation of the virus to pre-existing or virus-induced differences in the proportions of tRNAs in the infected cell (Subak-Sharpe, 1967; Subak-Sharpe et al., 1974; Roizman, 1980). Alphaherpesviruses like HSV, with a high G+C content, have CpG frequencies close to that expected at random possibly due to them undergoing latency in non-replicating cells (neurons). In contrast, their hosts have a deficiency in CpG doublets (although the GpC doublet is present at

the expected frequency), probably due to their potentially mutagenic effects (Bird, 1980). Thus, CpG doublets are present in viral coding sequences at a higher frequency than in the host cell coding sequences (Subak-Sharpe, 1967). Although the prediction that herpesviruses might encode tRNAs for CpG-containing codons (Subak-Sharpe, 1967) has not been borne out (Morris et al., 1970; Bell et al., 1971), the idea that the G+C content of herpesvirus genomes is a consequence of the different abundances of tRNAs encoding the same amino acid is still favoured.

Analogous situations are found in both eukaryotes and prokaryotes, where there is a striking correspondence between codon usage and tRNA abundance (Ikemura, 1981; Bennetzen and Hall, 1982). This model proposes a role for codon/tRNA ratios in gene expression. Thus, genes whose polypeptide products would be toxic to the cell, if present in excess, will contain many rare codons (for which the tRNAs are present at a low level) and hence be poorly expressed (Ikemura, 1981; Konigsberg and Godson, 1983).

There are three main objections to the selectionist theory regarding herpesviruses. Firstly, a theory suggesting optimization should lead to convergence, whereas with herpesviruses it is believed there is divergence from a presumably common ancestor (Honess, 1984). Secondly, while the advantage of codon usage as a means of gene regulation in polycistronic systems (ie., prokaryotes) is apparent, the advantage in eukaryotes, where mRNA is monocistronic, is less obvious. One point mutation in the promoter region could achieve a similar effect as the multiple changes required in a gene, if it is to be regulated by codon usage.

Thirdly, and perhaps most convincingly, the explanation could equally well be reversed : tRNA abundance being determined by the codon usage of ongoing protein synthesis.

Thus, a non-selectionist explanation for the varied base composition seems equally likely. The simplest explanation would be biased mutation rates (ie., [A-T]→ [G-T]≠[G-C]→ [A-T]) causing drift in overall base composition. This could be produced by the replication and recombination machinery of the cell and virus. The biasing mechanism would vary for different herpesviruses, and is required to impart direction. Mutator and antimutator variants of the HSV DNA polymerase have been isolated (Hall et al., 1984; Honess et al., 1984). Differences in the substrate specificities of thymidine kinase from different herpesviruses, and modification in the specificity of this gene in HSV-1 by mutation, have been observed (Honess and Watson, 1977b; Honess et al., 1982, 1984; Larder et al., 1982). Differences in the pool of triphosphates between uninfected and HSV-1 infected cells (Jamieson and Bjursell, 1976) have been noticed. The discovery of a thymidylate synthetase gene in VZV and HVS is of interest (Davison and Scott, 1986; Honess et al., 1986; Thompson et al., 1987). These two A+T rich genomes thus have an enzyme which will increase the pool of A and T in the infected cell; the G+C rich HSV and EBV genomes lack this enzyme. It is highly probable that the herpesvirus replicative machinery is at least partly responsible for the drift in base composition.

Recombination between the repeat regions is likely to be responsible for the higher G+C content found in these regions in HSV (McGeoch et al., 1986). As the population

size of the repeats is double that of the unique sequences, then the drift to G+C would be faster in the repeats. An interesting case is HVS, where the G+C content in the unique sequence is 30%; this being called Light (L) DNA. In contrast, the G+C content of the repeated sequences is 71%; this being called Heavy (H) DNA (Stamminger et al., 1987). Biased gene conversion favouring G+C rich sequence retention would fix this high G+C content in the repeat and explain the sharp cut-off at the repeat/unique boundary. This is a similar method to that proposed by Dover (1982) for multigene family evolution in eukaryotes. The mode of transmission of herpesviruses, including long term infection (latency) and periodic recurrences, would tend to favour the fixation of mutator phenotypes. In this type of infection selective pressures will be less active than in a short acute infection (Koch, 1971; Kubitshik, 1974).

It is claimed that rather than cell tropism determining the virus G+C content, the virus G+C content is determining tissue tropism (Honess, 1984). One possible mechanism would be through regulatory sequences, which have a marked compositional bias, with examples of both AT-rich (poly-adenylation site (Proudfoot and Brownlee, 1976)) and GC-rich (Spl-binding sites (Jones and Tjian, 1985)) sequences. Such cis-acting sequences have been shown to exhibit tissue or species tropism (Queen and Baltimore, 1983; Spandidos and Wilkie, 1983; Linney et al., 1984; Picard and Schaffner, 1984), presumably representing differences in the availability of trans-acting factors (Ephrussi et al., 1985; Meriolar et al., 1985; Queen et al., 1985). Thus, differences in cell specific trans-acting

factors might provide a basis for the biological consequences of a generally-biased base composition. It is unlikely that the G+C content of coding sequences in HSV affects their temporal regulation, as genes of all temporal classes have a similar composition (Honess, 1984; Dr. D. J. McGeoch, personal communication). In addition, gene U_S11, a true-late gene (Johnson et al., 1986), has an atypical codon usage for HSV genes, presumably as a consequence of its coding sequence overlapping that for U_S10 (Rixon and McGeoch, 1984), yet it is regulated in a similar manner to another true-late gene, gC (Homa et al., 1986), which displays typical codon usage.

I.12.c Structure of the repeats

Herpes simplex virus consists of two unique segments each bounded by a pair of inverted repeats, with the 'a' sequence present as a direct repeat at the termini and as an inverted repeat at the L-S junction (see section I.6.c). Isomerization about the L-S junction leads to the presence of four equimolar isomers (Sheldrick and Berthelot, 1974; Wadsworth et al., 1975; Delius and Clements, 1976). The role of the repeated sequences and inversion is unknown. Genes contained within the repeats are diploid. However, since different genes may be contained within the repeats of related viruses (Davison and McGeoch, 1986), it is possible that these genes do not require to be diploid, and are only so as a consequence of repeat movement during evolution, mediated by recombination (see below). Not all herpesviruses have inverted repeats and thus not all herpesviruses are capable of isomerization. Indeed, viruses with similar

biological properties may have totally different repeat structures (Honess, 1984).

At least three of the four isomers of HSV appear to be biologically active (Davison and Wilkie, 1983b; Poffenberger and Roizman, 1985; Jenkins and Roizman, 1986; Longnecker and Roizman, 1986), with any one isomer capable of giving rise to all four isomers at high frequency (Roizman, 1979; Roizman et al., 1979).

At one time it was suggested that segment inversion might play a role in gene regulation and the virus lifecycle (Roizman, 1979). However, this idea was negated by the discovery that no genes or regulatory sequences cross the L-S junction (Clements et al., 1979; Mackem and Roizman, 1980). It has also been suggested that genome segments are relics of an ancestral fusion of two separate pieces of DNA. Available evidence would dispute this, despite the fact that the S segment of herpesviruses appears more variable than the L segment (McGeoch, 1987), and most of the genes in U_S of HSV, with the possible exception of gD, are dispensible for growth in tissue culture (Longnecker and Roizman, 1986, 1987; Umene, 1986; Brown and Harland, 1987; Longnecker et al., 1987; Weber et al., 1987). For example, Wilkie et al. (1978) showed that dinucleotide frequencies were the same in different parts of the genome, suggesting at least a recent common evolutionary history. It has also been suggested that the reversible dissociation and reassociation of genome segments may represent a method of controlling the establishment of latency and reactivation (Ritchie and Timbury, 1980). However, this has been ruled out by the finding of joints, but no ends, in latent HSV genomes (Rock

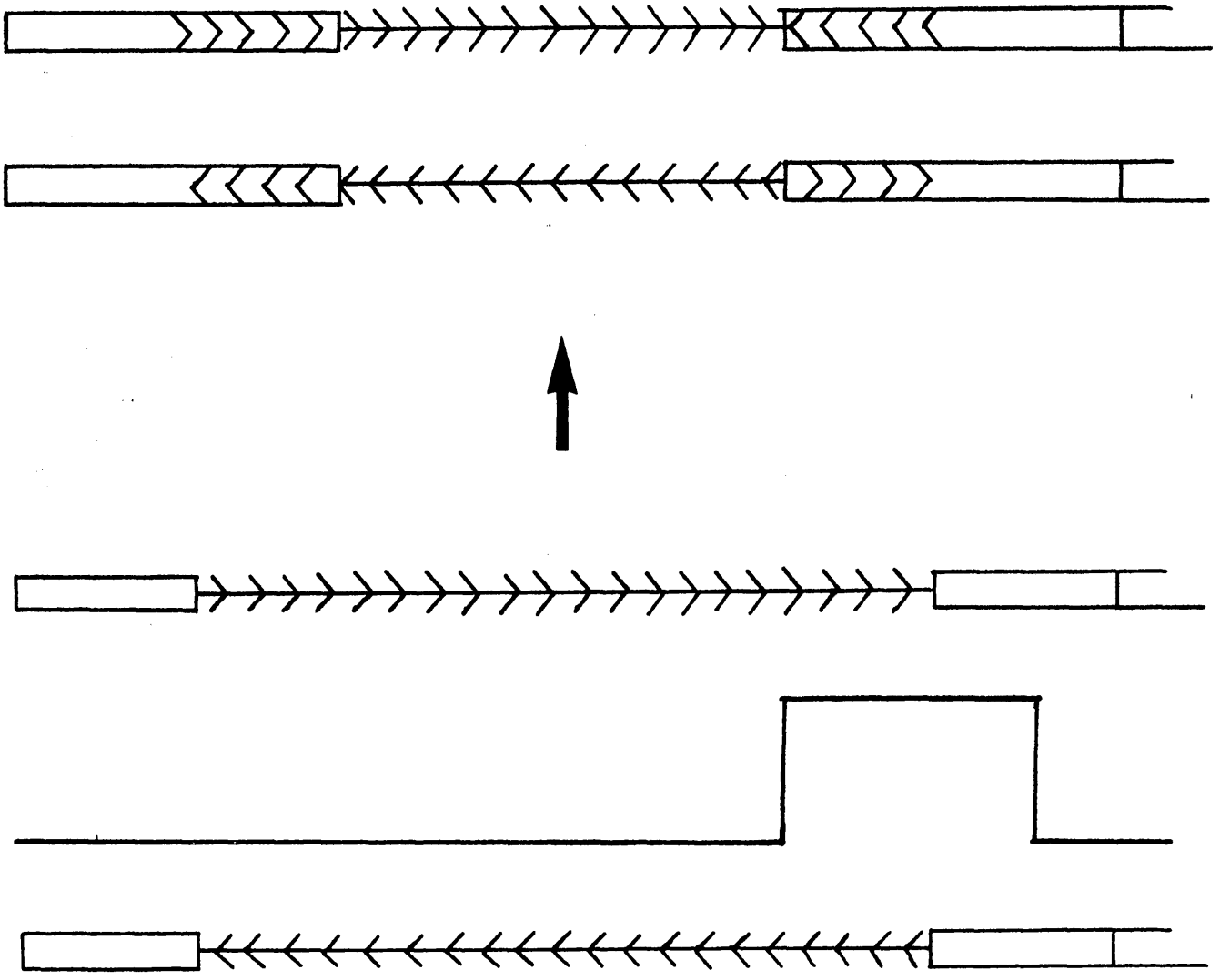
and Fraser, 1983, 1985).

I.12.d Movement of the repeats

It has been suggested that the inverted repeats in herpesviruses are dynamic structures, capable of contraction or expansion (Honess, 1984; McGeoch, 1984; Whitton and Clements, 1984b; Davison and McGeoch, 1986). In HSV, the short repeat contains the entire gene (IE3) encoding VmwIE175 (McGeoch et al., 1976; Clements et al., 1979) and the shared 5' untranslated leader and intron of the genes (IE4 and IE5) encoding VmwIE68 and VmwIE12, respectively, whose coding sequences are contained entirely within the opposite ends of U_S (Whitton and Clements, 1984b). However, the position of the repeat/unique junction differs in HSV-1 and HSV-2. In HSV-1 the ATGs of VmwIE12 and VmwIE68 are 8 bp and 40 bp, respectively, into U_S (Murchie and McGeoch, 1984), whereas in HSV-2 they are 1 bp and 33 bp, respectively, into U_S (Whitton and Clements, 1984b). Expansion of the short repeat in HSV-2 by 7 bp would lead to this arrangement, with the ATG of VmwIE12 in HSV-2 acting as a further barrier to expansion. Whitton and Clements (1984b) suggested that repeat expansion could have occurred by recombination between two wild-type genomes, one of which has U_S inverted relative to the other, homologously in the repeat and non-homologously, or illegitimately, in the unique sequences (Figure 17), to give an extended repeat arising from one end of U_S with the other end of U_S being deleted. This would lead to no change in the overall genome size. Unequal non-homologous recombination could also occur, leading to an increase or decrease in genome size.

Figure 17. Expansion of repeated sequences in HSV.

A simple recombination scheme to account for movement of the junctions between unique and repeat sequences in HSV. The upper part of the figure shows the S segment aligned in opposite orientations, as indicated by the arrow-heads. The heavy lines between them denote two crossovers, the first between homologous sequences in the repeats and the second illegitimately between non-homologous sequences in the unique sequences. The lower part shows the two progeny molecules with expanded repeats and reduced unique sequences.



The above authors suggest that factors limiting expansion of the repeats would be the position of essential transcriptional regulatory or translated sequences, which would be either altered or deleted by the proposed mechanism. Thus, one could imagine a situation where IE4 and IE5 were, at one time, separately regulated, not necessarily both as immediate-early genes; repeat expansion may then have led to both sharing a common promoter, 5' untranslated region and intron, but having unrelated coding sequences. In HSV-2, repeat expansion has proceeded as far as is possible without affecting the coding region of these polypeptides.

This mechanism has been proposed to explain the isolation of HSV genomes with expanded short repeats; in these genomes, sequences from the left of U_S have been duplicated, accompanied by the deletion of sequences from the right of U_S. Thus, some genes at the left of U_S, including IE4, are now present in diploid amounts, while genes from the right, including IE5, are now deleted (Umene, 1986; Brown and Harland, 1987).

In the long repeat of HSV-1 (the long repeat of HSV-2 has not yet been sequenced), the situation is somewhat different. The long repeats contain the gene (IE1) encoding VmwIE110 (Clements et al., 1979; Perry et al., 1986) and, in strain F, a 43,500 mol. wt. protein ICP34.5 (Chou and Roizman, 1986). However, strain 17 does not contain this gene (Dr. D. J. McGeoch, personal communication). There is no strong evidence for any genes near the unique/repeat junction (Perry, 1986). However, near the junction, at both ends of U_L, there is a gene being transcribed away from the repeats (Perry, 1986); it has been suggested that at least some of

the regulatory signals for these genes are contained in the repeats and thus are common. In this case, transcriptional initiation signals may have acted as barriers to expansion. Little is known about these genes. MacLean and Brown (1987b,c; this thesis) have isolated HSV strain 17 genomes where part of the long internal repeat and adjacent unique sequences have been deleted. Here the repeat has effectively contracted, as the sequences in the terminal repeat which were deleted in the internal repeat will now be part of U_L. A genome with an expanded long repeat has also been isolated (MacLean and Brown, 1987c; this thesis); in this genome sequences from the left end of U_L have been duplicated, accompanied by deletion of sequences from the right of U_L. A mechanism similar to that previously proposed, involving illegitimate recombination between genomes whose U_L regions are in inverted orientation relative to one another, has been proposed to account for this.

Different strains of HCMV have different R_L/U_L boundaries (Spector et al., 1983). In PRV, which usually contains no long repeat, genomes with long inverted repeats (and four isomers) have been isolated following passage in tissue culture (Lomnicizi et al., 1984, 1987). Here, sequences from one end of U_L have been duplicated in inverted orientation at the other end, presumably again by illegitimate recombination.

As well as expanding, the repeats can also contract. One mechanism would be by simple deletion of sequences in one repeat up to, and possibly including, unique sequences (see above). An alternative mechanism would be for sequences to be lost from both repeats by illegitimate recombination.

However, this would involve two different parental genomes (one containing a deleted repeat), and therefore appears less likely than expansion, which would involve only a pair of identical parents (Whitton and Clements, 1984b). Contraction would predict that the functional element acting as a barrier would be contained within the repeat rather than the unique sequence (Whitton and Clements, 1984b).

Davison and McGeoch (1986) have proposed a model of repeat contraction/expansion to explain the relationship between the short regions of HSV-1 and VZV. The final structures of both regions and related genes are illustrated in Figure 18. All the genes in VZV have homologues in HSV-1, but six of the HSV-1 genes have no homologues in VZV. It is assumed that the two regions are related via descent from a common progenitor. However, the arrangement of genes in the repeats and in the unique region varies, with several genes not being colinear. The relationship between the two could be explained by a complicated pattern of repeat expansion and contraction. Figure 19 shows a scheme for repeat movements which could explain the relationship between HSV-1 and VZV. HSV-1 is arbitrarily at the start and VZV at the end, but no temporal relationship is implied. The diagram represents the retracing of steps from HSV-1 towards an ancestral herpesvirus and hence to VZV. Gene loss in one direction could also be seen as gene gain from an outside source in the other direction. These steps are only hypothetical, and are the simplest explanation involving repeat movement :

(a) Gene 1 is taken into the repeats and genes 11 and 12 are lost from opposite ends of U_S;

Figure 18. Relationship between genes in the S segments of HSV-1 and VZV

The location and orientation of predicted polypeptide coding regions in the S segment of HSV-1 and VZV are indicated. R_S is represented by an open box and U_S by a solid line. Homologous genes are indicated by diagonal lines between them. For clarity relationships are indicated for only one copy of the genes in the inverted repeats.

Adapted from Davison and McGeoch (1986).

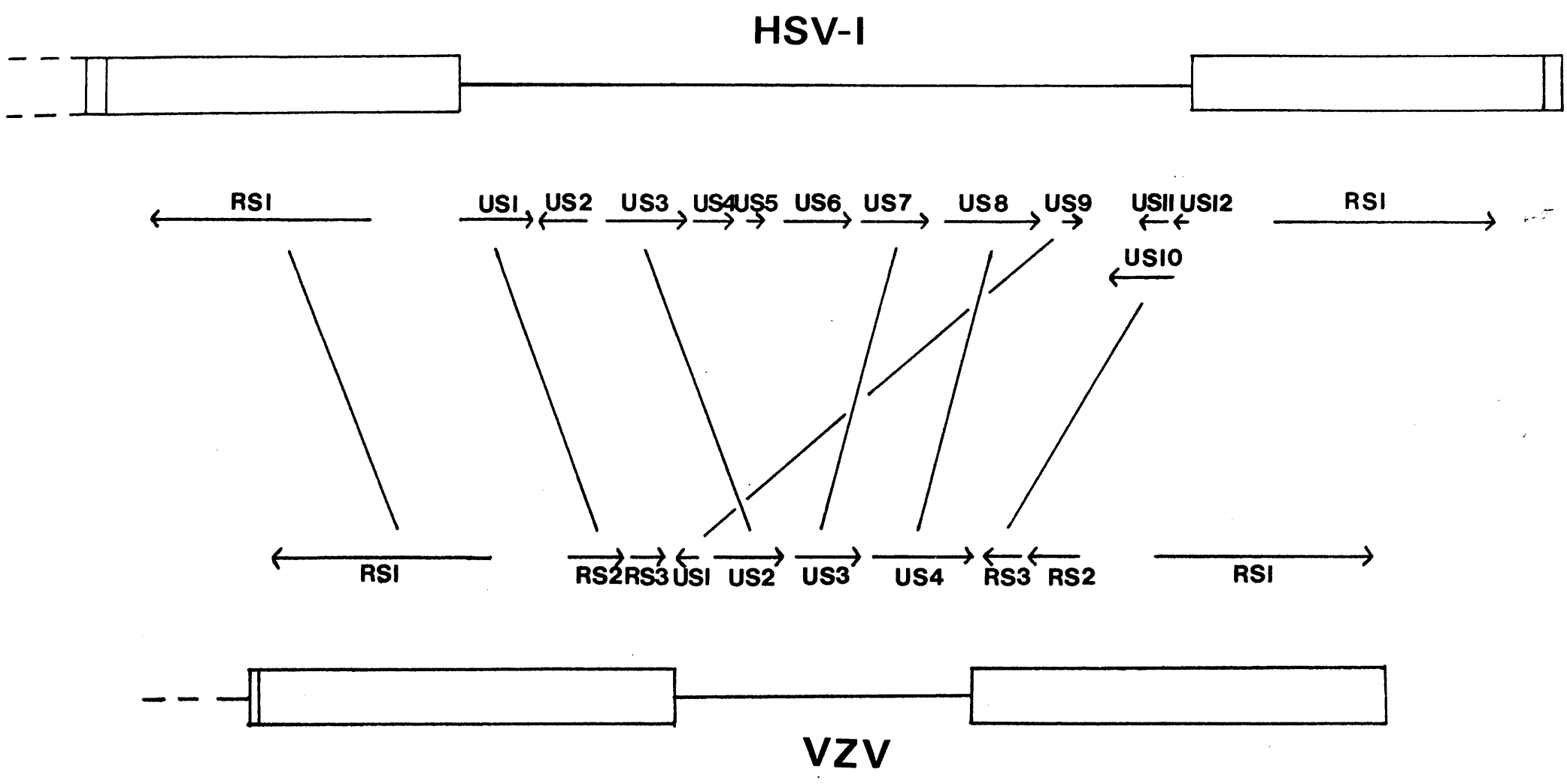

HSV-I
RSI
USI
US2
US3
US4
US5
US6
US7
US8
US9
USII
USI2
RSI
USI0
RSI
RS2
RS3
USI
US2
US3
US4
RS3
RS2
RSI
VZV

Figure 19. Descent of the S segments of HSV-1 and VZV from that of an ancestral herpesvirus.

The numbering refers to HSV-1 genes U_S1 to 12 and this has been retained in the remainder of the figure. No commitment to direction in time has been made although HSV-1 is arbitrarily at the start and VZV at the end.

A. Gene 1 is taken into the repeats and genes 11 and 12 are lost from the opposite end of U_S.

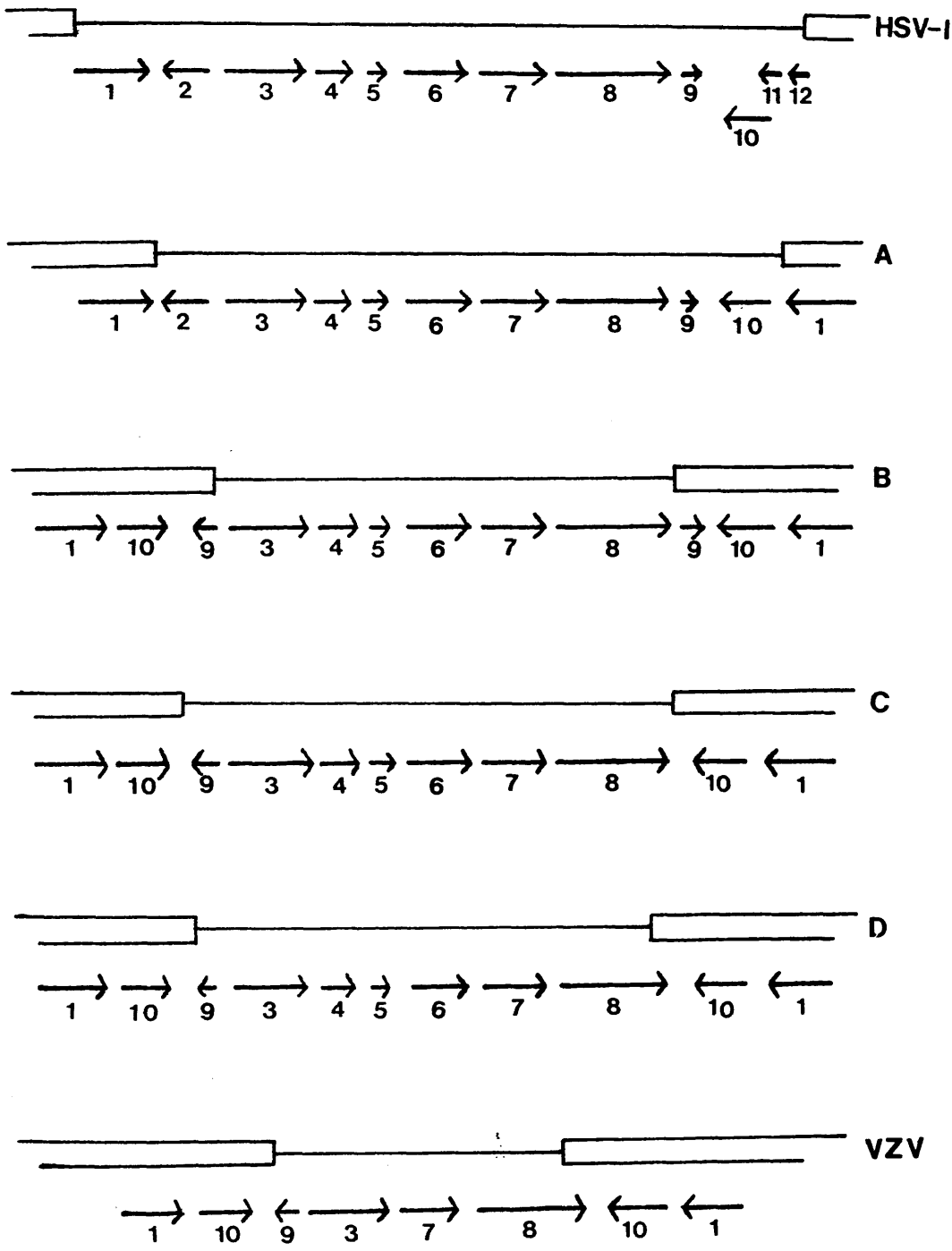
B. Genes 9 and 10 are taken into the repeats and gene 2 is lost from the opposite end of U_S.

C. Gene 9 is lost from the repeats, the remaining copy appearing at the opposite end of U_S from its position in HSV-1.

D. The downstream end of gene 8 is taken into the repeats and the termination codon of gene 9 is moved adjacent to the junction.

E. Genes 4, 5 and 6 are deleted. This step could also be accomplished by a more elaborate movement of the repeats and need not necessarily occur after steps A to D. This is now the gene arrangement in VZV.

Adapted from Davison and McGeoch (1986).


HSV-I
1
2
3
4
5
6
7
8
9
11
12
10
A
1
2
3
4
5
6
7
8
9
10
1
B
1
10
9
3
4
5
6
7
8
9
10
1
C
1
10
9
3
4
5
6
7
8
10
1
D
1
10
9
3
4
5
6
7
8
10
1
VZV
1
10
9
3
7
8
10
1

(b) Genes 9 and 10 are taken into the repeats and gene 2 is lost from the opposite end of U_S;

(c) Gene 9 is lost from the repeats, the remaining copy appearing at the opposite end of U_S from its position in HSV-1;

(d) The downstream end of gene 8 is taken into the repeats and the termination codon of gene 9 is moved adjacent to the junction;

(e) Genes 4, 5 and 6 are deleted. This step could also be accompanied by a more elaborate movement of the repeats and need not necessarily occur after steps (a) to (d).

It is clear that the inverted repeats are dynamic structures, capable of variation, mediated by illegitimate recombination, during evolution. The causes of this illegitimate recombination are largely unidentified and probably complex. However, it is clear that the coding and regulatory sequences near the repeat/unique junction play an important role in defining the extent of repeat movement. Movement beyond these limits would alter the coding potential or pattern of gene expression of the virus, and hence its biological properties.

The flexible nature of the repeats indicates that the presence of two copies of those genes in the repeat may not be of primary importance in determining virus properties, and suggests that it is unlikely that the repeat is an element in which certain distinct functions are compartmentalized.

I.13 GENETICS OF HERPES SIMPLEX VIRUS

One of the most useful approaches to elucidation of HSV gene function has been the isolation and analysis of mutants of HSV. These have been used to map many polypeptides and functions. The main class are conditional lethal, temperature-sensitive (ts) mutants. Conditional lethal mutants are able to grow under certain (permissive) conditions, but not under other (non-permissive) conditions. More recently, deletion mutants have been useful in determining essential and non-essential genes. A brief summary of the classes of HSV mutants isolated is given below.

I.13.a Drug-resistant mutants

The majority of HSV drug-resistant mutants have lesions in either the virus tk or DNA polymerase, although it is possible that mutations exist in other polypeptides, such as the MDB (Honess et al., 1984; Chion et al., 1985; Larder et al., 1987).

Anti-viral drugs, such as nucleoside analogues like 5-bromo-2'-deoxyuridine (BUdR) (Kit and Dubbs, 1963; Dubbs and Kit, 1964) and 5-bromo-2'-deoxycytidine (BCdR) (Brown and Jamieson, 1977; Stow et al., 1978) are phosphorylated by viral but not cellular tk and inhibit the DNA polymerase in their phosphorylated form. Mutants resistant to these compounds are tk-negative and thus use either the cellular tk or the de novo pathway of thymidine synthesis and avoid phosphorylating the nucleoside analogues, which are thus not recognized by the DNA polymerase. Acyclovir (ACV) (acycloguanosine) is also

phosphorylated by tk, and is thus recognized by the DNA polymerase. The majority of ACV-resistant mutants are tk-negative. However, some ACV-resistant viruses which are tk-positive have been isolated. Here, the lesion has been mapped to the DNA polymerase, which fails to recognize the phosphorylated form of ACV (Crumpacker et al., 1980; Field et al., 1980; Darby et al., 1981; Larder and Darby, 1985; Larder et al., 1987).

The anti-viral drug phosphonoacetic acid (PAA) inhibits the virus DNA polymerase (Leinbach et al., 1976) by binding to the pyrophosphate-binding site. PAA-resistant mutants, which map to the DNA polymerase, have been isolated (Hay and Subak-Sharpe, 1976; Purifoy and Powell, 1977).

I.13.b Plaque morphology mutants

Most HSV-1 strains, for example HSV-1 (mP) and HSV-1 strain 17 syn$^+$, cause individual virus-infected cells to round up. Variants have been isolated which, in contrast, cause extensive fusion of virus infected cells or produce a mixture of both rounded and multinucleated cells in tissue culture. This is known as syncytium (syn) formation or cell fusion (Hoggan and Roizman, 1959; Brown et al., 1973). Several syn mutants have been isolated and these have been mapped to seven or eight loci (section I.9.e.i) (reviewed in Marsden, 1987).

I.13.c Immune cytolysis-resistant mutants

Immune cytolysis-resistant mutants exhibit altered synthesis, processing or incorporation of glycoproteins into infected cell membranes (Glorioso et al., 1980; Pancake et

al., 1983). These mutants render virus infected cells resistant to complement-mediated immune cytolysis with antisera or monoclonal antibodies directed against HSV-specific glycoproteins. This has allowed the identification of genes controlling the synthesis and processing of HSV glycoproteins. Monoclonal antibody resistant (mar) mutants may also possess mutation(s) affecting the antigenic sites of glycoprotein exposed on the virion envelope, and hence are resistant to neutralization by monoclonal antibodies (Holland et al., 1983; Buckmaster et al., 1984; Marlin et al., 1985). These mutants provide information on the structure and function of glycoproteins and the position and importance of the antigenic sites within a particular glycoprotein.

I.13.d Temperature-sensitive mutants

The majority of conditional lethal mutants of HSV are temperature-sensitive (ts). Ts mutants have defects in essential genes, which allow them to replicate at the permissive temperature (PT), usually 31°C-34°C, but not at the non-permissive temperature (NPT), usually 38°C-39.5°C. Normally a ts lesion is caused by a point mutation, leading to an amino acid substitution, for example, the ts lesion in VmwIE175 in tsK (Davison et al., 1984). This makes the protein either unstable, or unable to assume a functional configuration, at the NPT.

There is an extremely low level of spontaneous ts mutation in wild-type virus stocks (Coates, 1982). However, most ts mutations have been induced either by treatment of replicating virus with BUdR (a thymidine analogue which

causes transitions) or by mutagenesis of virions with nitrous acid, hydroxylamine, nitrosoguanidine or uv light (Schaffer et al., 1970, 1973; Timbury, 1971; Esparza et al., 1974; Manservigi, 1974). These methods tend to induce multiple mutations in the one genome. This can be resolved by recombining the ts lesion of interest back into a wild-type virus, to create a mutant with only a single lesion (Preston V. et al., 1984). Using the above approach it was, however, not possible to induce ts lesions in many regions of the virus genome. Therefore, cloned HSV fragments were mutated in vitro and recombined into a wild-type virus genome (Chu et al., 1979; Sandri-Goldin et al., 1981). Although, in theory, ts lesions can be introduced into any gene, ts lesions will only be identified in those genes with an essential function in vitro. Complementation analysis has recognized over 35 complementation groups, implying that there are at least this number of essential genes in HSV (Schaffer et al., 1978; 1987).

If two ts mutants complement one another for growth at the NPT, then they are regarded as being in different complementation groups and, hence, in different genes (Brown et al., 1973). Low levels of complementation may imply intragenic complementation, as has been found for 2 tk-negative mutants (Jamieson and Subak-Sharpe, 1974)

As well as determining essential genes, ts mutants have also yielded information on many stages of the HSV lytic cycle. These include penetration (Sarmiento et al., 1979; Little et al., 1980; Addison et al., 1984), regulation of gene expression (Preston, 1979b; DeLuca et al., 1984) and DNA replication (Schaffer et al., 1973) and encapsidation

(Preston et al., 1983).

Marker rescue of ts mutants by gel purified from total HSV or cloned restriction endonuclease fragments of wild-type HSV DNA has been used to accurately map ts lesions (Wilkie et al., 1974, 1978; Stow et al., 1978). Marker rescue can be carried out using fragments as small as 320 bp (Preston, 1981), thus allowing the ts lesion to be assigned to a specific gene. Interestingly, no ts mutants have been described in U_S.

A different type of ts mutant where the PT is the high temperature (38.5°C) and the NPT the low temperature (31°C), has also been described. Only one cold-sensitive (cs) mutant of HSV, which maps to U_S, has been described to date (Tognon et al., 1981).

I.13.e Host-range mutants

Mutants of HSV exist which are able to grow in some cell lines but not others. These are known as host-range mutants. HSV-1 (MP) fails to replicate efficiently in dog kidney cells (Aurelian and Roizman, 1964). Blind passage in these cells allowed the isolation of variants able to grow in them. Ts host-range mutants of HSV-2 have been isolated, which are able to replicate in rabbit kidney cells at both 33°C and 39°C, whereas in hamster embryo fibroblasts they are ts, being able to replicate at 33°C but not 39°C (Koment and Rapp, 1975a,b). Interestingly, their ts, host-range phenotype corresponded with their in vivo properties, as they were virulent in mice and rabbits, but attenuated in hamsters. (The internal temperature of animals is at the NPT for ts mutants).

A virus containing a 500 bp deletion in IE4 also exhibits host-range dependence. It is capable of growth in

Vero cells and dividing HFL cells, but not in BHK21 C13 cells or non-dividing HFL cells. It has been proposed that in permissive cells a host factor substitutes for VmwIE68 (Post and Roizman, 1981; Ackermann et al., 1985; Sears et al., 1985).

A modification of the conventional definition of host-range dependence has come about by the introduction of cloned HSV genes into mammalian cells. These biochemically-transformed cell lines then carry stably-integrated HSV genes. Thus, a ts mutant in VmwIE175 has been shown to replicate at the NPT in BHK21 C13 cells transformed with IE3, but not in normal, untransformed BHK21 C13 cells (Davidson and Stow, 1985). This approach has been used to construct viruses containing deletions in putative essential genes : deletion variants have been constructed in VmwIE175 (DeLuca et al., 1985; Smith and Schaffer, 1987), gB (Cai et al., 1987) and VmwIE110 (Stow and Stow, 1986; Sacks and Schaffer, 1987), confirming the essential nature of the former two polypeptides, while in the latter case the viruses proved able to replicate in normal cells. The above approach should allow the construction of deletions in any HSV gene, allowing the essential nature of such genes to be assessed.

I.13.f Deletion mutants

Although ts mutants have been useful in determining essential HSV genes, they have a number of drawbacks. Firstly, as previously stated, they will only be isolated in essential genes; secondly, they may be leaky or revert, making their analysis difficult. The use of deletion mutants

in conjunction with complementing cell lines overcomes these problems. Removal of the entire or a large part of the open reading frame of a gene will unambiguously determine whether or not that gene is essential. Deletion variants which arose spontaneously or by deliberate construction have determined several non-essential genes, at least in vitro.
Non-essential genes include VmwIE110 (Stow and Stow, 1986; Sacks and Schaffer, 1987), gC (Little et al., 1981; Holland et al., 1984), two genes at the right of U_L (U_L55 and U_L56) (Perry, 1986) coding for predicted polypeptides of 20K and 22K, respectively (MacLean and Brown, 1987b, c, this thesis), tk (Sanders et al., 1982) and all the genes in U_S with the exception of gD (Longnecker and Roizman, 1986, 1987; Umene, 1986; Brown and Harland, 1987; Longnecker et al., 1987; Weber et al., 1987; Harland and Brown, 1988).

Genomes deleted in restriction enzyme sites for use in recombination have also been isolated (Brown et al., 1984; Harland and Brown, 1985, 1988; Brown and Harland, 1987; MacLean and Brown, 1987a; this thesis).

I.14 RECOMBINATION

I.14.a General recombination

Recombination has been most extensively studied in bacteria, bacteriophage, fungi and yeast. It is from these systems that models of recombination have been derived. This work has been extensively reviewed by Stahl (1979) and Szostak et al. (1983). Three main models of recombination have been proposed, and these are described below. Some work has also been carried out to analyse general recombination

in higher eukaryotes and this will be discussed later (section I.14.c).

Recombination between two DNA molecules leads to the exchange of genetic material or DNA. An odd number of cross-over events occurring between two genes will lead to their recombination; the greater the distance between the genes the greater the probability of recombination (Haldane, 1919). The maximum recombination frequency between any two genes will be 50%, since once the markers are far enough apart then there is a 50% chance of an odd number of crosses occurring leading to recombination, and a 50% chance of an even number of crosses occurring leading to no recombination between these genes. Recombination frequency is defined as the percentage of recombinants in the population of progeny.

Cross-overs are not usually distributed evenly along the chromosome; the existence of one cross-over interferes with others in its vicinity, decreasing recombination frequency in the affected area; this is known as positive interference. Negative interference has also been observed. Here recombination between two close markers will promote recombination between one of these two markers and a flanking marker 50% of the time. Thus, one exchange promotes another. This was included into a model for recombination proposed by Pritchard (1955), who suggested that chromosomes pair for recombination discontinuously, and that in any one pairing region recombination is frequent.

From tetrad analysis, (Holliday, 1964) it was assumed that recombination would lead to a Mendelian segregation of alleles 4:4. However, aberrant ratios were observed, with information being transferred non-reciprocally from one duplex to

another. This was known as gene conversion, which involves the duplication of an allele from one chromatid to another, ie. AB x ab→Ab + AB, as opposed to the expected Ab + aB. This leads to a 6:2 segregation pattern. Another class of aberrant segregation is observed involving post-meiotic segregation : this occurs when two strands of a duplex carry different genetic information, so that the spore divides to give two genetically distinct daughter cells following mitosis (Kitani et al., 1962). This gives rise to both 5:3 and aberrant 4:4 segregation patterns. All these segregation products are illustrated in Figure 20. These types of aberrant segregation are associated with recombination of flanking markers at a frequency of up to 50%.

I.14.a.i The Holliday model

Holliday (1964) has proposed a model of recombination which explains the results from tetrad analysis. In this model (Figure 21) : (a) two homologous duplexes align and recombination is initiated by the symmetrical cutting of one strand of the same polarity in each of the duplexes; (b) The single-stranded ends are reciprocally exchanged; (c) Branch migration occurs along the chromatids, generating a cross-strand structure known as a half-chiasma, chi-form or Holliday intermediate; (d) This is resolved by cleavage of two of the four connecting strands of the same polarity and ligation to the other strand. Cleavage will occur randomly and 50% of the time in each direction, indicated 1 and 2. If cut 1 is made, leading to cleavage of the initiating strands, then there is a reciprocal exchange of strands between duplexes with no recombination of outside markers.

Figure 20. Tetrad analysis.

The genetic consequences of the involvement of a single heterozygous site in symmetrical heteroduplex DNA as explained by the Holliday model. When a marked site does not fall within the region of heteroduplex DNA there is normal 4:4 segregation. When a marked site (solid and open circles) falls within the region of heteroduplex DNA, mismatches are created and aberrant segregation can occur. If the mismatch on one heteroduplex is corrected, 5:3 segregation occurs; if the mismatches on both duplexes are corrected in the same way 6:2 segregation occurs. If neither mismatch is corrected aberrant 4:4 (ab 4:4) segregation occurs.

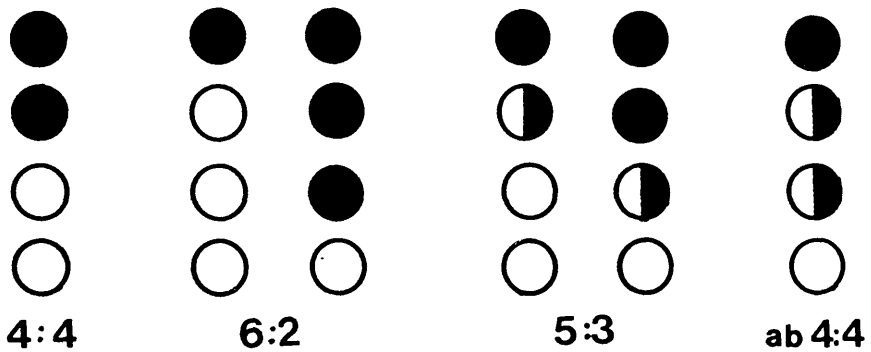

4:4
6:2
5:3
ab 4:4

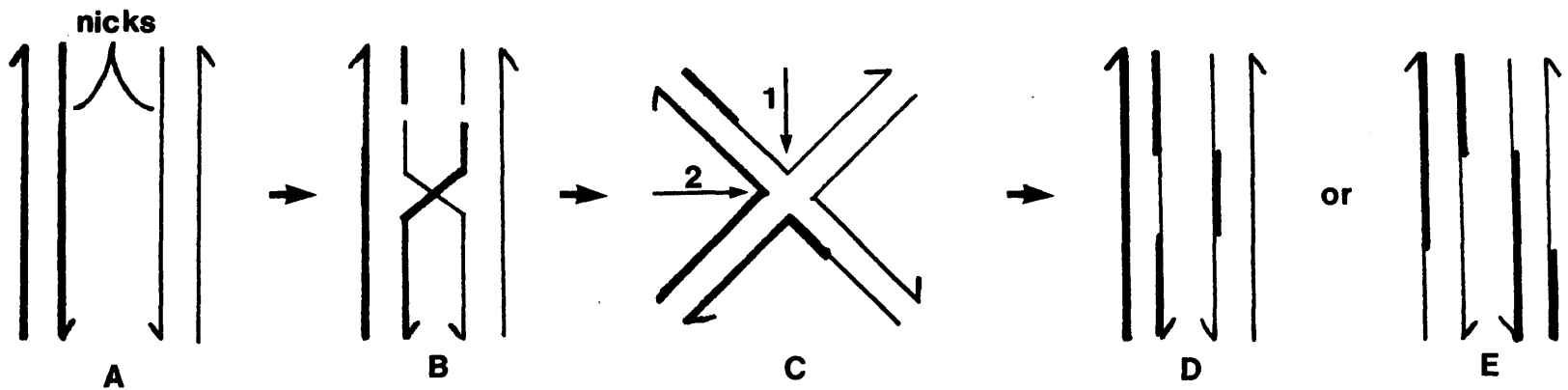
Figure 21. The Holliday model.

a) Strands of the same polarity are nicked at homologous sites. One duplex is represented by thick lines and the other by thin lines.

b) The nicked strands are exchanged on one side of the nick, forming symmetric heteroduplex DNA and a Holliday junction.

c) The Holliday junction can isomerize through a symmetrical intermediate, allowing resolution to occur by cutting of (1) the originally crossed strands to generate d) a crossover; or (2) the non-crossed strands to generate e) a non-crossover.

The Holliday Model



In contrast, if cut 2 is made, then in addition to the reciprocal exchange of strands within the exchange region, the flanking markers will also recombine. These two possibilities are referred to as a patch and a splice, respectively (Stahl, 1979).

The Holliday model accounts for the data produced by tetrad analysis. When a patch or splice occurs, the single-strand exchange results in the formation of heteroduplex DNA which contains two different genetic markers at the same locus. These markers are segregated into separate cells following mitosis; this is post-meiotic segregation. Such heteroduplexes may be converted to homoduplexes by the cell. Gene conversion (6:2 segregation) arises from the repair of mismatches to the same product on both chromatids in a region of symmetric heteroduplex DNA. Correction of only one heteroduplex leads to 5:3 segregation, correction of neither leads to aberrant 4:4 segregation. Obviously, correction of both heteroduplexes in opposite directions will lead to correct 4:4 segregation. The two possible resolutions of the Holliday junction explain the 50% frequency in the correlation between crossing-over of flanking markers and aberrant segregation. The Holliday model also predicts distance-dependent recombination frequencies, and outside of the splices and patches, reciprocal recombination.

I.14.a.ii The Meselson-Radding model

Evidence from various systems has shown that duplexes interact and can form heteroduplexes, as predicted by the Holliday model. Meselson and Weigle (1961) carried out

density labelling on replication-blocked lambda. Lambda DNA was labelled in vivo with ^{13}C and ^{18}N 'heavy' isotopes. Cells were then doubly-infected with 'heavy' and normal 'light' lambda. The DNA isolated was composed not only of 'heavy' and 'light' molecules, but also many intermediates, indicating that heteroduplexes had been formed.

However, it became apparent that not all heteroduplexes were symmetric, and that asymmetric heteroduplexes were also formed. In yeast, the frequency of double exchanges occurring on both chromatids at the same locus was much less than expected if heteroduplex formation was symmetric (Fogel and Mortimer, 1971, 1974).

Meselson and Radding (1975) have proposed a model which accounts for both symmetric and asymmetric heteroduplex DNA (Figure 22). Recombination is initiated (a) by a single-stranded nick on one of the two interacting duplexes. The 3' end of the nicked strand acts as a primer for DNA synthesis which displaces the strand ahead of it. The displaced single strand invades the other duplex at a homologous site displacing a D-loop (b) and forming a small region of asymmetric heteroduplex DNA. The single-stranded D-loop is degraded and the invading strand is ligated in place. The limited region of heteroduplex is expanded (c) by concerted DNA synthesis on the first (donor) duplex and by exonucleolytic degradation on the second (recipient) duplex. After the enzymatically driven production of asymmetric heteroduplex DNA stops, either branch migration or isomerization (d) can bring the 5' and 3' single-stranded ends into apposition so that they can be ligated. The resulting Holliday junction can move along the duplex by the

Figure 22. The Meselson-Radding model.

a) Recombination is initiated by a nick on one of the two interacting duplexes (thick lines). The 3' end of the nicked strand acts as a primer for DNA synthesis (dotted line) which displaces the strand ahead of it.

b) The displaced single strand invades the other duplex (thin line) at a homologous site, displacing a D-loop and forming a region of asymmetric heteroduplex DNA.

c) The single-stranded D-loop is degraded and the invading strand is ligated in place. The limited region of asymmetric DNA is expanded by concerted DNA synthesis on the first (donor) duplex and by exonucleolytic degradation on the second (recipient) duplex.

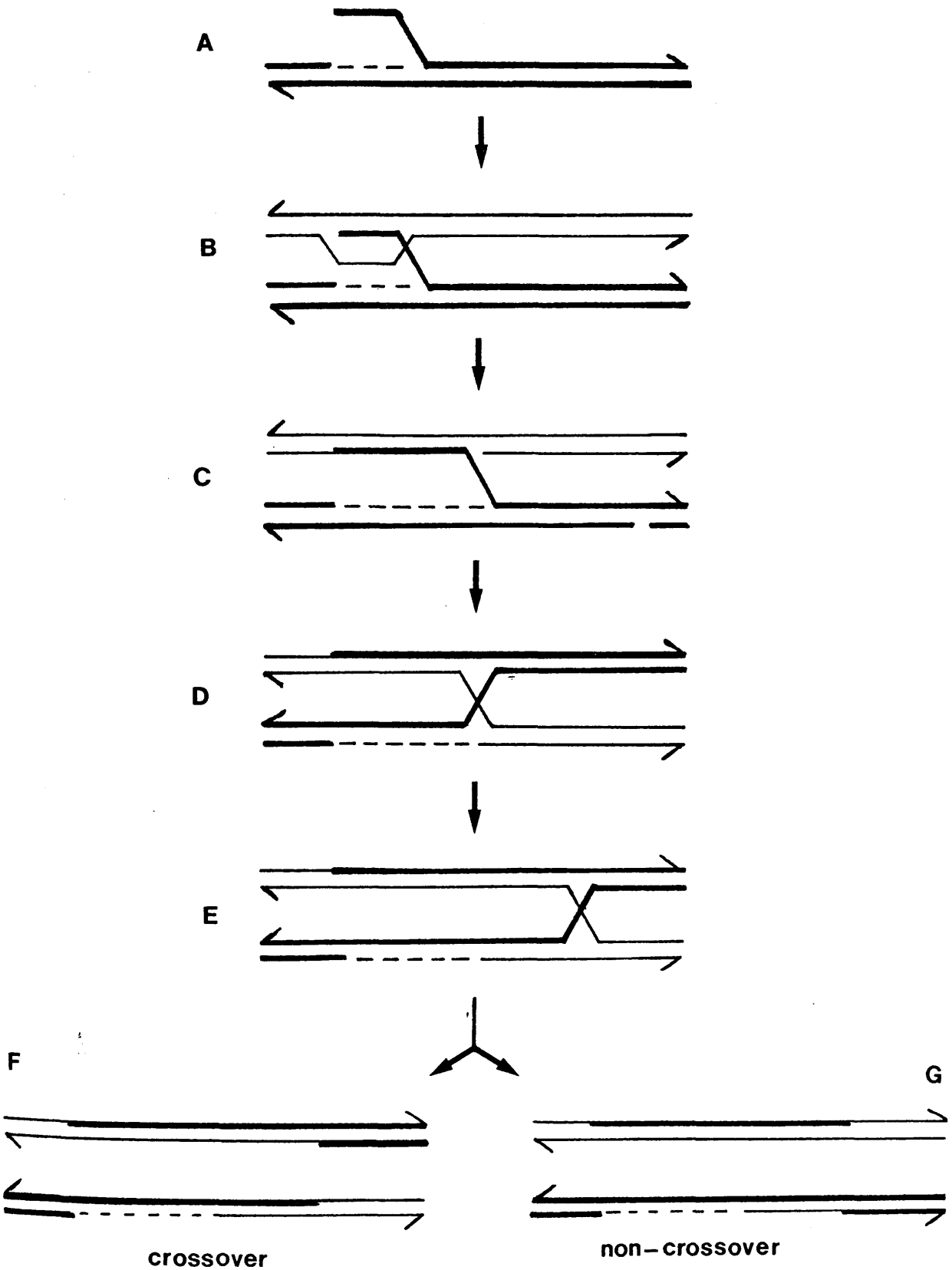
d) After the enzymatically driven production of asymmetric heteroduplex DNA stops, either branch migration or isomerization can bring the 5' and 3' single-stranded ends into apposition so that they can be ligated to form a Holliday junction.

e) This Holliday junction can move along the duplex by the process of branch migration generating symmetric heteroduplex DNA. Resolution as in Figure 21 can yield either :

f) The crossover configuration, or

g) The non-crossover configuration.

The Meselson–Radding Model



process of branch migration (rotary diffusion : Meselson, 1972) generating symmetric heteroduplex DNA. The Holliday junction is resolved as before (e) to yield either the cross-over configuration (f) or the non-cross-over configuration (g). This model, unlike the Holliday model, involves DNA synthesis and initiation, in this case on one strand only.

I.14.a.iii The double-strand break repair model

Several observations from yeast suggest that the Meselson-Radding model may not be completely correct. These include the location of cross-over events relative to conversion events and initiation sites; the apparent strand specificity of mismatch repair and the fact that the invading strand is the recipient of genetic information (Stahl, 1979).

Using a yeast-plasmid transformation system, an alternative model has been proposed (Szostak et al., 1983). Orr-Weaver et al. (1981) have shown that the introduction of a double-stranded break into the yeast sequences in the plasmid is highly recombinogenic. If two yeast sequences are present in the plasmid, which is thus equally likely to recombine in two places, then the introduction of a double-stranded break into one of these sequences always leads to integration of this sequence at its homologous position in the chromosome. Double-stranded gaps in the recombining sequence are also highly recombinogenic, the deletion always being repaired (this is not the case if a circular plasmid with a gap is used). Although a double-stranded nick in the area of recombination is highly

recombinogenic, cleavage outside the area of recombination has little effect.

Based on these observations, a double-strand break repair model of recombination has been proposed (Szostak et al., 1983; Figure 23). (a) A double-stranded cut is made in one duplex and a gap flanked by 3' single-stranded termini is produced by exonuclease activity; (b) One of the free 3' ends invades a homologous duplex, displacing a D-loop; (c) The D-loop is enlarged by repair synthesis from the 3' end until the other 3' end can anneal to complementary single-stranded sequences; (d) Repair synthesis from the second 3' end completes the process of gap repair, and branch migration results in the formation of two Holliday junctions. Resolution of the two junctions, by cutting either the inner or outer strands, leads to either the cross-over (e), or non-cross-over (f) configuration, with respect to flanking markers. Thus, in this model DNA synthesis is again involved, this time on two strands, and two regions of heteroduplex are formed.

The model accounts for gene conversion in two ways : if the locus falls within a double-stranded gap it will be converted by a double-stranded transfer of information without the direct involvement of heteroduplex DNA; if the locus falls within either of the flanking regions of asymmetric heteroduplex then gene conversion occurs, as in the Meselson-Radding model, by mismatch repair. Heteroduplex DNA in which a mismatch repair does not occur will lead to post-meiotic segregation. The model explains the high frequency correction of deletions in yeast (Fogel et al., 1978).

Figure 23. Double-strand break repair model.

a) A double-strand cut is made in one duplex (thick line), and a gap flanked by 3' single strands is formed by the action of exonucleases.

b) One 3' end invades a homologous duplex (thin line), displacing a D-loop.

c) The D-loop is enlarged by repair synthesis until the other 3' end can anneal to complementary single-stranded sequences.

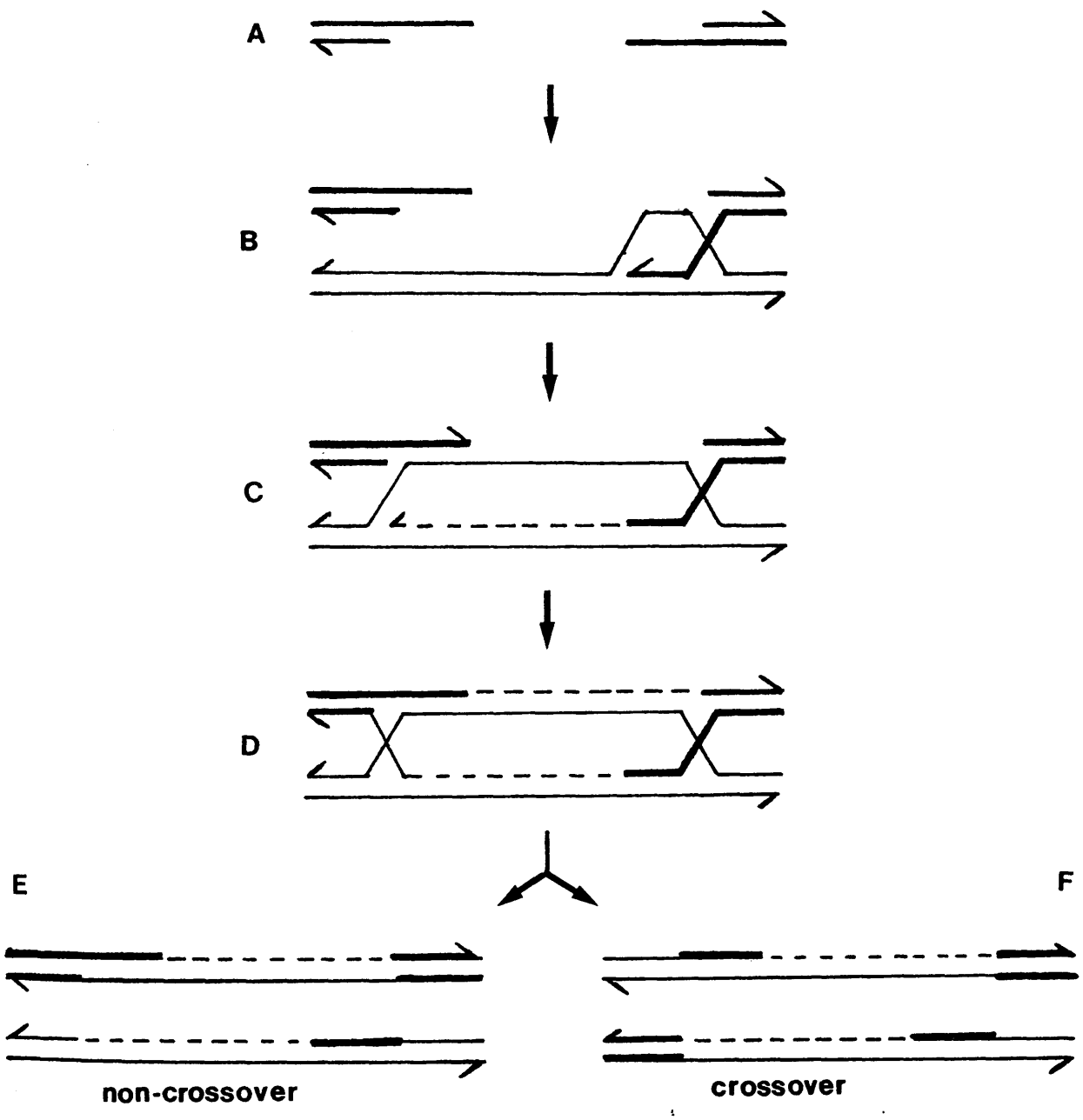
d) Repair synthesis from the second 3' end completes the process of gap repair, and branch migration results in the formation of two Holliday junctions. Resolution of two junctions by cutting either inner or outer strands leads to :

e) Two possible non-crossover configurations, or

f) Two possible crossover configurations.

In the illustrated resolutions, the right hand junction was resolved by cutting the inner, crossed strands.

Double-Strand Break Repair Model



All the above models make various predictions, which have been demonstrated in at least some recombination systems. Chi structures have been visualized under the electron microscope in *E. coli* plasmids (Potter and Dressler, 1976), yeast plasmids in meiotic cells (Bell and Byres, 1979, 1982) and adenovirus genomes from infected cells (Wolgemuth and Hu, 1980).

I.14.b Non-homologous recombination

Non-homologous recombination may be subdivided into two overlapping categories. Firstly, site-specific recombination, involving short stretches of homology. Examples of this are bacteriophage lambda integration into the *E. coli* host DNA (Landy and Ross, 1977), immunoglobulin rearrangements (Sakano *et al.*, 1981), transposition (Reed, 1981) and site-specific inversion via the 'a' sequence in HSV (Mocarski and Roizman, 1981; Smiley *et al.*, 1981; see section I.7.d)

The second type is known as illegitimate recombination, which involves little or no sequence homology (Roth *et al.*, 1985). This type of recombination appears to be rare in bacteria and yeast (Orr-Weaver *et al.*, 1981) but common in mammalian cells with targeted integration of exogenous DNA at its homologous chromosomal site being masked by 1,000 fold higher frequency of random integration (Thomas *et al.*, 1986). Illegitimate recombination is thought to involve a two-step process of breakage of DNA molecules followed by end joining (Wilson *et al.*, 1982), with short stretches of homology (1-7 bp) possibly being involved (Albertini *et al.*, 1982; Stringer, 1982; Ruley and Fried, 1983; Bullock *et al.*,

1984; Nalbantoglu et al., 1986; Roth and Wilson, 1986). The mechanism of illegitimate recombination and its relationship to general homologous recombination is unclear, but may involve similar mechanisms to those involved in site-specific recombination (Wilson et al., 1982).

I.14.c Recombination in higher eukaryotes

Although sister chromatid exchange has been shown to occur in mammalian cells by radioactive labelling and autoradiography (Wolff, 1977; Latt, 1981), it is extremely difficult to study recombination between chromosomes; therefore most work on recombination in higher eukaryotes has involved viruses or extrachromosomal plasmids in the hope that these will give an insight into the cellular processes of recombination.

Recombination has been shown to occur in HSV (section I.14.d), polyoma virus (Miller and Fried, 1976) and adenovirus (Frost and Williams, 1978). Recombination in adenovirus has been extensively studied and provides a good model system. Using Ad2 and Ad5, two closely homologous adenoviruses, Boursnell and Mautner (1981) and Mautner and Boursnell (1983) showed that recombination occurred only in homologous regions of the genome. This was supported by work of Young and Silverstein (1980).

There is evidence that adenovirus recombination starts during the eclipse phase and continues into the late phase of viral replication. Individual genomes appear capable of undergoing several recombinational events, ie. progeny as well as parental molecules appear to take part in recombination. Young and Silverstein (1980) have shown that

recombination frequency is 10 fold higher at late times than at early times. Comparison of the restriction enzyme digestion patterns of recombinants between ts mutants of Ad2 and Ad5 showed that the complexity, ie. the number of cross-overs, of the ts^+ recombinants was greatly increased at late times compared to early times (Sambrook et al., 1975; Young and Silverstein, 1980). This finding was also confirmed by triparental crosses of ts mutants. Munz et al. (1983) showed, by superinfection studies, that recombination continued late into the exponential phase of virus growth. If one ts mutant was used to infect cells and a second ts mutant superinfected at various times, ts^+ recombinants could still be detected if superinfection was delayed until the late exponential phase. Recombination and replication appear to be linked, although it is unclear how. Recombinants can be first detected just after the onset of DNA replication. If replication is blocked then no recombination occurs; reversal of the block in replication allows recombination to occur (Young et al., 1984). Using ts mutants in the adenovirus DNA polymerase and DNA-binding protein, it was shown that no recombination could be detected until 24 h post infection - at which time a limited amount of DNA replication could be detected (Stillman et al., 1982).

Two possibilities exist to explain the close link between recombination and replication in the adenovirus system. Firstly, recombination might involve a protein(s) involved in replication, such as the DNA polymerase or DNA-binding protein. Alternatively, the single strands of DNA produced during replication might serve as substrates

for recombination (Flint et al., 1976). This would be analogous to the situation in T4 bacteriophage, where DNA replication and recombination are tightly linked (Mosey, 1983). These single strands would serve as recombination substrates, as suggested by Meselson and Radding (1975).

Adenovirus can also undergo marker rescue (Frost and Williams, 1978; Volkert and Young, 1983). The relationship to the previous form of recombination is unclear, and little is known about the mechanism.

Overlap recombination, between terminal overlapping subgenomic fragments, has also been demonstrated (Chinnadurni et al., 1979). Recombination has been shown to occur at any point in the overlapping region, being no more frequent at the ends (Volkert and Young, 1983). If Ad2 and Ad5 are used, it has been shown that recombination occurs in areas of highest homology (Mautner and MacKay, 1984). As the adenovirus fragments are incapable of replication prior to recombination, this homologous recombination, unlike the situation with intact viruses, must be occurring independently of replication, and may involve host recombination functions.

Young et al. (1984) have shown that recombination requires at least one viral function, and that at higher temperatures replication proceeds normally, but no recombination is observed. Therefore, one component of the recombination process is thermolabile for this purpose.

The role of cellular functions in adenovirus recombination is unclear. Comparison of recombination frequencies between ts mutants in normal cells and cells deficient for DNA repair shows that there is no apparent

difference. Thus, in contrast to E. coli, where recA protein plays a role in DNA repair and recombination (West et al., 1981), adenovirus recombination does not appear to involve the host cell repair system (Young and Fisher, 1980). In contrast, in overlap recombination, recombination frequency is stimulated 20-100 fold by uv irradiation of the cells 16 h prior to transfection (Babiss et al., 1984), suggesting a role for the host cell DNA repair/recombination system.

Recombination between transfected plasmid molecules has also been shown to occur, again presumably using cellular enzymes (Folger et al., 1984; Kucherlapari et al., 1984; Song et al., 1985; Anderson and Eliason, 1986; Chakrabarti and Seidman, 1986; Weiss and Wilson, 1987). Double-stranded breaks within the recombining sequences promote recombination (Song et al., 1985; Chakrabarti and Seidman, 1986), thus resembling the double-strand break repair model (Szostak et al., 1983). Work on these systems may help understanding of the processes of homologous recombination in eukaryotic cells. Interestingly, an in vitro recombination system using nuclear extracts has been developed (Kucherlapari et al., 1985; Rauth et al., 1986), which may allow the individual proteins involved in eukaryotic recombination to be determined.

I.14.d Herpes simplex virus recombination

Recombination in HSV was first demonstrated by Wildy (1955) and confirmed by Subak-Sharpe (1969); the latter worker isolating ts$^+$ recombinants from crosses between pairs of ts mutants. Linkage maps of ts lesions have been constructed (Brown et al., 1973; Schaffer et al., 1974;

Brown and Ritchie, 1975; Timbury and Calder, 1976; Parris et al., 1980). While these were generally correct in the order of the lesions, the genetic distances between the markers did not accord with their physical map locations, possibly due to multiple cross-overs between distant markers (Stow et al., 1978; Wilkie et al., 1978). One exception to this was in HSV-2 strain HG52, where the genetic and physical maps were found to be in close correlation (Timbury and Calder, 1976; Wilkie et al., 1978).

Intertypic recombinants between HSV-1 and HSV-2 have been of use in mapping viral polypeptides (Timbury and Subak-Sharpe, 1973; Halliburton et al., 1977; Morse et al., 1977, 1978; Wilkie et al., 1977; Marsden et al., 1978; Preston et al., 1978), but of little use in understanding recombination per se, due to the limiting factor of homology between the two genomes.

Herpes simplex virus recombination is still relatively poorly understood. Little is known about the role of viral or host functions (Dasgupta and Summers, 1980), the possible effects of particular base sequences (ie. whether there are recombinational hotspots in the genome) on recombination, the role of the different isomers (Honess et al., 1980) or whether recombination involves both progeny as well as parental molecules as suggested by Ritchie et al. (1977).

Umene (1985), following the suggestion of Honess et al. (1980) and Brown et al. (1984), carried out recombination between two HSV-1 strains differing in eight restriction enzyme sites. Using these sites as unselected markers, he could find no area of the genome that had an excess of recombination, and calculated an overall recombination

frequency of 0.007 per kbp which concords with the values previously found for HSV (Roizman, 1979) and adenovirus (Young and Silverstein, 1980). Honess et al. (1980), investigating recombination between pairs of selected and unselected markers, showed two-factor recombination frequencies of 2-40%, and again found no satisfactory correlation with distance. Here again, different HSV-1 strains were used. One problem with this could be lack of homology in different areas of the genome, or incompatibility of gene products from different strains.

Analysis of HSV recombination is complicated by the structure of the virus genome and consequent presence of four genome isomers. While this does not affect the analysis of recombination between markers within either U_L or U_S alone, it complicates analysis of recombination between markers in different segments of the genome. Preliminary analysis by Honess et al. (1980) suggests a circular recombination map. This could indicate either involvement of all four isomers, or recombination beteen the circular molecules formed following HSV infection (Davison and Wilkie, 1983a; Poffenberger et al., 1983; Poffenberger and Roizman, 1985) or between the long concatamers of head-to-tail HSV molecules formed during replication (Jacob et al., 1979). The data of Honess (1984) suggests that more than one isomer and probably either circular or concatameric molecules are involved.

Whether progeny as well as parental genomes take part in recombination is controversial. On the strength of triparental crosses and time-course studies, Ritchie et al. (1977) suggested that, for HSV-1, progeny virus contribute

to recombination. In contrast, in PRV, Ben-Porat et al. (1982) concluded from density-labelling studies that only parental molecules take part.

Herpes simplex virus also contains a site-specific recombination system involving the 'a' sequence. This was discussed in section I.7.d.

I.15 AIMS OF PROJECT

To determine the role of parental and progeny molecules, the contribution of specific viral genes and the role of specific sequences in recombination, HSV-1 and HSV-2 genomes lacking restriction enzyme sites have been constructed (Brown et al., 1984; Harland and Brown, 1985, 1988; MacLean and Brown, 1987a).

The original aim of this project was to isolate a HSV-1 strain 17 variant lacking all four wild-type Xba I sites; these sites could then be used as unselected markers to study intratypic recombination in HSV-1. To introduce selectable markers, a ts lesion would be inserted into the genome lacking Xba I sites and recombination carried out between two ts mutants of HSV-1 strain 17 differing in the number of Xba I sites. Time-courses of recombination would be carried out, with the appearance of ts^+ recombinants being monitored. The distribution of Xba I sites on molecules known to have recombined between the two ts markers (ie. the ts^+ progeny) would then be analyzed. This might allow us to determine the effect of DNA replication on recombination frequency. The role of progeny as well as parental molecules could be determined by analyzing the

complexity, or number of cross-overs, of the *ts*$^+$ recombinants at different times. Calculating the recombination frequency between each marker within the area between the *ts* lesions might allow us to determine whether there is an over- or under-representation of recombination in certain areas of the genome, although the large distance between the Xba I sites would probably make any small differences in recombination frequency undetectable. Recombination frequency between the Xba I sites outwith the *ts* lesions would also be analyzed.

Previously it had been observed, from the analysis of single plaque isolates of HSV-2 strain HG52 and the HSV-1 strain 17/ HSV-2 strain HG52 recombinant R12-5, that the frequency of viable spontaneous deletion variants within a population of these strains was extremely high (Brown *et al.*, 1984; Harland and Brown, 1985). Thus, it was envisaged that similar variants of HSV-1 strain 17 might arise during the isolation of a HSV-1 strain 17 genome lacking Xba I sites; it was intended that any such potentially interesting variants would be further analyzed and characterized.

MATERIALS AND METHODS

MATERIALS

Cells

Unless otherwise stated, baby hamster kidney 21 clone 13 (BHK21 C13) cells (Macpherson and Stoker, 1962) were used for this work. Human foetal lung (HFL), Flow 2002 (Flow laboratories) and African green monkey kidney (Vero) cells were used where stated.

Virus

Herpes simplex virus type 1 Glasgow strain 17 (Brown *et al.*, 1973) was the wild-type virus used throughout this study. The Xba I restriction enzyme variants X2 (Brown *et al.*, 1984) and 1707 (KB11/45) (Cook and Brown, 1987) have been previously described. Temperature-sensitive mutants used in this study were *ts*1201 (Preston *et al.*, 1983) and *ts*1206 (Dr. V. G. Preston, personal communication).

Bacteria

The bacteria used were *E. coli* K12 strain HB101 (Boyer and Roulland-Dussoix, 1969) and CSH26, a dam$^-$ strain [F$^-$ *ara*B/(*lac pro*)*thi str*A]. These were grown in L-broth, with ampicillin (100 ug/ml) or tetracycline (15 ug/ml) being added where appropriate.

HSV-1 recombinant plasmids

The following recombinant plasmids carrying restriction enzyme fragments of HSV-1 cloned into pAT153 (Twigg and Sherratt, 1980) were used in this work. These were supplied

by Drs. A. J. Davison, C. M. Preston, V. G. Preston and F. J. Rixon.

Plasmid	map location
Kpn I m	0.285-0.322
Kpn I c	0.435-0.518
Kpn I t	0.322-0.343
Kpn I n	0.343-0.373
Kpn I p	0.373-0.401
Kpn I v	0.401-0.413
Kpn I a'	0.413-0.421
Kpn I x	0.421-0.433
Kpn I o	0.700-0.730
Kpn I k	0.951-1.000
Eco RI f	0.312-0.414
Eco RI f from ts1201	0.312-0.414
Bam HI b	0.741-0.811
Hpa I r	0.767-0.791
pMC9(1)	*
pMC9(2)	*

* pMC9 contains HSV-1 DNA from the Kpn I o/g site (0.730 m.u.) to the Hpa I v/r site (0.767 m.u.) cloned into the Eco RI site of pAT153 via an Eco RI linker (Davison and Rixon, 1985). Probe pMC9(1) was constructed by digesting the plasmid with Bam HI and Eco RI and using the fragment running from the Bam HI l/b site (0.741 m.u.) to the Hpa I v/r site (an Eco RI site in this plasmid). This procedure was carried out because the whole plasmid gave a high background level of hybridization. pMC9(2) consists of the whole of pMC9.

Tissue culture media

BHK21 C13 cells were routinely cultured in Glasgow modified Eagle's medium (Busby et al., 1964) supplied by Gibco Biocult, supplemented with 100 units/ml penicillin, 100 ug/ml streptomycin, 0.2 ug/ml amphotericin B and 0.002% (w/v) phenol red. To this was added 10% (v/v) tryptose phosphate broth and 10% calf serum (ETC_{10}). HFL and Vero cells were cultured in the same medium with the exception that the 10% calf serum was replaced by 10% foetal calf serum (ETF_{10}). Variants of the basic media used during this work were :

Emet/$5C_2$	Eagle's medium containing one-fifth the normal concentration of methionine and 2% calf serum.
Emet/$5F_{C2}$	Eagle's medium containing one-fifth the normal concentration of methionine and 2% foetal calf serum.
PIC	Phosphate-free Eagle's medium containing 1% calf serum.
EHu5	Eagle's medium containing 5% human serum.

Stock solutions

Phosphate buffered saline-A (PBS-A)

170mM NaCl, 3.4mM KCl, 1mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.2 (Dulbecco and Vogt, 1954).

Phosphate buffered saline (PBS)

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

PBS/calf serum

PBS containing 5% calf serum.

Tris-saline

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

Trypsin

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

Versene

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

Trypsin-Versene

One volume of trypsin plus four volumes of versene.

Giemsa stain

1.5% (w/v) suspension of Giemsa in glycerol heated at 56°C for 1.5-2 h and diluted with an equal volume of methanol.

L-broth

170mM NaCl, 10 g/l Difco bactotryptone, 5 g/l yeast extract.

L-broth agar

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

Chemicals

Most chemicals were supplied by BDH Chemicals UK or Sigma Chemical Co., and were of analytical grade. Ammonium persulphate and TEMED were supplied by Bio-Rad laboratories; caesium chloride, sodium hydroxide and boric acid by Koch-Light laboratories; nitrocellulose paper by Schleicher and Schuell; and unlabelled nucleotides by Pharmacia Ltd.

Radiochemicals

Radioisotopes were obtained from Amersham International plc. at the following specific activities :

5'[α-^{32}P]dNTPs	3000 Ci/mmol
[^{35}S]-methionine	800 Ci/mmol
[^{32}P]-orthophosphate	200 mCi/mmol
2-[^{3}H]-mannose	20 Ci/mmol
1-[^{14}C]-glucosamine	60 mCi/mmol

Enzymes

Restriction enzymes were obtained from Bethesda Research Laboratories (BRL), New England Biolabs or NBL Enzymes Ltd. DNA polymerase I, Klenow polymerase, T4 DNA ligase and T4 polynucleotide kinase were supplied by Boehringer Mannheim Corporation. Proteinase K, lysozyme and BSA were supplied by Sigma Chemical Co.

Restriction enzyme buffers

In general, the buffers used were either as recommended by BRL or Maniatis et al. (1982). These were prepared as 10x stock solutions (stored at -20°C). Commonly used examples

are :

10x restriction enzyme buffer for Xba I, Hpa I, Bgl II, Hind III, Eco RI.

60mM Tris.HCl (pH 7.4), 1M NaCl, 60mM $MgCl_2$, 0.1% (w/v) BSA.

10x restriction enzyme buffer for Bam HI.

200mM Tris.HCl (pH 8.0), 1M NaCl, 70mM $MgCl_2$, 20mM 2-mercaptoethanol, 0.1% (w/v) BSA.

10x restriction enzyme buffer for Sma I, Kpn I.

200mM Tris.HCl (pH 7.4), 500 mM KCl, 50mM $MgCl_2$, 0.1% (w/v) BSA.

Phenol

Phenol (BDH) used was saturated before use by mixing 2:1 with phenol saturation buffer (10mM Tris.HCl pH 7.5, 10mM EDTA, 100mM NaCl) and stored at either -20°C or 4°C for up to one month.

Chloroform

Chloroform was mixed 24:1 with isoamyl alcohol to reduce foaming during extraction and to facilitate the separation of the aqueous and organic phases.

Phenol:chloroform (1:1)

This is a 1:1 mixture of saturated phenol and chloroform:isoamyl alcohol (24:1).

Monoclonal antibodies and anti-peptide sera

Monoclonal antibody LP11 specifically precipitates gH of HSV-1 (Buckmaster *et al.*, 1984). Monoclonal antibody 2975

specifically precipitates gB of HSV-1 (Dr. A. C. Cross, personal communication). Monoclonal antibodies 1001, 4836 and 4916 specifically precipitate gC of HSV-1 (Dr. A. C. Cross, personal communication).

Anti-peptide serum 14713 was raised against the carboxy-terminus of VmwIE110 (Perry et al., 1986). Anti-peptide serum 14718 was raised against the amino-terminus of VmwIE63 by Dr. M. C. Frame (III.3b).

METHODS

II.1 Growth of cells

BHK21 C13 cells were grown in 80 oz roller bottles containing 100ml ETC_{10} at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Confluent cells (approximately $3x10^8$/roller bottle) were harvested by washing the monolayers twice with trypsin/versene, and the detached cells were resuspended in 20ml of ETC_{10}. Cells from one roller bottle were sufficient to seed a further 10. For experiments, cells were plated on 50mm or 35mm Petri dishes or Linbro trays at densities of $4x10^6$, $2x10^6$ or $2x10^5$ cells/plate, respectively. HFL and Vero cells were grown in a similar manner, except that ETF_{C10} was used and for routine passage the roller bottles were split 1 in 4.

II.2 Growth of virus stocks

Almost confluent BHK21 C13 cells in 80 oz roller bottles were infected at a multiplicity of infection (moi) of 0.003 plaque forming units (pfu) per cell, in 20ml ETC_{10} at 31°C. The infection was allowed to proceed until the cytopathic effect (cpe) was complete (usually 3-4 days) when the yield was harvested. The cells were shaken into the medium and pelleted by centrifugation at 2,000 rpm for 10 min in a Fisons Coolspin. The cell pellet was resuspended in 5ml of supernatant, sonicated in a bath sonicator (50W at 4°C) until homogeneous, and centrifuged as before. The supernatant was removed and stored on ice (supernatant 1). The pellet was resuspended in a further 5ml of the original supernatant, sonicated and centrifuged as above to give supernatant 2. The

cell pellet was discarded and supernatants 1 and 2 combined to give the cell-associated virus stock.

The supernatant from the original 2,000 rpm spin was centrifuged at 12,000 rpm for 2 h in a Sorvall GSA rotor, and the virus-containing pellet resuspended in 5-10ml of the supernatant and sonicated until homogeneous. This was termed the supernatant virus stock.

Virus stocks were checked for sterility, titrated at the appropriate temperature; 37°C for wild-type virus; 31°C and 38.5°C for ts virus; and stored in 1ml aliquots at -70°C.

II.3 Titration of virus stocks

Stocks to be titrated were serially diluted 10 fold in PBS/calf. Aliquots of 0.1ml were added to 70% confluent BHK21 C13 cell monolayers on 50mm Petri dishes, from which the medium had been removed. Following incubation at 37°C for 1 h, the plates were overlaid with 4ml EHu_5 and incubated for 2 days at 37°C or 38.5°C or for 3 days at 31°C. Monolayers were fixed and stained with Giemsa stain at room temperature (RT) for 30 min. After washing, plaques were counted using a dissection microscope.

II.4 Sterility checks on virus and cell stocks

Brain heart infusion agar (BHI) plates, and BHI plates containing 10% horse blood (BHI blood agar), were obtained from the cytology department. Cell or virus stocks were checked for fungal contamination by streaking on BHI plates, in duplicate. The plates were sealed with parafilm and incubated at RT for 7 days. Similarly, yeast or bacterial contamination was detected by streaking on BHI blood agar

plates and incubating at 37°C for 7 days. If no contamination became apparent within that time the stocks were considered sterile.

II.5 Preparation of virion DNA

This method is based on that described by Wilkie (1973) and Stow and Wilkie (1976). BHK21 C13 cells in 80 oz roller bottles were infected and harvested as described previously (section II.2). The cell supernatant was stored at 4°C. To extract cytoplasmic virus, the cell pellet was resuspended in RSB (10mM KCl, 1.5mM $MgCl_2$, 10mM Tris.HCl pH 7.5) containing 0.5% (v/v) Nonidet P40, incubated on ice for 10 min and centrifuged at 2,000 rpm in a Fisons Coolspin for 3 min. The supernatant from this spin was added to the previous cell supernatant. The pellet was resuspended in RSB/Nonidet P40, extracted as before, and the final supernatant added to the initial cell supernatant. The supernatant pool was centrifuged at 12,000 rpm in a Sorvall GSA rotor for 2 h. The virus pellet was resuspended in 8ml NTE (10mM Tris.HCl pH 7.5, 10mM NaCl, 1mM EDTA) by sonication and lysed by the addition of SDS and EDTA to a final concentration of 2% (v/v) and 0.8mM, respectively. The released virus DNA was carefully extracted twice with an equal volume of phenol and once with chloroform, and precipitated by the addition of 2 volumes of ethanol. The DNA was pelleted by centrifugation at 2,000 rpm in a Fisons Coolspin for 10 min, dried in a vacuum desiccator, redissolved in water containing 50ug/ml RNase A and quantitated by agarose gel electrophoresis. For quantitation, varying amounts of purified viral DNA were run, alongside a standard of known concentration, on an agarose

gel containing 0.5ug/ml ethidium bromide, and the intensities of the bands visualised under uv light compared.

II.6 Preparation of infected cell DNA

This method is based on that of Stow and McMonagle (1983). BHK21 C13 cell monolayers on 50mm Petri dishes were infected at a moi of 5 pfu/cell at 31°C. After 48 h the supernatant was removed and the cells lysed by incubating with 2ml of lysis buffer (0.6% SDS, 10mM EDTA, 10mM Tris.HCl pH 7.4) containing 500ug/ml pronase, for 4 h at 37°C. NaCl was added to a final concentration of 200mM and infected cell DNA was extracted twice with an equal volume of phenol and once with chloroform, precipitated by the addition of 2 volumes of ethanol, and dried in a vacuum desiccator. The DNA was dissolved in 400ul of water containing 50ug/ml RNase A. Restriction enzyme digests were usually carried out on 5% of the total sample (20ul).

II.7 Transfection of virus DNA by $CaPO_4$ precipitation/DMSO boost

To 50mm Petri dishes containing semi-confluent BHK21 C13 cells was added 400ul HEBS (130mM NaCl, 4.9mM KCl, 1.6mM Na_2HPO_4, 5.5mM D-glucose, 21mM HEPES) pH7.05 containing 10ug/ml calf thymus DNA, 0.2-2.0ug virus DNA (intact or Xba I cleaved) and 130mM $CaCl_2$. Following incubation at 37°C for 40 min, the plates were overlaid with 4ml ETC_{10} and further incubated for 4 h. After removing the medium, the plates were washed with ETC_{10}, 1ml of HEBS containing 25% (v/v) DMSO was added and the plates were incubated at RT for 4 min. The DMSO was removed and the plates washed twice with, and overlaid with,

4ml ETC_{10}. Incubation was continued at either 37°C (wild-type virus) or 31°C (ts virus) until the cpe was complete. The cells were scraped into the medium, pelleted by centrifugation at 2,000 rpm in a Fisons Coolspin for 10 min, then resuspended in 1ml medium, sonicated and the virus suspension stored at -70°C. The transfected plate stocks were titrated and single plaques isolated for further analysis (Graham and van der Eb, 1973; Stow and Wilkie, 1976).

For marker rescue of ts lesions, cloned HSV plasmids, linearized within the vector sequences, were added to the transfection mix at a genome ratio of 5-10:1. When the cpe was complete, the cells were harvested as described above and titrated at 31°C and 38.5°C to determine if the ts lesion had been rescued.

To introduce a restriction enzyme site variant on a cloned HSV fragment into the intact genome, a similar procedure was carried out with single plaques being isolated at 37°C and analyzed as described later (section II.9).

II.8 Selection enrichment of virus DNA

Virus DNA was digested with an excess of Xba I (approximately 5 units of enzyme per ug DNA) at 37°C, and samples were removed at various times, usually 1, 2, 4, and 16 h. Digested and undigested DNA was transfected onto BHK21 C13 cell monolayers (section II.7) - usually about lug DNA per plate. The plate receiving virus DNA digested for the longest time, but still showing cpe, was normally used for the isolation of single plaques. The reduction in virus titre on this plate compared to the undigested control was usually in the order of 100-1000 fold (see section III.1.c). By this

procedure, those genomes in the DNA population naturally lacking Xba I sites should have an increased chance of remaining intact following Xba I digestion, and thus their frequency in the population should be greatly increased. In those cases where it was believed that the frequency of genomes lacking Xba I sites would still be too low to be detected, the transfected plate stock was used to infect semi-confluent BHK21 C13 cell monolayers in 80 oz roller bottles, and virus DNA prepared as before (section II.5). This DNA was subjected to a further round of selection enrichment (Jones and Shenk, 1978; Brown et al., 1984; MacLean and Brown, 1987). This procedure is illustrated in section III.1.c, Figure 27.

II.9 Isolation of single plaque isolates

Virus stocks were titrated as previously described (section II.3), and the Petri dishes with the fewest plaques (usually less than 10) washed twice with PBS/calf. Using a 100ul eppendorf pipette, well separated plaques were picked, resuspended in 500ul PBS/calf in a small glass vial and sonicated for 30 sec.

To test a plaque isolate for temperature sensitivity, 100ul of sample was plated onto a linbro well in duplicate, one well being incubated at 31°C, the permissive temperature, and the other at 38.5°C, the non-permissive temperature. Plaques showing a titre significantly higher at 31°C than 38.5°C (at least 10 fold) were regarded as possibly temperature-sensitive.

To grow a plate stock, a 35mm Petri dish was infected with 100-250ul of the plaque at either 37°C (wild-type virus)

or 31°C (ts virus) until the cpe was complete. The supernatant from this plate was termed the plate stock of the appropriate plaque, and was stored at -70°C. The titre of these plate stocks was usually 10^7-10^8 pfu/ml.

In all cases where a plaque isolate either gave a different restriction enzyme profile from that of the wild-type, or appeared to be ts, it was plaque purified an additional 3 times prior to further analysis.

II.10 Preparation and isolation of ^{32}P-labelled virus DNA

This method is a modification of that described by Lonsdale (1979). Confluent monolayers of BHK21 C13 cells in linbro wells were washed with, and incubated overnight in PIC, then infected with either a titrated virus stock at 10 pfu/cell, or with 100ul of a plate stock, at an assumed moi of about 10 pfu/cell. After absorption for 1 h at 37°C, the virus was removed and the cells washed with, and maintained in, PIC for 2 h at 31°C. Fifty uCi of ^{32}P-orthophosphate (^{32}Pi) was added per well and incubation continued for a further 48 h at 31°C.

The cells were lysed by adding SDS to a final concentration of 2.5% (v/v) and incubating for 5 min at 37°C. The DNA was extracted once with an equal volume of phenol and precipitated by the addition of 2 volumes of ethanol. The DNA was dried at 37°C for 30-60 min (with the tubes in an inverted position) and redissolved in 200ul water. Usually 10% of each sample (20ul) was digested with the appropriate restriction enzyme and electrophoresed in an agarose gel (section II.21) for 16-24 h at 60-70V. Gels were air-dried and set up for autoradiography against Kodak X-Omat XS-1 film

(Eastman, Kodak) at RT.

II.11 Preparation and isolation of cytoplasmic ^{32}P-labelled virus immediate-early (IE) RNA

Confluent BHK21 C13 cell monolayers in 90mm Petri dishes were maintained in PIC overnight, and treated with 100ug/ml cycloheximide both 15 min prior to and continuously throughout infection. Virus was added at 50 pfu/cell, and allowed to absorb at 37°C for 1 h. The cells were washed twice in PIC and subsequently maintained in PIC containing 1mCi/ml ^{32}Pi (Clements et al., 1977). After a further 7 h at 37°C, the cells were washed twice with PBS, scraped into 5ml PBS and pelleted by centrifugation at 2,000 rpm in a Fisons Coolspin for 10 min. The cell pellet was resuspended in 1ml RSB (10mM KCl, 1.5mM $MgCl_2$, 10mM Tris.HCl pH 7.5) containing 0.5% Nonidet P40, and the nuclei removed by centrifugation at 2,000 rpm in a Fisons Coolspin for 10 min. The cytoplasmic supernatant was mixed with 1ml of a buffer containing 7M urea, 350mM NaCl, 10mM EDTA, 10mM Tris.HCl pH 7.8 and 1% SDS, and extracted three times with an equal volume of phenol:chloroform (1:1) and once with chloroform alone. The RNA was precipitated by the addition of 3 volumes of ethanol and 0.1 volume of 3M sodium acetate and incubation at -70°C overnight. The RNA was pelleted, dried in a vacuum desiccator and redissolved in 1ml water. For each hybridization experiment $2x10^7$ cpm of TCA precipitable RNA were used (Favaloro et al., 1980).

II.12 Virus growth properties

One-step growth experiments were carried out essentially

as described by Dargan and Subak-Sharpe (1985). Confluent BHK21 C13 cell monolayers in 35mm Petri dishes were infected at a multiplicity of 5 pfu/cell and virus absorbed for 1 h at 37°C. Following two washes with PBS/calf, the cells were overlaid with 2ml ETC_{10} and incubated at 37°C. Samples were harvested at 0, 2, 4, 6, 8, 12 and 24 h post infection. Virus was released by ultrasonic disruption and titrated (section II.3) and the titre expressed as pfu/10^6 cells (this is equivalent to pfu/ml, since $2x10^6$ cells were harvested into 2ml medium).

Longer term growth experiments involved infecting cells at a multiplicity of 0.001 pfu/cell, harvesting samples at 0, 2, 4, 12, 24, 36, 48 and 72 h post infection, and titrating as before. Twenty-four hour yield experiments were carried out by infecting cells at a multiplicity of 5 pfu/cell, harvesting after 24 h and titrating as before.

II.13 Assay for thymidine kinase (tk) negative virus

BHK21 C13 cell monolayers in 35mm Petri dishes were infected at a multiplicity of 5 pfu/cell in the presence or absence of 100ug/ml 5-bromo-2'-deoxycytidine (BCdR) in ETC_{10}. After 24 h incubation at 37°C, the virus was harvested and titrated on BHK21 C13 cells. BCdR is a cytidine analogue which is phosphorylated by the viral thymidine kinase enzyme (tk). The phosphorylated form inhibits the viral DNA polymerase, and thus reduces the yield of tk positive virus by 100-1000 fold. In contrast, tk negative virus, which will not phosphorylate BCdR, is unaffected by its presence (Stow et al., 1978).

II.14 Complementation yield test

Cells and medium were prewarmed to the non-permissive temperature (38.5°C) prior to use. Confluent BHK21 C13 cell monolayers in 35mm Petri dishes were infected at a multiplicity of 10 pfu/cell (either 5 pfu/cell of each *ts* mutant used in mixed infections, or 10 pfu/cell of a single *ts* mutant used in isolation). After 1 h at 38.5°C, unadsorbed virus was removed by washing the monolayer twice with ETC_{10}. The infected cells were further incubated in ETC_{10} for 24 h at 38.5°C, scraped into the growth medium, sonicated and the virus yield determined by titration at both 31°C and 38.5°C (section II.3). Complementation indices (C.I.) were calculated using the formula :

$$C.I. = \frac{(X+Y)^{31} - (X+Y)^{38.5}}{1/2[(X^{31}+Y^{31}) - (X^{38.5}+Y^{38.5})]}$$

where $(X+Y)^{31}$ and $(X+Y)^{38.5}$ represent the titres of progeny virus from a mixed infection of *ts*X and *ts*Y titrated at 31°C and 38.5°C, respectively. The denominator represents the sum of the yields obtained from the control single infections titrated at 31°C and 38.5°C; this value being halved to correct for the control cultures having received twice the multiplicity of a given mutant compared with the mixed infection. The $(X+Y)^{38.5}$ value corrects for any ts^+ recombinants in the progeny virus. The $(X^{38.5}+Y^{38.5})$ value corrects for any reversion or leak-through of mutant virus at 38.5°C. Normally, complementation indices of greater than 5 indicate that the *ts* lesions are in different complementation groups (Brown *et al.*, 1973).

II.15 Recombination experiments

Confluent BHK21 C13 cells on 35mm Petri dishes were infected at a multiplicity of 10 pfu/cell (either 5 pfu/cell of each ts mutant used in mixed infections or 10 pfu/cell of a single ts mutant used in isolation). After 1 h absorption at 37°C, the cell monolayers were washed twice with ETC_{10} and incubated in ETC_{10} at 31°C. At various time intervals (section III.2.c) the virus infected cells were harvested, sonicated and titrated at both 31°C and 38.5°C. The recombination frequency (RF) was calculated using the formula :

$$RF(\%) = 100 \times 2 \times \left[\frac{(X+Y)^{38.5}}{(X+Y)^{31}} - 1/2\left[\frac{X^{38.5}}{X^{31}} + \frac{Y^{38.5}}{Y^{31}} \right] \right]$$

where $(X+Y)^{38.5}$ and $(X+Y)^{31}$ represent the titres of progeny virus from a mixed infection of tsX and tsY titrated at 38.5°C and 31°C, respectively. $X^{38.5}$, X^{31}, $Y^{38.5}$ and Y^{31} represent the yield of the single infections titrated at 38.5°C or 31°C. The $1/2\left[\frac{X^{38.5}}{X^{31}} + \frac{Y^{38.5}}{Y^{31}} \right]$ factor corrects for reversion and leakiness of the parental ts mutants. The equation is doubled on the assumption that the total frequency of recombinants is twice that of the selected ts^+ progeny virus (Brown et al., 1973). Where the two ts parents had different numbers of Xba I sites, plaques were picked at 38.5°C and the distribution of Xba I sites in the recombinant ts^+ virus determined.

II.16 Glycerol stocks of bacteria

Bacterial stocks were prepared from 5ml shaking cultures grown overnight in L-broth in the presence of the appropriate

antibiotic at 37°C. The stocks were stored at both -20°C and -70°C, in 50% (v/v) glycerol.

II.17 Preparation of L-broth/agar plates

L-broth/agar was melted by placing a 350ml bottle in a boiling water bath for about 1 h and gradually allowed to cool until comfortable to hold. For L-broth/agar plates alone, approximately 20ml was poured onto 90mm bacterial Petri dishes, allowed to set at RT for about 30 min, and the plates dried for 20 min at 37°C in an inverted position with the lid loose. Plates were stored at 4°C for up to 1 month prior to use. For L-broth/agar plates containing antibiotic, the antibiotic was added to the appropriate concentration (100ug/ml ampicillin or 12.5ug/ml tetracyclin) just prior to pouring.

II.18 Transformation of bacterial cells with plasmid DNA

Twenty-five ul of a host bacterial glycerol stock was added to 5ml L-broth and grown overnight at 37°C in a rotary shaker. One ml of this was added to 19ml L-broth and grown with gentle shaking at 37°C until the optical density at 630nm (OD_{630}) was approximately 0.3 (90-100 min). The cells were pelleted by centrifugation at 8,000 rpm for 10 min in a Sorvall SS34 rotor, resuspended in 10ml of an ice-cold solution of 50mM $CaCl_2$ and incubated on ice for 20-30 min. The cells were repelleted and resuspended in 1ml of ice-cold 50mM $CaCl_2$. The cells were either used immediately or stored overnight on ice. This latter procedure usually gave higher transformation efficiency. Plasmid DNA (0.1-1ug) was incubated with the calcium chloride shocked cells on ice for

40 min; the cells were heat-shocked at 42°C for 5 min, transferred to 1ml L-broth and incubated at 37°C in a rotary shaker for 90-120 min. One hundred ul of varying dilutions of these cultures (usually 10^0, 10^{-1}, 10^{-2}) were spread on L-broth/agar plates containing the appropriate antibiotic. Following overnight incubation at 37°C, single bacterial colonies were picked using a cocktail stick and analysed (section II.19). (Maniatis et al., 1982)

II.19 Small-scale isolation of plasmid DNA by alkaline lysis

This method is essentially as described by Birnboim and Doly (1979). Colonies from an agar plate were inoculated into 5ml L-broth and shaken overnight at 37°C. 1.5ml of cells were added to an eppendorf tube and pelleted by centrifugation in a Beckman microfuge for 30 sec. The pellet was resuspended in 100ul of solution I (50mM glucose, 10mM EDTA, 25mM Tris.HCl, pH8.0) containing 4mg/ml lysozyme, and incubated at RT for 5 min. 200ul solution II (0.2M NaOH, 1% SDS(w/v)) was added and incubation continued on ice for 5 min. 150ul of ice-cold solution III (3M KAc, pH4.8) was added, and following incubation on ice for 5 min, the cell debris was pelleted by centrifugation in a Beckman microfuge for 5 min. DNA was extracted by mixing with an equal volume of phenol:chloroform (1:1), spinning for 2 min in a Beckman microfuge and removing the upper aqueous layer. This was added to 2 volumes of ethanol and the DNA precipitated at RT for 2 min, pelleted by centrifugation for 5 min in a Beckman microfuge, washed in 70% ethanol, dried in a vacuum desiccator and redissolved in 50ul water containing 50ug/ml RNase A. Usually 10ul of this was used per restriction digest.

II.20 Large scale isolation of plasmid DNA by alkaline lysis

This method is essentially that described by Birnboim and Doly (1979), and modified by Maniatis et al. (1982). Single colonies from an agar/L-broth plate or 25ul from a bacterial glycerol stock were inoculated into 5ml of L-broth containing the appropriate antibiotic and shaken at 37°C overnight. The culture was transferred into 500ml L-broth containing the appropriate antibiotic in a 2 litre flask and shaken at 37°C for 12-24 h.

The bacteria were pelleted by centrifugation at 8,000 rpm for 5 min in a Sorvall GSA rotor, the pellet resuspended in 7ml solution I (see above) containing 4mg/ml lysozyme and incubated at RT for 10 min. Freshly made solution II (14ml) was added and incubation continued for a further 10 min on ice. Ice-cold solution III (10.5ml) was added, incubation continued on ice for 10 min and the bacterial cell debris pelleted by centrifugation at 12,000 rpm for 30 min in a Sorvall SS34 rotor. DNA was extracted twice with an equal volume of phenol:chloroform (1:1) and once with an equal volume of chloroform. The DNA was precipitated at RT for 15 min by the addition of 2 volumes of ethanol, centrifuged at 12,000 rpm for 30 min in a Sorvall SS34 rotor at RT, washed in 70% ethanol, pelleted as before, and dried in a vacuum desiccator. The DNA pellet was dissolved in water containing 50ug/ml RNase A. The DNA was quantitated by running a small sample on an agarose gel containing 0.5ug/ml ethidium bromide and comparing its intensity against that of a known standard under uv light.

In some instances, to remove residual host cell DNA and

RNA, the DNA was further purified by isopycnic banding on caesium chloride gradients. In this case, the DNA was only extracted once with phenol:chloroform (1:1). Caesium chloride was added until the buoyant density was 1.56g/cm^3 and ethidium bromide was added to a final concentration of 0.5ug/ml. Centrifugation was carried out in a Sorvall TV865B rotor at 45,000 rpm for 16-24 h. The DNA was then visualized on a long-wave uv light box. Two bands were standardly seen - the upper containing host cell DNA and nicked-circular plasmid DNA, and the lower containing supercoiled plasmid DNA. The lower band was removed with a needle and the ethidium bromide removed by extracting 3 times with an equal volume of caesium chloride saturated isoamylalcohol. The DNA was precipitated by adding 2 volumes of ethanol and 0.1 volume of 3M sodium acetate and incubating at -20^{o}C for 30 min and treated as described above.

II.21 Agarose gel electrophoresis

Agarose gels (0.5-1.5% (w/v)) were prepared by boiling the appropriate quantity of agar in 250ml 1xTBE (89mM Tris, 89mM boric acid, 2mM EDTA) until dissolved. Once cool, the solution was poured onto a glass plate (152cm x 85cm) whose edges had been sealed with masking tape and onto which had been placed a teflon coated well-forming comb (15 or 12 tooth). Once set, the gel was placed in a horizontal gel tank containing 1xTBE. For non-radioactive samples, the TBE contained 0.5ug/ml ethidium bromide. For plasmid DNA, gels were electrophoresed at either 20-50V overnight or 80-120V for 3-6 h. For HSV DNA, gels were electrophoresed at 60-80V overnight. As a rule, to resolve DNA fragments of high mol.

wt., low % agarose gels were used, while for low mol. wt. fragments, higher % agarose gels were used (see section III).

II.22 In vitro ^{32}P-labelling of DNA by nick translation

This method was essentially that described by Rigby et al. (1977). DNA (0.5-1.0ug) to be used as a probe was labelled using 2 units of DNA polymerase I (containing 5'-3' polymerase activity and both 5'-3' and 3'-5' exonuclease activity) in a reaction containing 50mM Tris.HCl pH 7.8, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 10ug/ml BSA, 2uCi [α^{32}P]dCTP, 2uCi [α^{32}P]dGTP, 0.2mM cold dATP and 0.2mM cold dTTP, in a final volume of 30ul. After incubation at 15°C for 120 min the DNA was precipitated on dry ice for 15 min with 0.6 volumes of isopropanol and 0.1 volume of 3M sodium acetate. The DNA was pelleted by centrifugation in a Beckman microfuge for 5 min, resuspended in 30ul of water and reprecipitated with isopropanol and 3M sodium acetate. The resultant pellet was dried in a vacuum desiccator and redissolved in 100ul of 80% formamide.

II.23 Southern blotting and hybridization to ^{32}P-labelled DNA or RNA

Purified virion or infected cell DNA was digested with the appropriate restriction enzyme and run on an agarose gel for 16-24 h at 60-80V (see section III). The gel was visualized under short-wave uv light to confirm digestion of the samples and to partially fragment larger DNA molecules to aid their transfer. The gel was placed in 1 litre of Gel Soak I (200mM NaOH, 600mM NaCl) for 1 h and transferred into 1 litre of Gel Soak II (1M Tris.HCl pH 8.0, 0.59M NaCl) for 1

h. It was then placed on top of 2 sheets of Whatman 3mm filter paper, pre-soaked in 10xSSC (1xSSC is 15mM trisodium citrate, 150mM NaCl), and whose ends were suspended in 10xSSC. A sheet of nitrocellulose (BA85, Schleicher and Schuell), also pre-soaked in 10xSSC, and cut to the exact size of the gel, was placed on top of the gel, followed by 3 similarly sized sheets of pre-soaked Whatman 3mm filter paper, 3 sheets of dry Whatman 3mm filter paper, and finally a large stack of correctly sized Kleenex 'Hi-Dri' towels. A heavy weight was placed on top. This set-up acts as a wick to draw the SSC through the gel and nitrocellulose, transferring the DNA out of the gel and onto the underside of the nitrocellulose. After 12-24 h, the nitrocellulose was baked in a vacuum oven at 80°C for 2 h, to firmly attach the DNA.

The nitrocellulose was pre-hybridized in a sealed plastic bag containing hybridization buffer (6xSSC, 5xDenhardt's buffer (1xDenhardt's is 0.02% bovine serum albumin, 0.02% polyvinylchloride, 0.02% Ficoll), 0.1mg/ml salmon sperm DNA, 10mM Tris.HCl, pH7.5) at 75°C for 2-16 h. The hybridization buffer was replaced with fresh hybridization buffer containing nick-translated ^{32}P-labelled DNA or ^{32}P *in vivo* labelled RNA ($2x10^7$ cpm, in the latter case). Hybridization was allowed to proceed for 48 h at 75°C. The nitrocellulose was washed four times, for 1 h each, in a large volume (approximately 1 litre) of wash buffer (2xSSC, 0.1%SDS (w/v), 5mM Na_2HPO_4,pH7) at 60°C, allowed to air dry and either set up for autoradiography against Kodak XS-1 film at RT or for fluorography by using a Dupont image intensifying screen and exposing at -70°C. (Southern, 1975).

II.24 Dot blots

Plasmid DNA (1ug) was denatured by incubation in NaOH at a final concentration of 0.1M at RT for 25 min, neutralized by adding NH_4Ac to a final concentration of 1M and spotted onto nitrocellulose which had been pre-soaked in 1M NaAc. Once the spots had dried, the nitrocellulose was washed in 6xSSC and baked at 80°C in a vacuum oven. Pre-hybridization, hybridization to nick-translated DNA or ^{32}P *in vivo* labelled RNA, and washing, were carried out as previously described (II.23). The bound counts were quantitated in a scintillation counter. (Maniatis *et al.*, 1982).

II.25 Elution of restriction enzyme fragments from agarose gels

The DNA was digested with the appropriate restriction enzyme and electrophoresed on an agarose gel containing 0.5ug/ml ethidium bromide until the fragment to be isolated was well resolved. The gel was visualized under long-wave uv light and the relevant fragment removed using a sharp scalpel. The isolated gel slice was placed in dialysis tubing, pre-boiled for 15 min in TBE, along with a small volume of 1xTBE. Electrophoresis at 200V for 3-4 h transferred the fragment out of the gel slice. The current was reversed for 2-3 min to remove the DNA from the side of the dialysis tubing. The buffer containing the DNA fragment was removed, and the DNA extracted once each with phenol, phenol:chloroform (1:1) and chloroform, and then ethanol precipitated at -20°C by the addition of 2 volumes of ethanol and 0.1 volume of 3M sodium acetate. The purified DNA was rinsed with 70% ethanol, dried in a vacuum desiccator and

redissolved in water at approximately 1ug/5ul.

II.26 Restriction enzyme digestion

Restriction enzyme digests were carried out in the presence of 1x the appropriate restriction enzyme buffer (see Materials) containing 2-5 units of restriction enzyme per ug DNA. A typical reaction volume was 40ul. After digestion at 37°C for 2-16 h (usually 4 h), the reaction was stopped either by extraction with phenol:chloroform (1:1), or by adding one-sixth volume of Ficoll stop solution (5xTBE, 100mM EDTA, 10% (w/v) Ficoll, 0.1% (w/v) bromophenol blue) prior to agarose gel electrophoresis (Maniatis et al., 1982).

II.27 Filling in 5' overhangs with Klenow polymerase

Restriction enzyme digested DNA, containing 5' single strand extensions, was extracted once with phenol:chloroform (1:1), ethanol precipitated, washed in 70% ethanol, dried and redissolved in water at 1ug/10ul. 1ug of DNA was incubated in the presence of 1xNT buffer (50mM Tris.HCl pH 7.8, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 10ug/ml BSA) containing 0.2mM of the cold nucleotide triphosphates required to fill in the overhang, and 2 units of Klenow polymerase (the large fragment of E. coli polymerase, which contains the 5'-3' polymerase activity and 3'-5' exonuclease activity) at 15°C for 1 h. This is outlined diagramatically in III.1.a, Figure 26a. The reaction was stopped by extraction with phenol:chloroform (1:1) (Maniatis et al., 1982).

II.28 Ligation and linker insertion

One ug of blunt-ended DNA was extracted with

phenol:chloroform (1:1), ethanol precipitated, washed in 70% ethanol, dried and redissolved in 10ul water. This was incubated in a buffer of final concentration 66mM Tris.HCl pH 7.6, 6.6mM $MgCl_2$, 10mM DTT, 0.4mM ATP, containing 2 units of T4 ligase. For linker insertion, a 100 fold molar excess of non-phosphorylated linker was added; the linker being non-phosphorylated to minimize the possibility of greater than 1 linker being inserted. Ligated DNA was used to transform bacteria and the DNA from the resultant colonies subjected to restriction enzyme analysis. This is outlined diagramatically in III.1.a, Figure 26b (Maniatis et al., 1982).

II.30. Synthesis and purification of a synthetic oligonucleotide

A synthetic oligonucleotide was synthesized on a Biosearch 8600 DNA synthesizer. The DNA was eluted from the column by resuspending the beads in 1ml of ammonia and incubating at 55°C for 5 h. The ammonia was removed by lyophilization in the 'speedivac' vacuum desiccator, and the dried sample purified by denaturing polyacrylamide gel electrophoresis.

Seventy five ml of 16% sequencing gel mix (16% acrylamide, containing 1 part in 30 N-N'-methylene bisacrylamide, and 8.3M urea in 1xTBE), polymerized with 400ul of 10% ammonium persulphate and 40ul TEMED, was poured between two 20x22cm glass plates separated by 1.5mm spacers. Wells of approximately 1cm in width were formed using an 8-tooth teflon comb. The DNA samples were resuspended in 50ul of water by vortexing, then microfuged for 3 min. The supernatant was transferred to 50ul of sample buffer (28ul 10xTBE, 117ul water, 800ul deionized formamide), boiled for 10 min then quenched on ice and loaded immediately. Two ul of formamide-dye mix was loaded in a separate well to act as a mol. wt. marker. The gels were electrophoresed slowly, at 3.5-4mA overnight, in TBE.

To visualize the DNA the gel was removed, wrapped in cling film and viewed against a white chromatograph plate by

angled long-wave uv light. If the synthesis had been successful then a predominant, strong band with possibly a few minor lower mol. wt. bands was seen. The top band was cut out with a scalpel, mashed with a glass rod, and incubated at 42°C for 16 h in 1ml elution buffer (0.5M ammonium acetate, 1mM EDTA, 0.5% SDS). This was filtered through glass wool to remove the acrylamide, phenol:chloroform (1:1) extracted, ethanol precipitated, washed in 70% ethanol, dried and redissolved in water. To quantitate the DNA, the OD_{260} was read. The conversion factor for synthetic oligonucleotides is taken as 1 OD unit = 20ug/ml.

II.31 Adding 5' phosphate to non-phosphorylated DNA

DNA lacking a 5' phosphate group was incubated at 37°C for 1 h in a reaction mixture containing 1x kinase buffer (60mM Tris.HCl, pH7.8, 15mM 2-mercaptoethanol, 10mM $MgCl_2$), 250 mmoles ATP and 2 units polynucleotide kinase. It was extracted once with phenol:chloroform (1:1), ethanol precipitated, washed with 70% ethanol, dried and dissolved in water (Maniatis et al., 1982).

II.32 Site-directed mutagenesis using a synthetic oligonucleotide

Plasmid DNA was divided into two 5ug aliquots. One was cleaved with Kpn I, an enzyme with 2 sites surrounding the Xba I site to be mutated. The second was cleaved with Hind III, a single-cutting enzyme whose site is located outwith the Kpn I fragment containing the Xba I site. These two plasmid populations were extracted once with phenol:chloroform (1:1), ethanol precipitated, washed in 70%

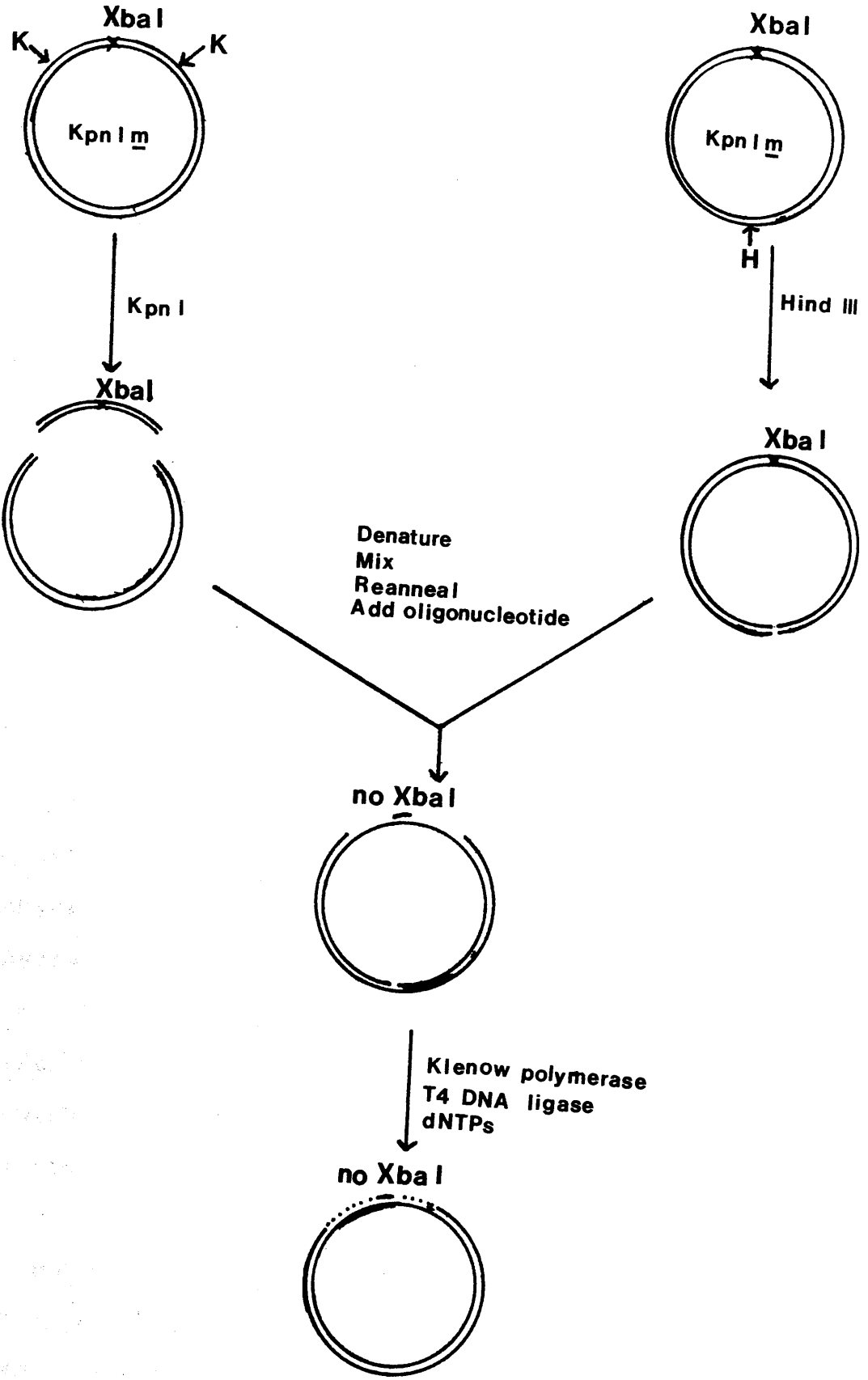
ethanol, dried and redissolved in 10ul water. They were then mixed and denatured by adding 5ul 1M NaOH (final concentration 0.2M) and incubating at RT for 20 min. The DNA was reannealed (to form `heteroduplexes') by sequential addition of 200ul water, 25ul 1M Tris.HCl pH 8.0 and 50ul 0.1M HCl, followed by incubation at 63°C for 3 h. A 100 fold molar excess of 5' phosphorylated oligonucleotide (with a 1 bp mismatch) was added to the gapped heteroduplex solution, and incubated for 2 h at RT to allow the oligonucleotide to anneal with the plasmid DNA. Incubation was carried out for 16 h at 15°C in a solution containing 1x ligase buffer (see II.29), 10 mmoles of all four deoxynucleotide triphosphates, 5 units Klenow polymerase and 2 units T4 DNA ligase. The DNA was extracted with phenol:chloroform (1:1), ethanol precipitated, washed with 70% ethanol, dried and redissolved in water. Half of the DNA was digested with Xba I to enrich for those molecules lacking Xba I sites prior to transformation of both digested and undigested DNA into E. coli (Oostra et al., 1983; Liang et al., 1986). This procedure is outlined diagramatically in Figure 24.

II.33 Preparation and analysis of HSV polypeptides

Confluent BHK21 C13 cell monolayers in 35mm Petri dishes were infected at a multiplicity of 20 pfu/cell. Virus was absorbed at 37°C for 1 h and the cells washed twice with and subsequently maintained at 37°C in Eagle's medium containing one-fifth the normal concentration of methionine and 2% calf serum (Emet/5C_2). At 4 h post infection [^{35}S]-methionine was added at 25uCi/ml. 24 h post infection, the infected cells were washed twice with PBS and harvested into 0.75ml sample

Figure 24. Site-directed mutagenesis.

Plasmid DNA (Kpn I m) was divided into two populations; one was digested with Kpn I (K) and the other with Hind III (H). The Xba I site is contained within the small Kpn I fragment which is not cleaved by Hind III. The two digested plasmid populations were denatured, mixed and allowed to reanneal. Several combinations of 'heteroduplexes' will be formed, including the one illustrated, which has a single-strand gap opposite the Xba I site. The synthetic oligonucleotide (complementary to the single strand) containing a 1bp mismatch is added and allowed to anneal. The single-strand gaps are filled in by Klenow DNA polymerase and dNTPs (dotted line) and the ends ligated with T4 DNA ligase. The resultant plasmids should no longer contain an Xba I site.


Xba I
K
K
Kpn I m
Kpn I
Xba I
Xba I
Kpn I m
H
Hind III
Xba I
Denature
Mix
Reanneal
Add oligonucleotide
no Xba I
Klenow polymerase
T4 DNA ligase
dNTPs
no Xba I

buffer (151mM Tris.HCl pH 6.7, 6.28% (w/v) SDS, 0.15% (v/v) 2-mercaptoethanol, 0.31% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and analysed by SDS-PAGE (Marsden et al., 1976, 1978). To label glycoproteins, infected cells were labelled with and maintained in EC_2 containing either 100uCi/ml [^{14}C]-glucosamine or 100uCi/ml [^{3}H]-mannose. For immunoprecipitation, cells were harvested as described later (II.35 and II.36).

II.34 Preparation and analysis of HSV immediate-early polypeptides

Confluent HFL or BHK21 Cl3 cell monolayers were treated with 100ug/ml cycloheximide in Emet/$5C_2$ 15 min prior to and continuously throughout infection. Cells were infected at 38.5^{o}C, at a multiplicity of 100 pfu/cell. Following absorption, the infected cells were washed twice with and subsequently maintained in Emet/$5C_2$. At 5 h post infection, actinomycin D was added at 2.5ug/ml. 15 min later, the monolayers were washed four times with PBS. Labelling was then carried out at 38.5^{o}C for 1 h in PBS containing 200uCi/ml [^{35}S]-methionine. Finally, the cells were washed twice with PBS, harvested into 0.75ml sample buffer (see II.33) and analysed by SDS-PAGE (Preston et al., 1978).

II.35 Immunoprecipitation of VmwIE110 and VmwIE63

Infected cells were lysed in extraction buffer (100mM Tris.HCl, pH 8.0, 10% (v/v) glycerol, 0.5% (v/v) Nonidet P40, 0.5% (w/v) sodium deoxycholate, 0.2mM PMSF) using 0.75ml per $2x10^{6}$ cells. Extracts were kept on ice for 1 h, sonicated and clarified by centrifugation for 5 min in a Beckman microfuge.

A standard number of counts were used in each experiment. Extracts (25ul) were incubated with 50ul of neat or diluted anti-peptide serum in the presence or absence of the relevant peptide (50ug) at 4°C for 3 h and for a further 1 h with 60ul of a 50% (v/v) suspension of protein A-Sepharose. Unbound proteins were removed by extensive washing with a buffer containing 60mM $LiCl_2$, 100mM Tris.HCl, pH 8.0, and 1% (v/v) 2-mercaptoethanol. Proteins were subsequently eluted from the pellet by boiling in sample buffer, and analysed by SDS-PAGE (Zweig et al., 1980).

II.36 Immunoprecipitation of virus glycoproteins

Infected cells were lysed in glycoprotein extraction buffer (50mM Tris.HCl, pH 7.2, 150mM NaCl, 1% (w/v) sodium deoxycholate, (w/v) 0.1% SDS, 1% (v/v) Triton X100, 0.2mM PMSF) using 0.75ml per $2x10^6$ cells. Extracts were kept on ice for 20 min and clarified by centrifugation for 5 min in a Beckman microfuge. A standard number of counts were used in each experiment. Extracts (500ul) were incubated with either 5ul of the relevant monoclonal antibody or control ascites fluid, and incubated at RT for 30 min, and for a further 2 h at 4°C with 60ul of a 50% (v/v) suspension of protein A-Sepharose. Unbound proteins were removed by extensive washing with the glycoprotein extraction buffer. Proteins were subsequently eluted from the pellet by boiling in sample buffer and analysed by SDS-PAGE (McLean et al., 1982).

II.37 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Slab gels were cast vertically in a sandwich consisting

of two glass plates separated by 1.5mm thick perspex spacers and sealed with teflon tape. Two types of resolving gel were used : (i) single concentration gels containing the appropriate concentration of acrylamide cross-linked with 2.5% (w/w) N,N'-methylene bisacrylamide in resolving gel buffer (375mM Tris.HCl, pH8.9, (w/v) 0.1% SDS), and (ii) gradient gels containing a 5%-12.5% linear gradient of acrylamide cross-linked with 5% (w/w) N,N'-methylene bisacrylamide in resolving gel buffer. The 12.5% acrylamide mixture also contained 15% glycerol to help stabilize the gradient. Polymerization was achieved by the addition of ammonium persulphate (APS) (0.006% (w/v)) and N,N,N',N',tetramethylenediamine (TEMED) (0.004% (v/v)) to the gel solutions prior to pouring the gels. Immediately after casting, the gel was overlaid with butan-2-ol in order to ensure a smooth interface on polymerization. Prior to addition of the stacking gel, the butan-2-ol was washed off with deionised water. The stacking gel contained 5% acrylamide (cross-linked with the same ratio of N,N'-methylene bisacrylamide used in the resolving gel) in stacking gel buffer (0.11mM Tris.HCl, pH 6.7, (w/v) 0.1% SDS), and was polymerized with APS and TEMED as above. After the stacking gel was poured, a teflon coated comb was inserted to form the wells.

Samples were prepared for analysis by boiling for 5 min in sample buffer, and gels electrophoresed in tank buffer (52mM Tris, 53mM glycine, 0.1% (w/v) SDS) at either 60mA for 3-4 h or 10-15mA for 18 h (Marsden et al., 1976, 1978).

Following electrophoresis, the gels were fixed or stained for 1 h in methanol:acetic acid:water 50:7:50, in the

absence or presence of 0.2% (w/v) Coomassie Brilliant Blue R250, respectively, and then destained for 3x30 min in methanol:acetic acid:water 5:7:88. The gels were either immediately dried under vacuum and exposed for autoradiography at RT, or were enhanced in 3 volumes of En^{3}Hance (New England Nuclear) for 1 h and washed in water for 30 min before being dried under vacuum and exposed for fluorography at -70°C.

RESULTS

RESULTS

III.1 ISOLATION OF HSV-1 GENOMES LACKING RESTRICTION ENZYME SITES

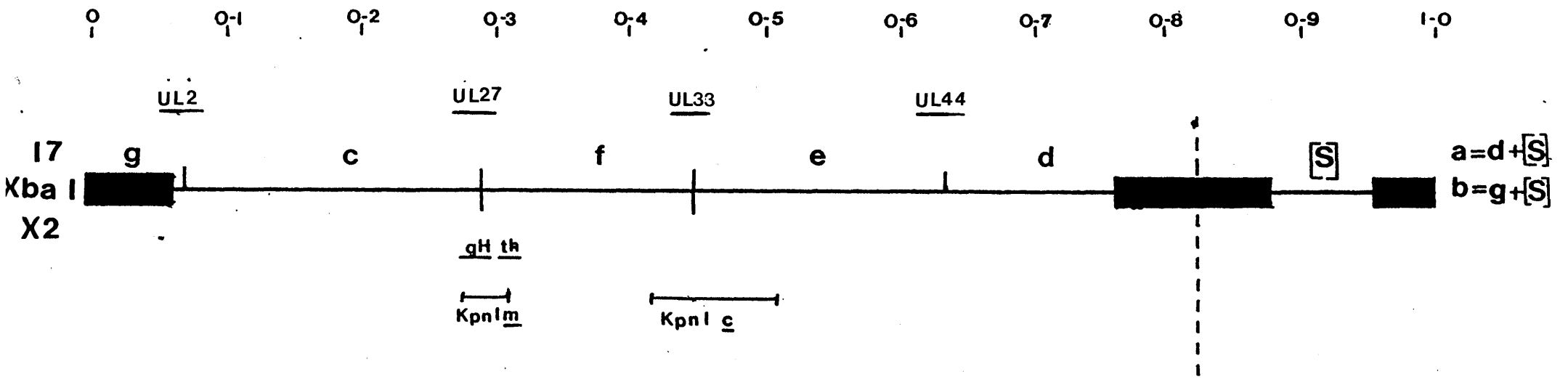
Wild-type HSV-1 strain 17 contains 4 Xba I restriction enzyme sites at 0.07, 0.29, 0.45 and 0.63 map units (m.u.) (Figure 25; Wilkie, 1976). Using a modification of the selection enrichment technique described by Jones and Shenk (1978), Brown et al. (1984) isolated a HSV-1 strain 17 genome lacking the 0.07 and 0.63 m.u. Xba I sites (Figure 25). In this virus, X2, the 0.07 m.u. site has been removed with no detectable deletion or insertion (the level of resolution being in the order of 150 bp) while the 0.63 m.u. site has been removed with a deletion of approximately 150 bp. X2 grows normally in one-step growth experiments (Brown et al., 1984) and high titre stocks have been obtained (10^9 pfu/ml) (see also section III.1.e). The polypeptide profile of X2 is discussed later (section III.1.d).

In order to remove the two remaining Xba I sites (those at 0.29 and 0.45 m.u.), two approaches were employed. First, plasmids containing HSV genomic segments spanning the 0.29 and 0.45 m.u. Xba I sites, Kpn I m and c, respectively (see Figure 25), were mutated by several means to remove the Xba I sites, and attempts were made to marker rescue them back into the intact virus (III.1.a, III.1.b). Second, selection enrichment was carried out, as described by Brown et al. (1984) (III.1.c).

Figure 25. Xba I map of HSV-1 DNA.

Xba I map for the DNA of HSV-1 strain 17 is shown above the line (Wilkie, 1976) and that for X2 below the line (Brown et al., 1984). Also illustrated below the line are the map positions of gH and tk and the regions cloned in Kpn I m and c. The 0.29 m.u. Xba I site is contained within gH and the distance between the Xba I site and the tk locus is greater than that between the tk locus and the end of Kpn I m.

The 0.07 m.u. Xba I site is contained within UL2 coding for the uracil DNA glycosylase; the 0.29 m.u. Xba I site is contained within UL27 coding for gH; the 0.45 m.u. Xba I site is contained within UL33 coding for a putative 14,000 molecular weight polypeptide; the 0.63 m.u. Xba I site is contained within UL44 coding for gC (McGeoch et al., 1988).


0
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-8
0-9
1-0
UL2
UL27
UL33
UL44
I7
Kba I
X2
g
c
f
e
d
[S]
a=d+[S]
b=g+[S]
gH th
Kpn I m
Kpn I c

III.1.a Loss of Xba I sites in cloned HSV fragments

Cloned HSV fragments spanning the 0.29 and 0.45 m.u. Xba I sites were obtained. The fragments chosen were Kpn I m (0.285-0.322 m.u.) and Kpn I c (0.435-0.518 m.u.), spanning the 0.29 and 0.45 m.u. sites, respectively (see Figure 25). Both of these had previously been cloned into the Pst I site of pAT153 by Dr Andrew Davison. Prior to use, both plasmids were digested with various restriction enzymes and shown to give the correct profile, with one exception : Kpn I m did not digest with Xba I (data not shown). One possible explanation for this was that the Xba I site was methylated and therefore resistant to cleavage with Xba I. The DNA had been isolated from E. coli strain HB101, which contains a dam methylase; this methylates A residues in the sequence 5'-GATC. As the Xba I cleavage site is 5'-TCTAGA, the sequence 5'-GATC will occur on average at 1/8 Xba I sites (1/16 on each strand). Therefore, a dam$^-$ strain of E. coli (CSH 26) was transformed with the plasmid Kpn I m and the DNA amplified. Using three enzymes which cut the sequence 5'-GATC differently according to the state of methylation (Sau 3A, which cuts both methylated and non-methylated DNA; Dpn I, which cuts only methylated (5'-G^{m}ATC) DNA; and Mbo I, which cuts only non-methylated DNA), we confirmed that Kpn I m DNA extracted from HB101 bacteria was methylated, whereas Kpn I m DNA extracted from CSH 26 cells was not (data not shown). The latter DNA was also cleaved by Xba I (data not shown). Subsequent sequence analysis (McGeoch and Davison, 1986b) has confirmed that this Xba I site is bordered by the sequence 5'-GATC, and hence is susceptible to methylation in dam$^+$ bacterial strains.

Although Kpn I c DNA from the dam$^+$ HB101 cleaved normally, indicating it was not methylated, this DNA was also transformed into the dam$^-$ CSH 26 cells as a precaution, in case manipulations created the sequence 5'-GATC which would falsely appear to lack the Xba I site. Thus, all further work with Kpn I m and c involved the use of CSH 26 rather than the standard HB101.

The first attempts to delete the Xba I sites from both plasmids involved selection enrichment. The DNA was cleaved with Xba I and then either transformed directly into E. coli or first religated, and then transformed into E. coli. It was hoped that this procedure would both enrich for natural mutants lacking the Xba I sites in the population (as linear DNA transforms much less efficiently than circular DNA) and mutate the Xba I restriction enzyme site in a small percentage of cases. Single colonies were isolated, small scale cultures grown up, and the DNA extracted and analyzed (by Xba I digestion) for loss of the Xba I site. Despite analyzing over 500 colonies for each plasmid, no plasmids lacking the Xba I site were obtained. Thus, the natural frequency of plasmid molecules lacking these Xba I sites in the population must be extremely low.

At this point it was decided to manipulate the two plasmids to delete the Xba I sites. The first approach chosen was to cleave the DNA with Xba I, fill in the 3' recessed ends with the Klenow fragment of DNA polymerase I, and ligate the two blunt ends (Figure 26a), prior to selection enrichment with Xba I. This procedure should result in a 4 bp insert which destroys the Xba I site, but which would lead to a frameshift in any polypeptide encoded

Figure 26. Manipulations of Xba I sites.

a) The use of Klenow DNA polymerase to fill in the 5' overhangs of an Xba I digested site. Religation leads to the destruction of the Xba I site with a 4bp insertion.

b) The addition of a Kpn I linker to a digested and filled in Xba I site. This leads to the destruction of the Xba I site with a 12bp insertion.

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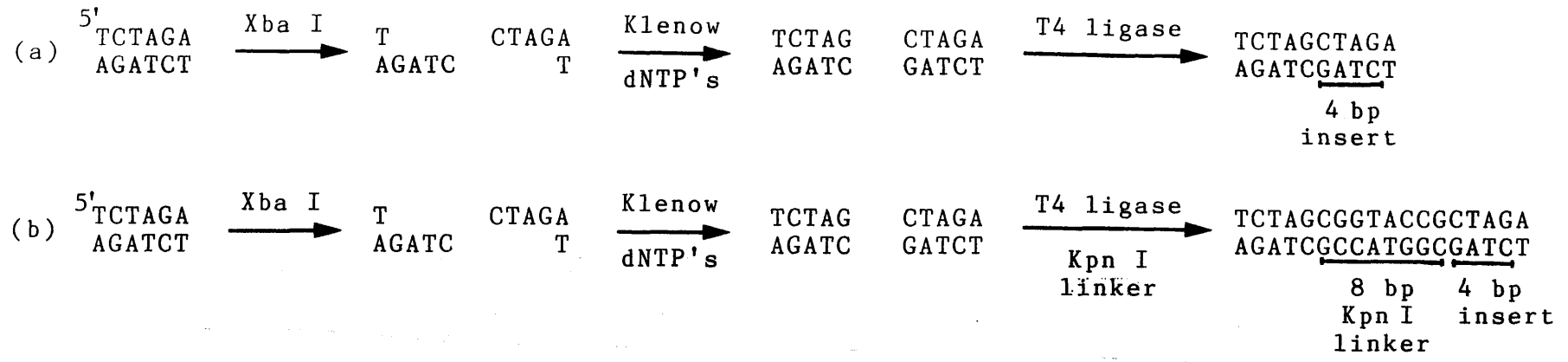
d) Amino acid and DNA sequence of gH around the 0.29 m.u. Xba I site. Underneath is written the sequence of the 18-mer synthetic oligonucleotide used to mutate the Xba I site. The left hand C and right hand GC are not part of the oligonucleotide but are included to show the amino acids around the Xba I site. The A (underlined) in the Xba I site has been mutated to a T (underlined) to destroy the Xba I site. This is in the 3rd base position of the codon and does not alter the amino acid, thus having no effect on gH.



(d) amino acid sequence : R F D L D E S

wild-type DNA sequence : CGC TTT GAT CTA GAC GAG AGC

mutated DNA sequence : CGC TTT GAT CTT GAC GAG AGC

by the DNA.

From this procedure a number of colonies were obtained for both Kpn I m and c. Of 5 colonies analyzed for Kpn I c, 4 had lost the Xba I site, with no apparent alteration in the size of the DNA around the site (data not shown). These were called pAC1, pAC2, pAC3 and pAC4. Attempts to marker rescue all 4 plasmids back into X2 were unsuccessful (see section III.1.b), suggesting that a frameshift mutation might have occurred within an essential polypeptide (the 0.45 m.u. Xba I site is contained within a 14,000 mol. wt. open reading frame - Dr. D. J. McGeoch, personal communication).

In the case of Kpn I m 20 colonies were analyzed, of which 16 had lost the Xba I site. Surprisingly, all of these had large deletions around the Xba I site, and only two retained sequences from both ends of the HSV fragment. These were pAM1 and pAM2, which had 0.6 and 0.7 kbp deletions, respectively (data not shown). No attempt was made to marker rescue these back into X2, as they would be liable to result in an inviable virus. A possible explanation for these deletions is that religation of the plasmid was unsuccessful and that recombination/deletion occurred during circularization within the bacteria. Alternatively, there was a contaminating exonuclease in one of the reagents used. In the second attempt with Kpn I m, 6 colonies were obtained of which 2 (pAM3 and pAM4) had lost the Xba I site with no apparent deletion (data not shown). Once more, marker rescue was unsuccessful, suggesting that again a frameshift mutation may have been introduced into an essential polypeptide (section III.1.c). It was subsequently learned

that glycoprotein H, an essential glycoprotein, is encoded by the region spanning the 0.29 m.u. Xba I site (Buckmaster et al., 1984; McGeoch and Davison, 1986b).

In an attempt to compensate for the putative frameshift, insertion mutagenesis was carried out on both plasmids. The plasmids were cleaved with Xba I, blunt ended with Klenow polymerase and then a nonphosphorylated 8 bp Kpn I linker ligated between the two ends (Figure 26b). A nonphosphorylated linker was used to minimize the possibility of more than one linker being inserted. Insertion of only one linker would result in an overall addition of 12 bp, ie. an in-frame insertion of four amino acids. This would hopefully have less effect on an encoded protein than a frameshift mutation. Prior to transformation, selection enrichment was again carried out using Xba I.

With Kpn I m, 50 colonies were screened. Of these, 41 had lost the Xba I site, but only one of these plasmids (pAM5) contained a Kpn I linker. (A Kpn I digest of a linker-containing plasmid should give the same profile as an Xba I/Kpn I double digest of the original plasmid - data not shown). The low frequency is not surprising due to the use of a phosphorylated plasmid (which would easily self-ligate) and a nonphosphorylated linker, even at the 100 fold molar excess of linker used.

With Kpn I c, a similar pattern was seen. Eighty-nine colonies were analyzed, of which 85 lacked the Xba I site. Of these again only one (pAC5) contained a Kpn I linker. Attempts made to marker rescue these plasmids back into X2 were unsuccessful (section III.1.b).

While deleting the Xba I sites from the plasmids,

selection enrichment was also being carried out on intact viral DNA (section III.1.c). In this way the 0.45 m.u. Xba I site was deleted. Therefore, all further plasmid manipulations were carried out using only Kpn I m.

These 3 plasmids were again used in marker rescue experiments, this time into X3 or 1701 (an HSV-1 strain 17 genome

containing only the 0.29 m.u. Xba I site - see III.1.c). Again this was unsuccessful (III.1.b).

At this point it was learned that the 0.29 m.u. Xba I site was contained within the essential glycoprotein, gH. The sequence around this region was kindly supplied to us by Drs A. C. Minson and D. J. McGeoch. This allowed the synthesis of an 18-mer oligonucleotide with a one base alteration which destroyed the Xba I site but, because it was in the third base position, did not alter the amino acid sequence of gH (see Figure 26d). The oligonucleotide was inserted into Kpn I m, to construct plasmid pAM9 which was marker rescued into 1701. This time virus lacking the 0.29 m.u. Xba I site was isolated (see III.1.b). Thus, of all the HSV-1 plasmids from which the Xba I sites had been deleted, only the construct (pAM9) which had no effect on the polypeptide coding potential of the virus, was successfully marker rescued back into the virus genome.

III.1.b Marker rescue of HSV plasmids lacking Xba I sites into intact HSV

All the Kpn I m and c plasmids lacking Xba I sites with no detectable deletions (pAM3-9 and pAC1-5 (III.1.a), respectively) were used in cotransfection experiments with intact HSV DNA (see method, section II.7). The plasmids were individually marker rescued into either X2 DNA, containing the 0.29 and 0.45 m.u. Xba I sites (Figure 25) or, in the case of pAM6-9, into 1701 DNA, containing only the 0.29 m.u. Xba I site (III.1.c). Following transfection, the virus was harvested when cpe was complete, titrated and single plaques isolated. In general, no more than fifty plaques were

analyzed from the one transfection. From these plaque isolates, virus stocks were generated in 35mm Petri dishes. The isolates were analyzed by restriction enzyme analysis of ^{32}P *in vivo* labelled DNA (see method, section II.10). At this stage only the results of the analysis and not the description of the digestion pattern expected or observed is given; this description will be given in section III.1.c. Table 2 shows that no successful marker rescue was obtained with any plasmid except Kpn I *m* containing the synthetic oligonucleotide (pAM9), where just under 10% of the plaques were recombinants; a figure close to the expected frequency for this size of plasmid (Stow *et al.*, 1978).

From the marker rescue experiments 1251 virus plaques were analyzed for the loss of the 0.29 m.u. Xba I site and 1017 for the loss of the 0.45 m.u. Xba I site, with no success. To ensure that the marker rescue experiments were working, control marker rescue experiments were carried out. The mutant, *ts*1201 was marker rescued by a fragment spanning its *ts* lesion in p40 at 0.33 m.u (Preston *et al.*, 1983). The fragments chosen were Eco RI *f* (0.312-0.415 m.u.) and, as a control, Kpn I *c* (0.435-0.518 m.u.). The results are illustrated in Table 3. The marker rescue was carried out 3 times on the different batches of BHK21 C13 cells used for marker rescue of the plasmids lacking Xba I sites. Each time, no wild-type virus was detected when *ts*1201 DNA alone, or in conjunction with the control plasmid Kpn I *c*, was used for the transfection. Only one set of the control values are shown in Table 3. Recombination frequencies with Eco RI *f* ranged from 5-7%, indicating that failure to lose the Xba I site was not a failure of marker rescue, but probably due to

Table 13 : BCdR sensitivity of 1703

Virus	Yield* +BCdR	Yield* -BCdR
strain 17	1.9×10^5	3.2×10^8
X2	3.3×10^5	2.1×10^8
1703	3.2×10^8	3.6×10^8

* expressed as the virus titre per 4×10^6 BHK21 C13 cells, following infection for 24 h in the presence or absence of BCdR at 100ug/ml.

Table 2 : Marker rescue of Xba I sites

Plasmid used	Recipient DNA	Number of plaques analyzed	Number of positive plaques
pAM3	X2	95	0
pAM4	X2	96	0
pAM5	X2	592	0
pAM6	1701	96	0
pAM7	1701	182	0
pAM8	1701	190	0
pAM9	1701	96	8
pAC1	X2	94	0
pAC2	X2	95	0
pAC3	X2	95	0
pAC4	X2	96	0
pAC5	X2	631	0

Table 3 : Marker rescue frequency of ts1201

Fragment used	Titre at 31°C*	Titre at 38.5°C*	Recombination frequency
-	5.4×10^7	$<10^2$	0%
Kpn I c	9.2×10^7	$<10^2$	0%
Eco RI f (1)	7.1×10^7	5.1×10^6	7.18%
Eco RI f (2)	8.1×10^6	4.2×10^5	5.18%
Eco RI f (3)	2.3×10^7	1.2×10^6	5.22%

* pfu/ml

lethal mutations being introduced into essential viral polypeptides.

The eight plaques which had lost the 0.29 m.u. Xba I site following marker rescue with pAM9 all arose from the one transfection plate and are thus likely to be clonally related. The polypeptide profiles of these variants support this conclusion, see later (III.1.e).

III.1.c Selection enrichment of HSV DNA with Xba I

Selection enrichment was carried out on X2 DNA in order to enrich for those genomes existing within the DNA population which lacked either the 0.29 and/or 0.45 m.u. Xba I site. Such natural variants should be more resistant to cleavage by Xba I, and hence more likely to cause a successful infection following transfection. DNA was digested with an excess of enzyme (5 units/ug DNA) and samples removed at various times, usually after 1, 2, 4 and 16 h. About 1ug digested DNA from each time point and undigested DNA as a control were transfected onto BHK21 C13 cell monolayers. The plates were harvested, usually after 3 days at 37°C, the virus yield titrated and plaques isolated from the virus-positive plate which had received DNA digested for the longest period of time. This was usually the 16 h plate, although occasionally, when the transfection efficiency was low, no virus was found on this plate. By 16 h, there was normally a 100-1000 fold reduction in virus titre, compared to the undigested control. The results of a typical experiment are shown in Table 4. There could be several reasons for the survival of some infectivity even after apparently complete digestion : (i) genomes lacking

Table 4 : Effect of Xba I treatment on X2 DNA infectivity

Time (h)*	Titre (pfu/ml)ϕ
0	1.3×10^7
1	2.5×10^6
2	9.9×10^5
4	1.1×10^4
16	5.2×10^3

* Time (h) of Xba I digestion of X2 DNA.

ϕ Plates were harvested 3 days post-transfection (37°C).

Xba I sites not being cleaved by Xba I (this, in general, was not the case); (ii) a small amount of incomplete digestion, which was not seen by gel electrophoresis; and (iii) religation of the Xba I digested DNA within the transfected cells (Dr. F. J. Rixon, personal communication).

In general, from each Xba I digestion and transfection no more than 100 plaques were isolated for analysis. Virus stocks were grown from these plaque isolates in 35mm Petri dishes and analyzed by restriction enzyme digestion of ^{32}P *in vivo* labelled DNA. As well as isolating plaques, the transfected plate stocks were also used to prepare a large scale preparation of virion DNA which was subjected to further rounds of Xba I digestion and transfection (serial selection enrichment) (Figure 27). In this way, it was hoped to enrich for minor populations which lacked one or both Xba I sites. In general, 50-200 plaque isolates were analyzed per round.

In order to identify genomes lacking either the 0.29 or 0.45 m.u. Xba I sites, Xba I/Bgl II double digests were carried out. Due to the large sizes of the Xba I fragments present in X2 (Figure 28) it was felt that Xba I digestion alone would not clearly distinguish variants lacking either or both of the Xba I sites.

The 0.29 and 0.45 m.u. Xba I sites are contained within the Bgl II *m* and *d* bands, respectively (Figure 29). Thus, Bgl II *m*, a $4x10^6$ mol. wt. band, is cleaved by Xba I to two *m'* bands of around $2.1x10^6$ and $1.9x10^6$ mol. wt., while Bgl II *d*, a $17x10^6$ mol. wt. band, is cleaved to two *d'* bands of around $13x10^6$ and $4x10^6$ mol. wt., which comigrate with Bgl II *f* and *n*, respectively (Figure 30, lanes 1 and 2).

Figure 27. Serial selection enrichment.

Diagram of the procedure used for selection enrichment of HSV genomes lacking Xba I sites. A DNA population containing a few molecules lacking one or more Xba I sites was cleaved with Xba I prior to transfection. This should enrich for those molecules lacking Xba I sites. Single plaques were isolated and analyzed. To enrich for minor populations lacking Xba I sites, the transfected plate harvest was used to grow up a burrler preparation of HSV DNA which was subjected to further rounds of selection enrichment (serial selection enrichment).

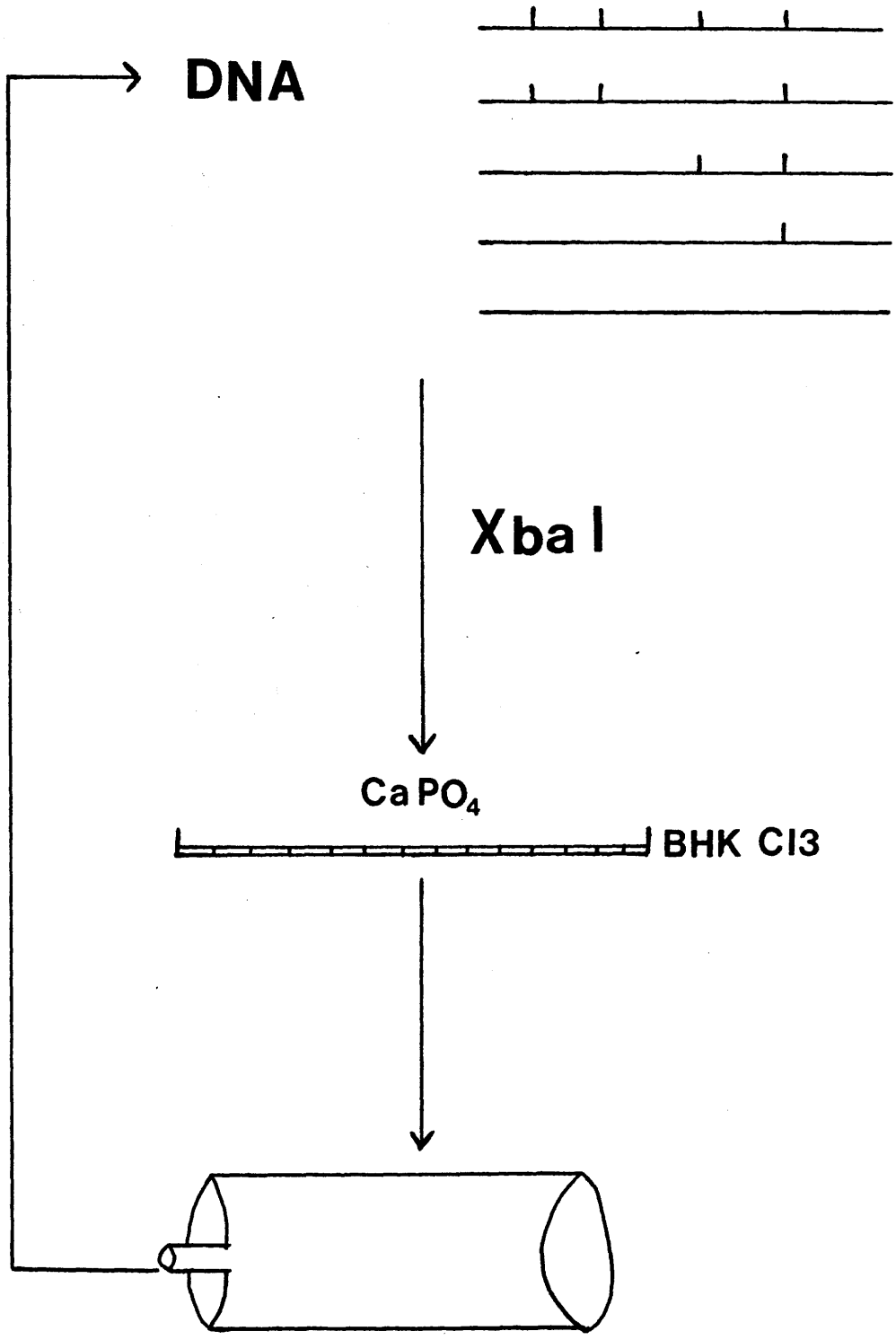

DNA
Xba I
CaPO4
BHK C13

Figure 28. Xba I profile of HSV-1 DNA.

Autoradiographs of Xba I restriction digests of HSV-1 DNA ^{32}P-labelled *in vivo* (0.5% agarose). Lane 1, strain 17; lane 2, B1/2; lane 3, B9/6; lane 4, X2.

B1/2 lacks the 0.07 m.u. Xba I site; B9/6 lacks the 0.63 m.u. Xba I site; X2 lacks both the 0.07 and 0.63 m.u. Xba I sites (Brown *et al.*, 1984).

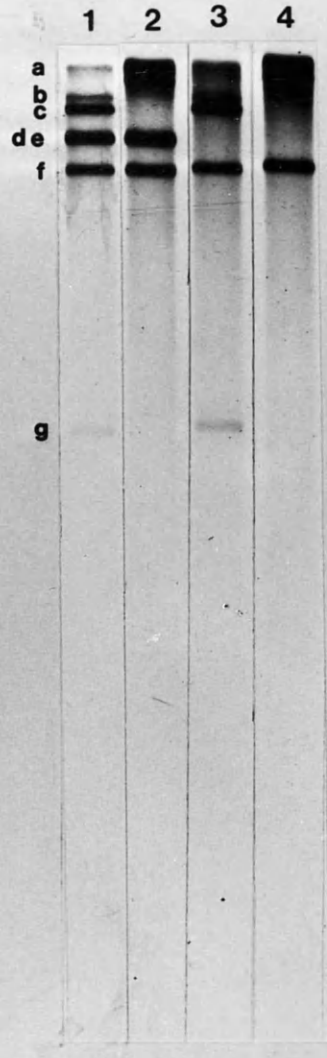

1
2
3
4
a
b
c
d
e
f
g

Figure 29. Bgl II/Xba I map of X2 DNA.

Bgl II map of HSV-1 strain 17 (and X2) DNA is shown, with the two Xba I sites (X) in X2 marked below the line.

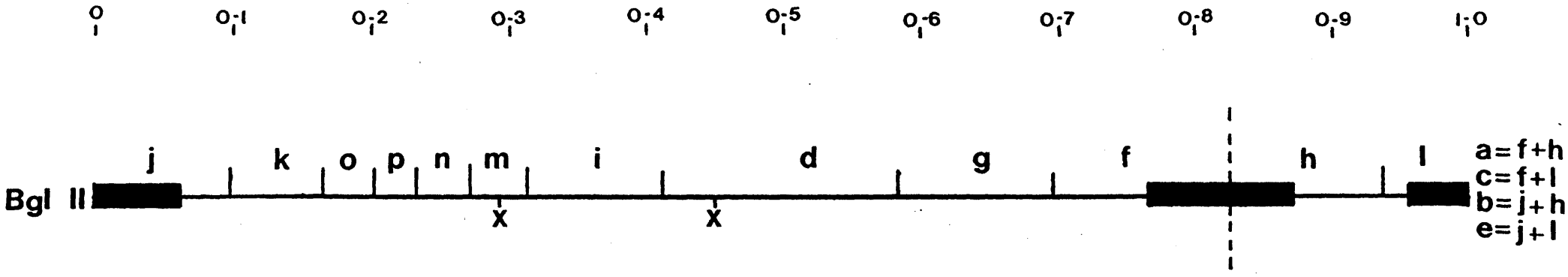
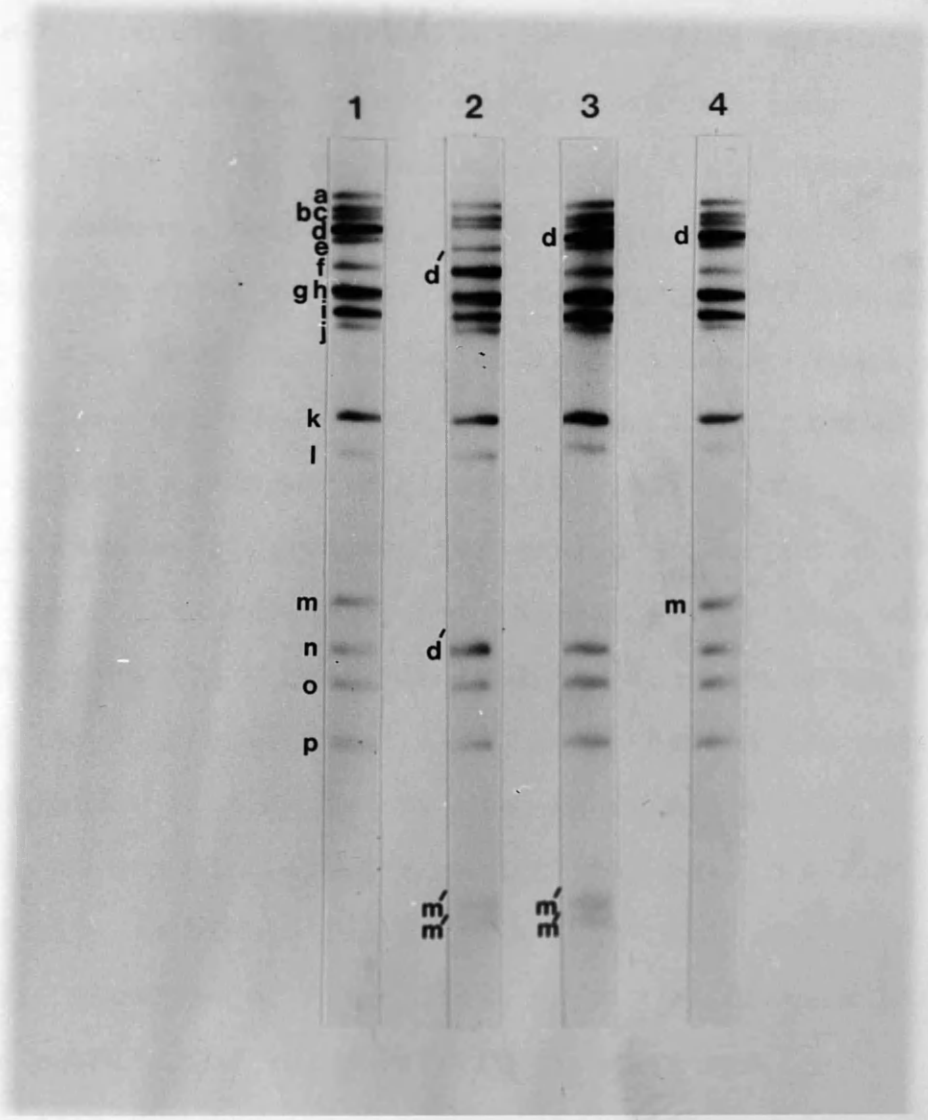

0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Bgl II
j
k
o
p
n
m
i
d
g
f
h
l
a=f+h
c=f+l
b=j+h
e=j+l

Figure 30. Bgl II profile of HSV-1 DNA.

Autoradiographs of HSV-1 DNA ^{32}P-labelled *in vivo* (0.5% agarose). Lane 1, X2 digested with Bgl II; lane 2, X2 digested with Bgl II/Xba I; lane 3, 1701 digested with Bgl II/Xba I; lane 4, 1702 digested with Bgl II/ Xba I. In the Bgl II/Xba I digest of X2, the m and d bands are absent and are replaced by two m' and two d' bands. In the Bgl II/Xba I digest of 1701 the d band has returned but the two m' bands are still present. In the Bgl II/Xba I digest of 1702 both the d and m bands are now present. Variation in the mobility of the l band is due to variation in the 'a' sequence between isolates.


1
2
3
4
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
d´
d
d
m
d´
m´
m´

Variability in the mobility of the terminal fragment Bgl II l (Figures 29 and 30) is due to variation within the 'a' sequence (Davison and Wilkie, 1981).

Several rounds of selection enrichment were carried out. Prior to the seventh round, 419 plaques had been isolated, of which none had lost either Xba I site (Table 5). From the seventh round 192 plaques were isolated of which 14 had identical gel profiles, but which differed from that of X2. They were assumed to be clonally related and one (X3 or 1701) was selected as the prototype. A Bgl II/Xba I digestion of 1701 is shown in Figure 30, lane 3. The two Bgl II m' bands were still present, indicating retention of the 0.29 m.u. Xba I site. However, the two Bgl II d' bands were absent and the Bgl II d band was present, indicating the absence of the 0.45 m.u. Xba I site. To determine the nature of the alteration leading to the removal of the 0.45 m.u. Xba I site, an Eco RI digestion was carried out. The 0.45 m.u. Xba I site is within Eco RI o, a $1x10^6$ mol. wt. band (Figure 31). Cleavage of X2 and 1701 by Eco RI (Figure 32) showed the mobility of the o band to be unaltered, indicating that the site loss had arisen with no detectable deletion or insertion (greater than 150 bp). Again, variation in the mobility of the terminal fragment, Eco RI k, is probably due to variation within the 'a' sequence (Figures 31 and 32).

Attempts to delete the remaining 0.29 m.u. Xba I site from 1701 by selection enrichment were totally unsuccessful, despite over 2,000 plaques being analyzed from 11 rounds of selection enrichment (Table 6).

At this stage, it was decided to abandon selection

Table 5 : Serial selection enrichment of X2 DNA using Xba I

Round	Number of plaques analyzed	Number of plaques lacking an Xba I site
1	48	0
2	92	0
3	91	0
4	92	0
5	48	0
6	192	0
7	192	14*
overall	755	14

* All 14 isolates lacked the 0.45 m.u. Xba I site.

Figure 31. Eco RI/Xba I map of X2 DNA.

Eco RI map of HSV-1 strain 17 (and X2) DNA is shown, with the two Xba I sites (X) in X2 marked below the line.

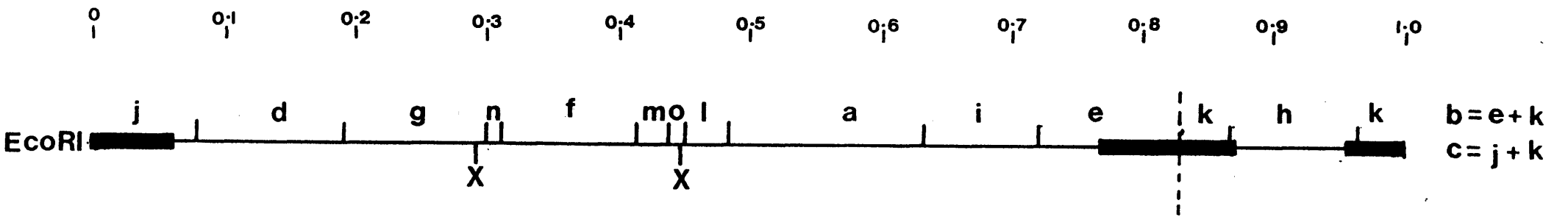
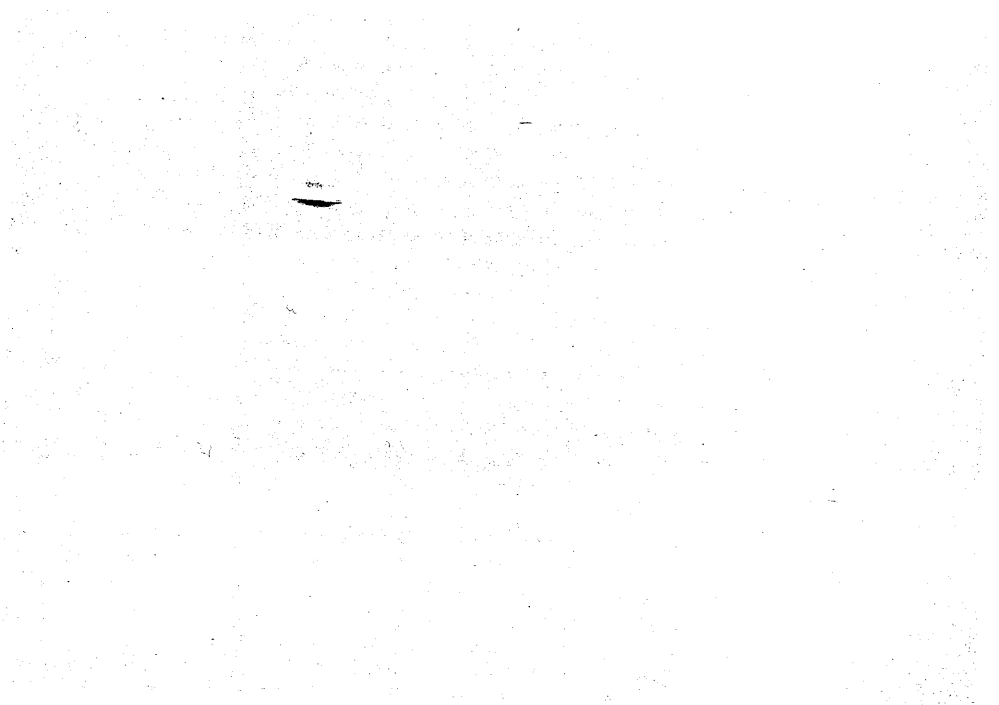

0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
EcoRI
j
d
g
n
f
m
o
l
a
i
e
k
h
k
X
X
b = e + k
c = j + k

Figure 32. Eco RI profile of HSV-1 DNA.

Autoradiographs of HSV-1 DNA ^{32}P-labelled *in vivo* and digested with Eco RI (0.6% agarose). Lane 1, X2; lane 2, 1701. Longer exposures of the *o* band are shown in lanes 1a and 2a. The mobility of the *o* band in 1701 is identical to that in X2, indicating the absence of any apparent deletion/insertion caused by the loss of the 0.45 site. The altered mobility of the *k* band is due to variation in the 'a' sequence.



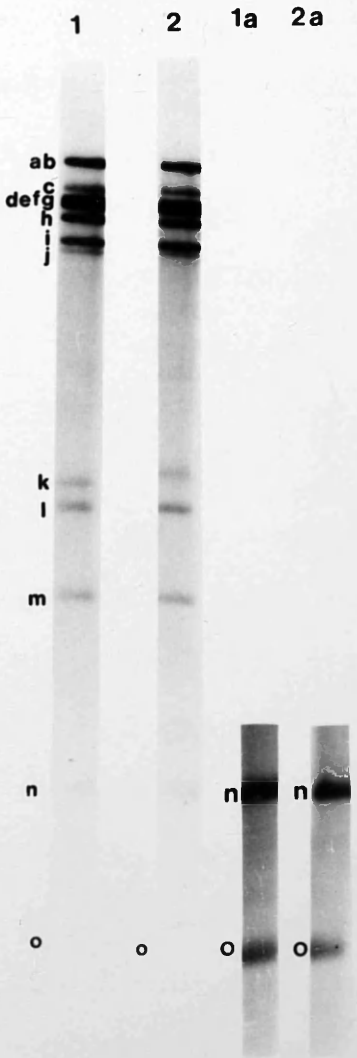

1
2
1a
2a
ab
c
def
g
h
i
j
k
l
m
n
o
o
n
n
o
o

Table 6 : Serial selection enrichment of 1701 DNA using Xba I

Round	Number of plaques analyzed	Number of plaques lacking an Xba I site
1	192	0
2	192	0
3	192	0
4	190	0
5	192	0
6	191	0
7	192	0
8	192	0
9	191	0
10	192	0
11	189	0
overall	2105	0

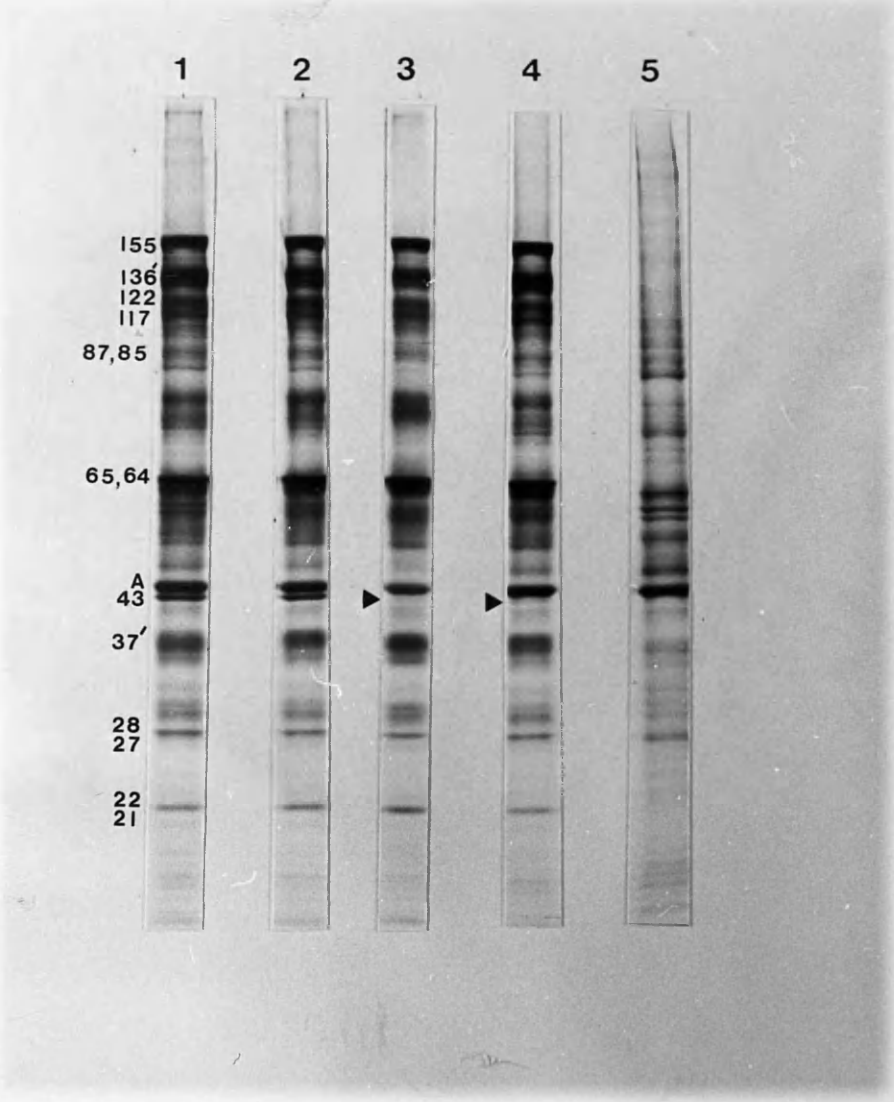
enrichment and delete this Xba I site by site-directed mutagenesis (III.1.a, III.1.b). Marker rescue was carried out (III.1.b) and 96 plaques isolated and analyzed by Xba I/Bgl II digestion. Of these, 8 gave identical gel profiles, but which differed from that of 1701. They were assumed to be clonally related and one (X4 or 1702) was designated as the prototype. It differed from 1701 in that the two Bgl II m' bands were absent and the Bgl II m band was present (Figure 30, lane 4). Thus, 1702 had lost the 0.29 m.u. Xba I site and contained no Xba I sites. A Bgl II/Xba I digestion of 1702 produced an identical profile to a Bgl II digestion of X2 (Figure 30, lanes 1 and 4) and wild-type strain 17 (data not shown). The mobility of the Bgl II m band in 1702 was the same as in X2 (Figure 30, lanes 1 and 4), indicating that the 0.29 m.u. Xba I site had not been lost by a detectable deletion or insertion (greater than 150 bp).

III.1.d Polypeptide profile of HSV-1 variants, X2, 1701 and 1702

The general polypeptide profiles of X2, 1701 and 1702 were compared with that of wild-type strain 17 (Figure 33). X2 (lane 2) had an identical profile to 17 (lane 1), whereas 1701 (lane 3) and 1702 (lane 4) both failed to induce a 43K polypeptide assumed to be the tk protein (see below). Apart from this, the polypeptide profiles of 1701 and 1702 were essentially similar to 17 and X2. Towards the end of the project, it was discovered that the 0.63 m.u. Xba I site was situated in the 3' end of gC. As this site had been removed by a deletion of around 150 bp, it was expected that gC would be affected. Preliminary experiments suggested that

Figure 33. Polypeptide profiles of X2, 1701 and 1702.

Whole cell extracts labelled with [^{35}S]-methionine from 4-24 h post infection were prepared as described (section II.33) and run on a 5-12.5% gradient SDS polyacrylamide gel. Lane 1, strain 17; lane 2, X2; lane 3, 1701; lane 4, 1702; lane 5, mock infected. The molecular weight x 10^{-3} of predominant HSV-1 polypeptides is marked on the left hand side. A is actin. The absent 43,000 mol. wt. polypeptide in 1701 and 1702 is marked ► .


1
2
3
4
5
155
136′
122
117
87,85
65,64
A
43
37′
28
27
22
21

X2, 1701 and 1702 synthesized a truncated form of gC (data not shown). The immediate-early polypeptide profiles of 17, X2, 1701 and 1702 were all identical (data not shown).

The tk-negative phenotype of 1701 and 1702 was confirmed by BCdR yield experiments. BCdR reduces the yield of tk-positive virus by 2-3 $\log_{10}$ pfu/ml but has little effect on the yield of tk-negative virus. Twenty-four hour yield experiments with 17, X2, 1701 and 1702 were carried out in the presence and absence of BCdR and the progeny titrated. The results (Table 7) show that while the yield of wild-type strain 17 and X2 is markedly reduced by BCdR, there is little effect on the yield of 1701 and 1702. Thus, 17 and X2 are tk-positive as expected, while 1701 and 1702 are tk-negative.

Since 1702 had been derived from 1701, which is tk-negative, then it would be expected to be tk-negative. As the mutated Kpn I m plasmid used to remove the 0.29 m.u. Xba I site also spans the tk locus (Wagner et al., 1981) it has the potential to rescue this mutation. However, a crossover is more likely to have occurred between the 0.29 m.u. Xba I site and the tk lesion than between the tk lesion and the right end of the fragment, as the distance between the 0.29 m.u. Xba I site and the tk gene is much larger than that between tk and the right end of the fragment (Figure 25), thus giving a greater possibility of recombination (Szostak et al., 1983).

The mutation in tk in 1701 and 1702 was believed to be a secondary mutation, unrelated to the loss of the Xba I sites. This was confirmed by the ability to marker rescue the tk lesion in 1702 with HSV-1 fragments spanning this

Table 7 : BCdR sensitivity of Xba I site variants

Virus	Yield* +BCdR	Yield* -BCdR
strain 17	1.9×10^5	3.2×10^8
X2	3.3×10^5	2.1×10^8
1701	1.65×10^8	1.9×10^8
1702	2.6×10^8	2.8×10^8

* expressed as the virus titre per 4×10^6 BHK21 C13 cells, following infection for 24 h in the presence or absence of BCdR at 100ug/ml.

gene, without affecting its restriction enzyme profile (Dr. F. J. Rixon, personal communication).

III.1.e Growth properties of 1701 and 1702

The growth characteristics of 1701 and 1702 are similar to those of strain 17 and X2. A one-step growth experiment over 24 h showed no marked differences in the growth properties of 17, X2, 1701 and 1702 (Figure 34). The lag periods of 1701 and 1702 appear slightly longer than that of 17 and X2, but as this is a very variable property, the significance is uncertain (Dr. D. Dargan, personal communication). The growth characteristics of 17, X2, 1701 and 1702 over several rounds of replication are almost identical (Figure 35). High titre stocks (>10^9 pfu/ml) of 1701 and 1702 have been obtained.

Both 1701 and 1702 gave a similar titre at 31°C and 38.5°C, indicating that they are not temperature-sensitive (data not shown).

III.2 GENERATION OF HSV-1 GENOMES FOR USE IN RECOMBINATION AND THEIR USE THEREIN

The object of the construction of a HSV-1 genome lacking Xba I restriction enzyme sites (1702) was to carry out recombination studies using the Xba I sites as multiple unselected markers in recombination crosses. To increase the number of markers, a HSV-1 genome containing 5 Xba I sites (1708; III.2.a), as opposed to the normal 4 Xba I sites in HSV-1 strain 17, was constructed. Existing ts lesions were then introduced into 1702 and 1708 (III.2.b), prior to

Figure 34. One-step growth curves of X2, 1701 and 1702.

One step growth curves of HSV-1 strain 17△, X2 ○, 1701 □ and 1702 ● in BHK21 C13 cells. Cells were infected at a multiplicity of 5 pfu/cell, the monolayers washed twice with PBS/calf serum, overlaid with ETC10 and incubated at 37°C. Plates were harvested at 0, 2, 4, 6, 8, 12 and 24 h post infection (section II.12).

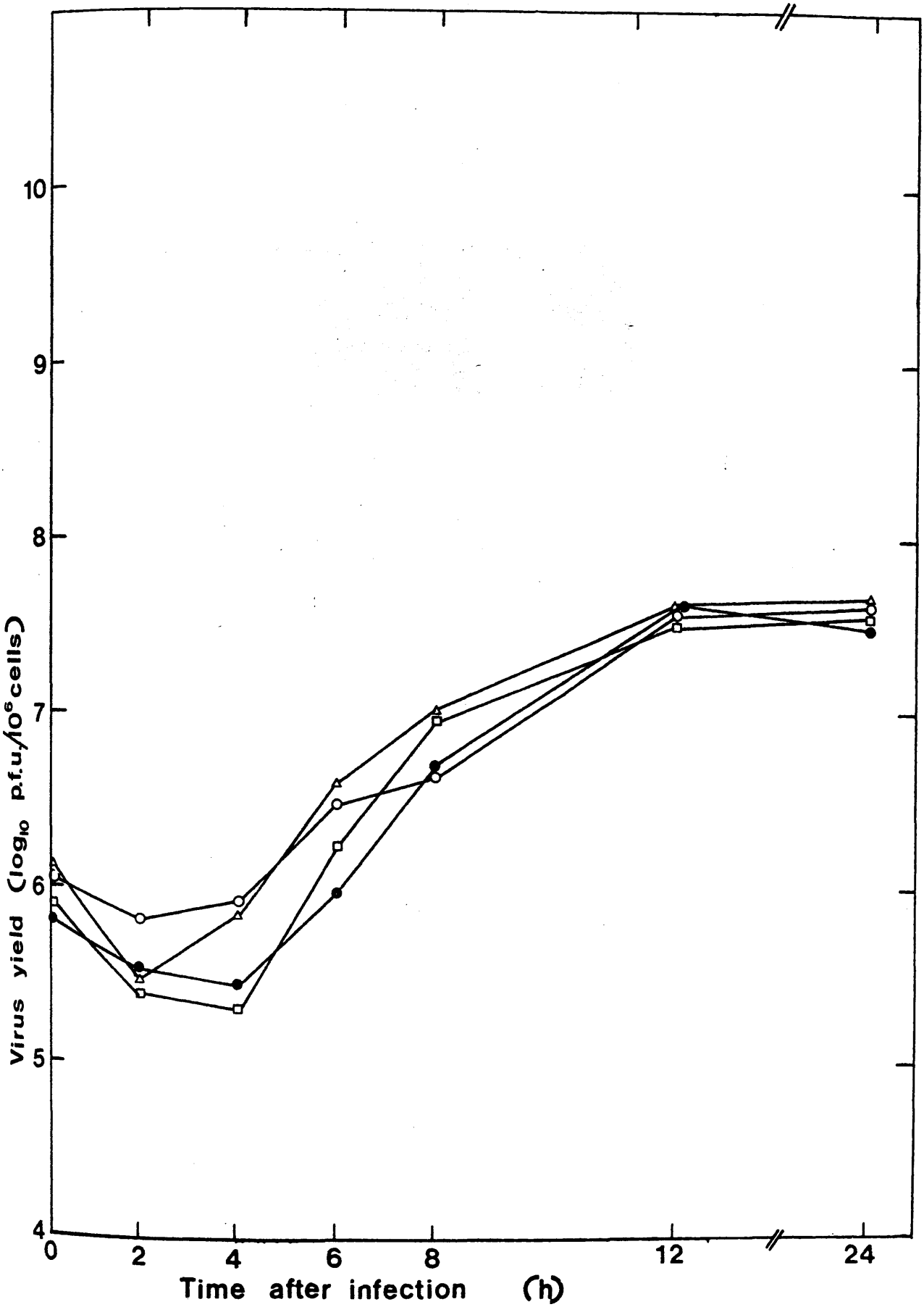
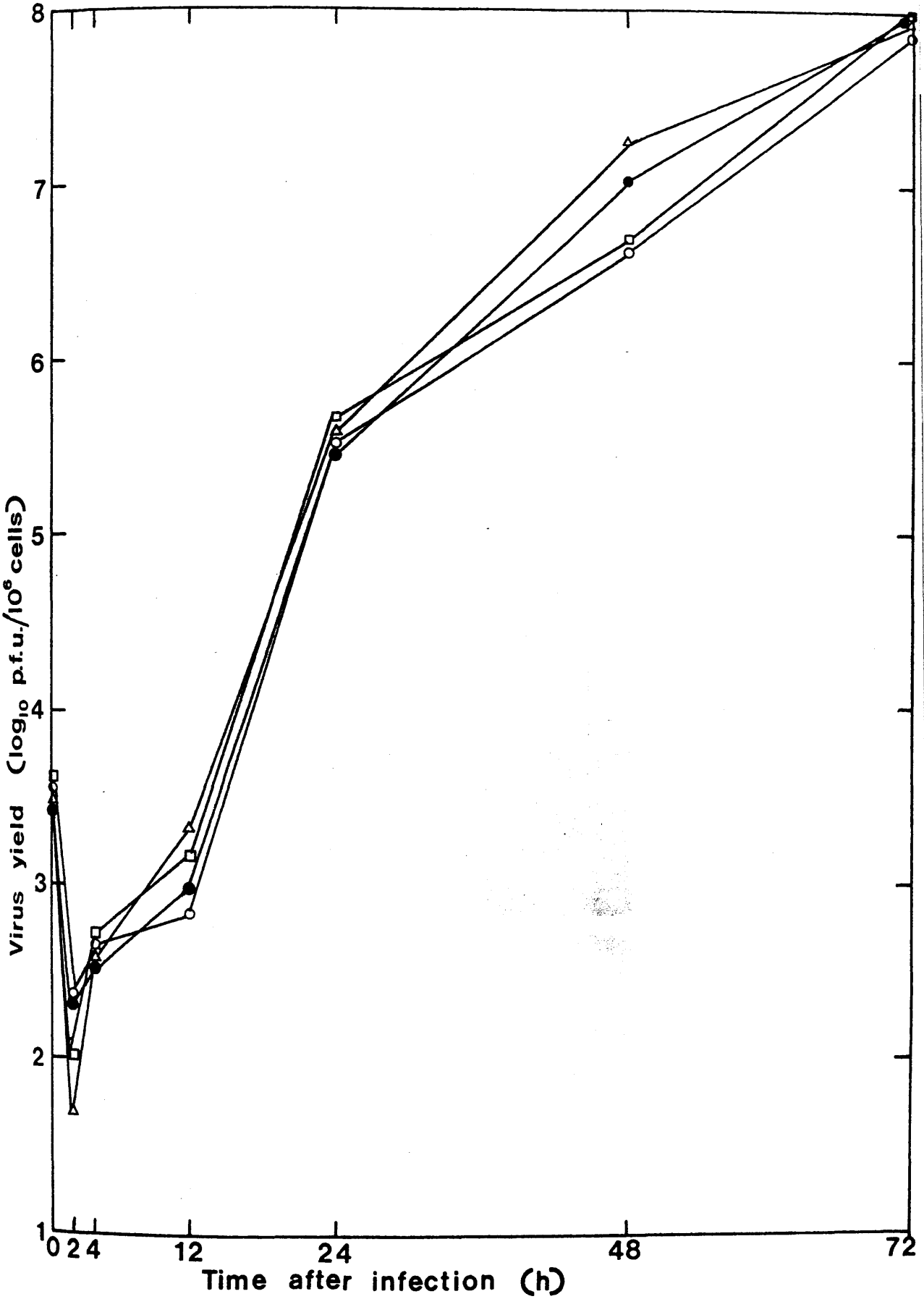

10
9
8
7
6
5
4
Virus yield (log10 p.f.u./10^6 cells)
0
2
4
6
8
12
24
Time after infection (h)

Figure 35. Long-term growth curves of X2, 1701 and 1702.

Long-term growth curves of HSV-1 strain 17Δ, X2 ○, 1701 □ and 1702 ● in BHK21 C13 cells. Cells were infected at a multiplicity of 0.001 pfu/cell, the monolayers washed twice with PBS/calf serum, overlaid with ETC10 and incubated at 37°C. Plates were harvested at 0, 2, 4, 12, 24, 48 and 72 h post infection (section II.12).


8
7
6
5
4
3
2
1
Virus yield (log10 p.f.u./10^6 cells)
0 2 4 12 24 48 72
Time after infection (h)

recombination analysis (III.2.c), so that the distribution of the Xba I sites could be analyzed in genomes known to have recombined between a pair of selected markers.

III.2.a Generation of a HSV-1 variant, 1708, containing 5 Xba I sites

In order to generate a variant containing 5 Xba I sites, recombination experiments were carried out between the wild-type strain 17, which contains 4 Xba I sites at 0.07, 0.29, 0.45 and 0.63 m.u. (Figure 36), and the variant 1707, which contains 3 Xba I sites at 0.29, 0.45 and 0.74 m.u. (Figure 36; see Materials for the origin of 1707). After 24 h at 37°C, virus was harvested from the infected cells, titrated, single plaques isolated and stocks prepared from 35mm Petri dishes. ^{32}P *in vivo* labelled DNA was prepared from these isolates and subjected to Xba I restriction enzyme analysis to identify recombinant genomes. A number of recombinants were isolated, some of which gave a pattern indicating the presence of 5 Xba I sites at 0.07, 0.29, 0.45, 0.63 and 0.74 m.u. One of these, 1708, was regarded as the prototype. Figure 36 indicates where a crossover would have occurred to generate this genome, assuming a single crossover event. Figure 37 shows the DNA profile of 1708 following Xba I digestion. The *d* band is cleaved into two novel bands, d_1 and d_2; d_1 is a 1 M band of about 9×10^6 mol. wt., while d_2 is a 0.5 M band of about 7×10^6 mol. wt. The joint fragment *a*, which in strain 17 contains the *d* fragment, is reduced in size by about 9×10^6 mol. wt. in 1708, and comigrates with *b* and *c* (see Figures 36 and 37). A Hpa I/Xba I double digest confirmed the

Figure 36. Xba I map of HSV-1 strain 17 and 1707.

Xba I map for the DNA of HSV-1 strain 17 (above the line) and 1707 (Cook and Brown, 1987 - below the line). The region marked 1 between the 0.63 m.u. site in strain 17 and the 0.74 m.u. site in 1707 is where recombination must occur to give rise to the variant 1708 with 5 Xba I sites.

Variant 1707 was generated from X2 by the spontaneous gain of an extra Xba I site at 0.74 m.u. (Cook and Brown, 1987).

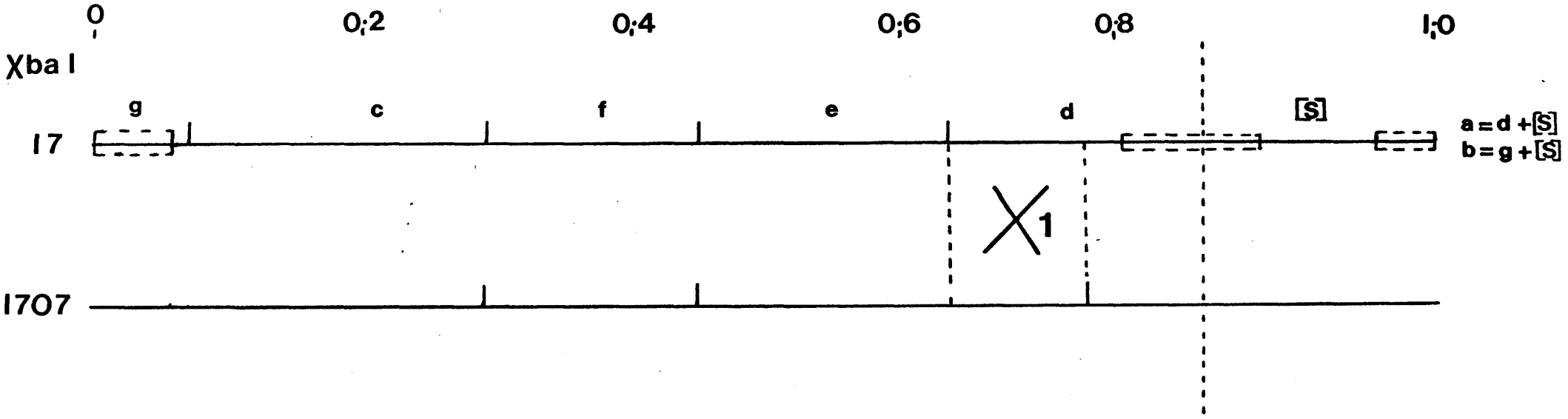
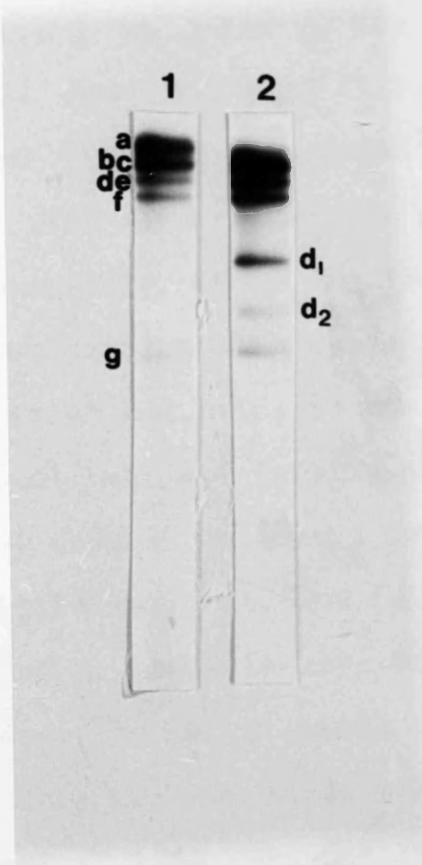

0
0;2
0;4
0;6
0;8
1;0
Xba I
g
c
f
e
d
[S]
17
a = d + [S]
b = g + [S]
1
1707

Figure 37. Xba I profile of 1708.

Autoradiographs of HSV-1 DNA ^{32}P-labelled *in vivo* and digested with Xba I (0.5% agarose). Lane 1, strain 17; lane 2, 1708, $\underline{d}_1$ and $\underline{d}_2$ refer to the products of cleavage of $\underline{d}$ at the 0.74 m.u. Xba I site.


1
2
a
b
c
d
e
f
g
d_1
d_2

presence of the extra Xba I site in 1708, compared to strain 17, and showed that this occurred in Hpa I s, as it does in 1707 (data not shown; Cook and Brown, 1987).

A Bgl II/Xba I digest also confirmed the presence of the extra Xba I site and showed it to be in Bgl II f (Figures 38 and 39 lane 4). The 4 Xba I sites in strain 17 are contained within Bgl II j, m, d and g, respectively (Figure 38 and 39 lane 2). Thus, Bgl II j (9.8×10^6 mol. wt.) is cleaved by Xba I to two j' bands of approximately 7×10^6 mol. wt. and 2.8×10^6 mol. wt. which comigrate with Bgl II k and below Bgl II p, respectively, while the j containing joints b and e are now comigrating with c and f, respectively; Bgl II m (4×10^6 mol. wt.) is cleaved to two m' bands of around 2.1×10^6 mol. wt. and 1.9×10^6 mol. wt., running below p; Bgl II d (17×10^6 mol. wt.) is cleaved to two d' bands of around 13×10^6 mol. wt. and 4×10^6 mol. wt. which comigrate with f and n, respectively; and Bgl II g (11×10^6 mol. wt.) is cleaved to two g' bands of around 6×10^6 mol. wt. and 5×10^6 mol. wt., running below Bgl II l.

A Bgl II/Xba I digest of 1707 (Figures 38 and 39 lane 3) results in cleavage of Bgl II m and d and also of Bgl II f due to the presence of the 0.74 m.u. Xba I site. Bgl II f (13×10^6 mol. wt.) is cleaved into two f' bands of around 8.5×10^6 mol. wt. and 4.5×10^6 mol. wt., running above k and m, respectively; the f containing joints a and c are also deleted and now migrate between b and c and above d, respectively. A Bgl II/Xba I digest of 1708 (Figures 38 and 39 lane 4) results in cleavage of Bgl II j, m, d, g and f, indicating the presence of all 5 Xba I sites.

The virus (1708) exhibits wild-type growth

Figure 38. Bgl II/Xba I maps of HSV-1 strain 17, 1707 and 1708.

Bgl II maps of HSV-1 strain 17, 1707 and 1708 are shown above the line, with the positions of the Xba I sites (X) in each genome shown below the line, to indicate which Bgl II bands are cleaved by Xba I.

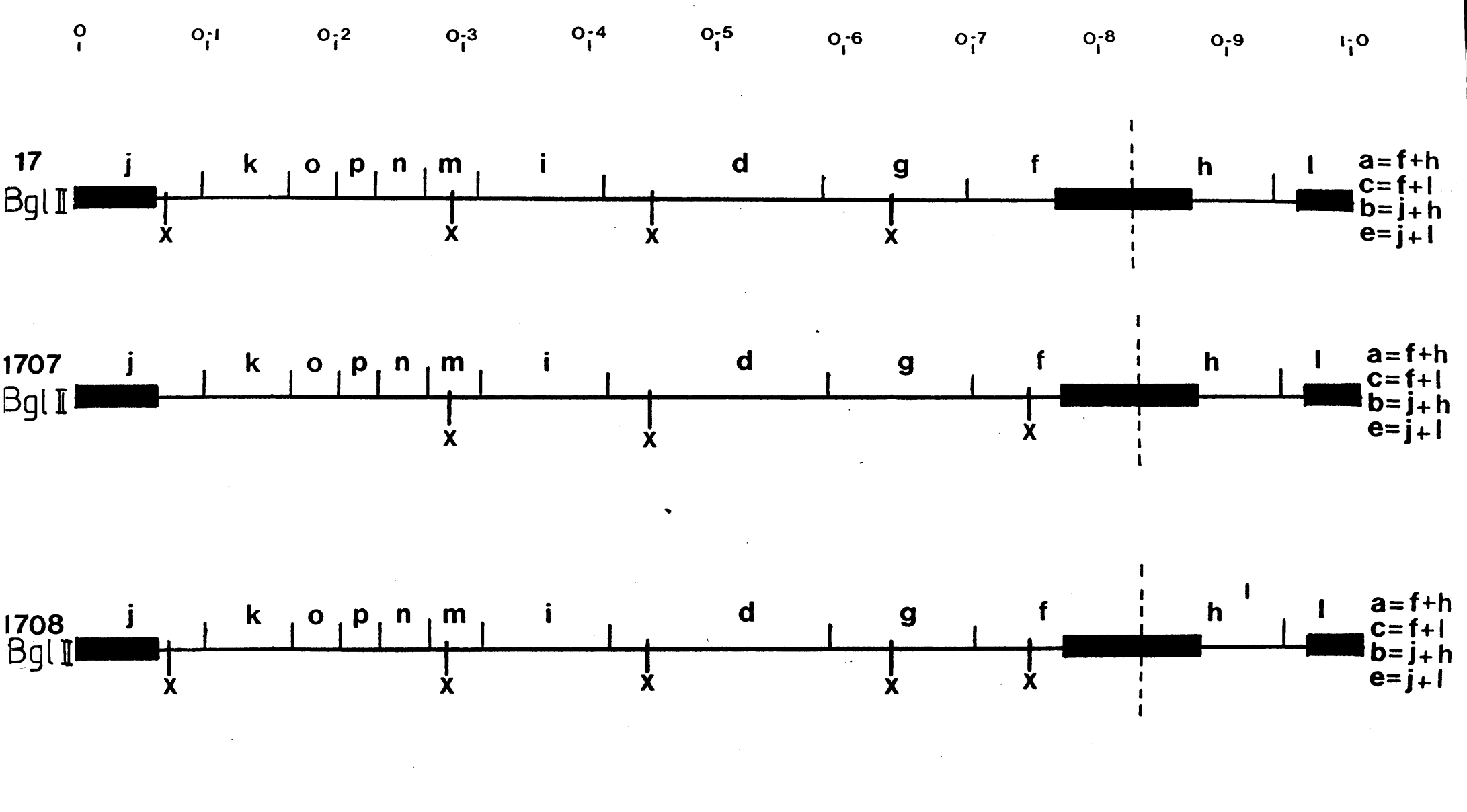
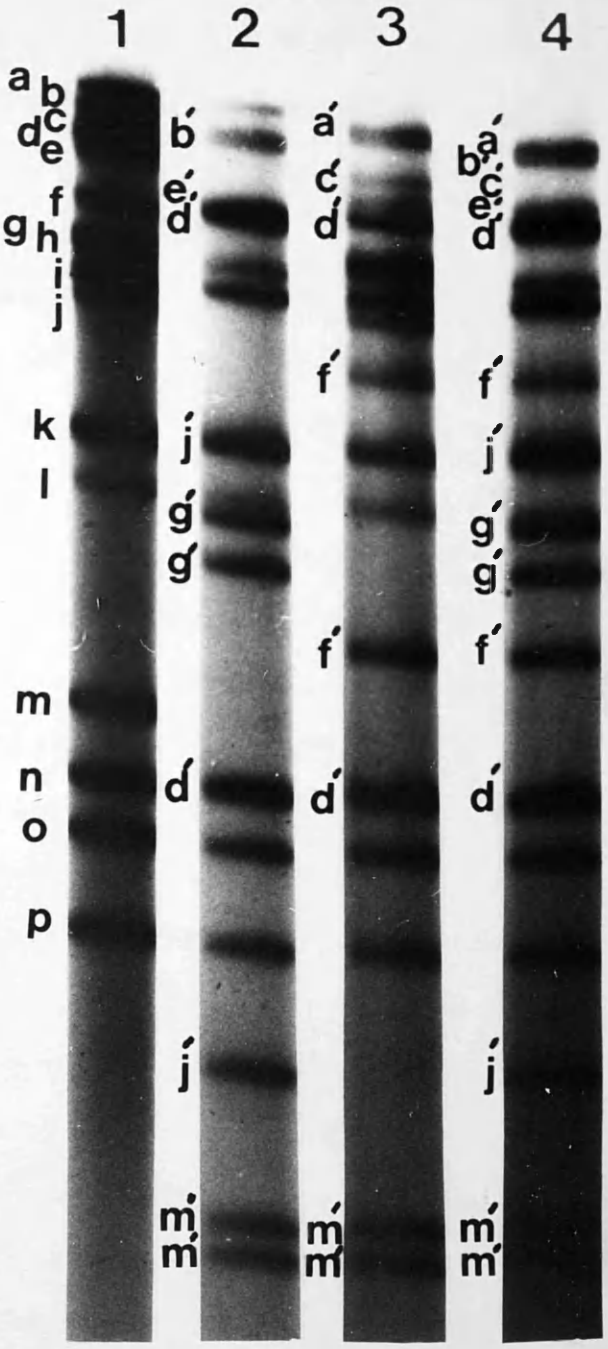

0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
17
Bgl II
1707
Bgl II
1708
Bgl II
j
k
o
p
n
m
i
d
g
f
h
l
X
a=f+h
c=f+l
b=j+h
e=j+l

Figure 39. Bgl II/Xba I profile of HSV-1 strain 17, 1707 and 1708.

Autoradiographs of HSV-1 DNA ^{32}P-labelled *in vivo* (0.5% agarose). Lane 1, strain 17 digested with Bgl II; lane 2, strain 17 digested with Bgl II/Xba I; lane 3, 1707 digested with Bgl II/Xba I; lane 4, 1708 digested with Bgl II/Xba I. The nomenclature of the Bgl II bands is given in lane 1. In lanes 2, 3 and 4 the products of Xba I cleavage of the Bgl II bands are marked by the letter of the fragment from which they are derived plus a prime symbol (').


1
2
3
4
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
b′
e′
d′
j′
g′
g′
d′
j′
m′
m′
a′
c′
d′
f′
f′
d′
d′
j′
m′
m′
a′
b′
c′
e′
d′
f′
j′
g′
g′
f′
d′
j′
m′
m′

characteristics on both a one-step growth experiment (Figure 40) and growth experiments over several rounds of replication (Figure 41). High titre stocks of 1708 were obtained (>10^9 pfu/ml). 1708 gave a very similar titre at 38.5°C and 31°C, indicating that it was not temperature-sensitive (data not shown).

The general polypeptide profile and immediate-early polypeptide profile of 1708 is identical to that of wild-type HSV-1 strain 17 (data not shown).

III.2.b Insertion of ts lesions into 1702 and 1708

Temperature-sensitive (ts) lesions were introduced into 1702, containing no Xba I sites (III.1.c) and 1708, containing 5 Xba I sites (III.2.a).

The ts lesion from ts1201 was introduced into 1702. This ts lesion maps to 0.33 m.u. and is within the capsid protein p40. At the non-permissive temperature ts1201 fails to package DNA into capsids (Preston et al., 1983). The Eco RI f fragment of ts1201 (0.312-0.414 m.u.) was marker rescued into 1702, at 31°C. Single plaques were isolated and analyzed for temperature-sensitivity. Out of 100 plaques analyzed, one was ts. The restriction profile of this isolate was identical to that of 1702, and it was named ts1702 (data not shown). This would be expected as Eco RI f does not span any Xba I sites.

The ts lesion from ts1206 (Dr. V. G. Preston, personal communication) was introduced into 1708. This ts lesion maps to 0.72 m.u. and is situated within gene U_L52, which specifies a 115x10^3 mol. wt. open reading frame (Dr. D. J. McGeoch, personal communication). This gene is essential for

Figure 40. One-step growth curves of 1704, 1705, 1706 and 1708.

One-step growth curves of HSV-1 strain 17○, 1704●, 1705△, 1706■ and 1708□ were carried out in BHK21 C13 cells. Cells were infected at a multiplicity of 5 pfu/cell, the monolayers washed twice with PBS/calf serum, overlaid with ETC10 and incubated at 37°C. Plates were harvested at 0, 2, 4, 6, 8, 12 and 24 h post infection and titrated as normal.

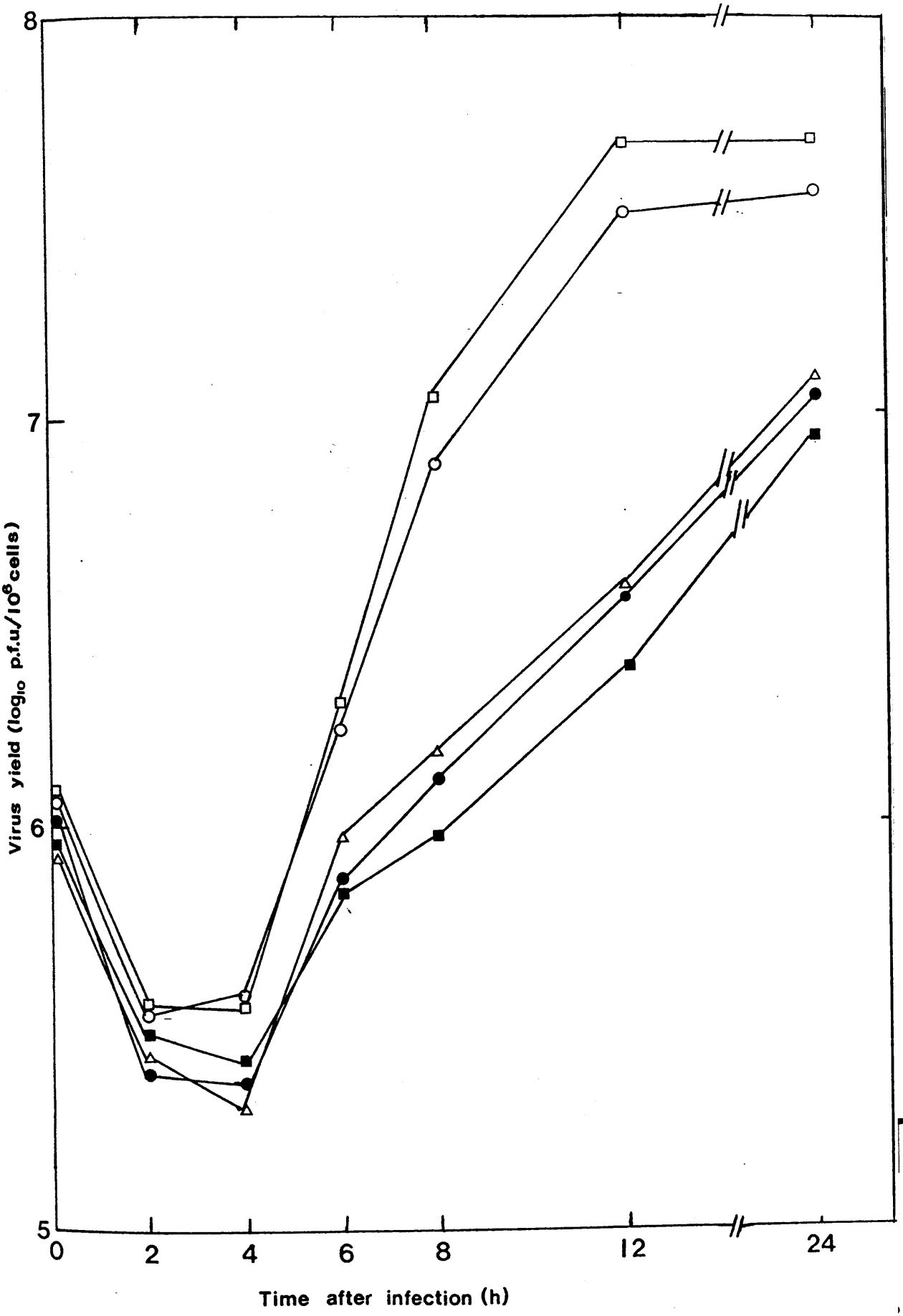
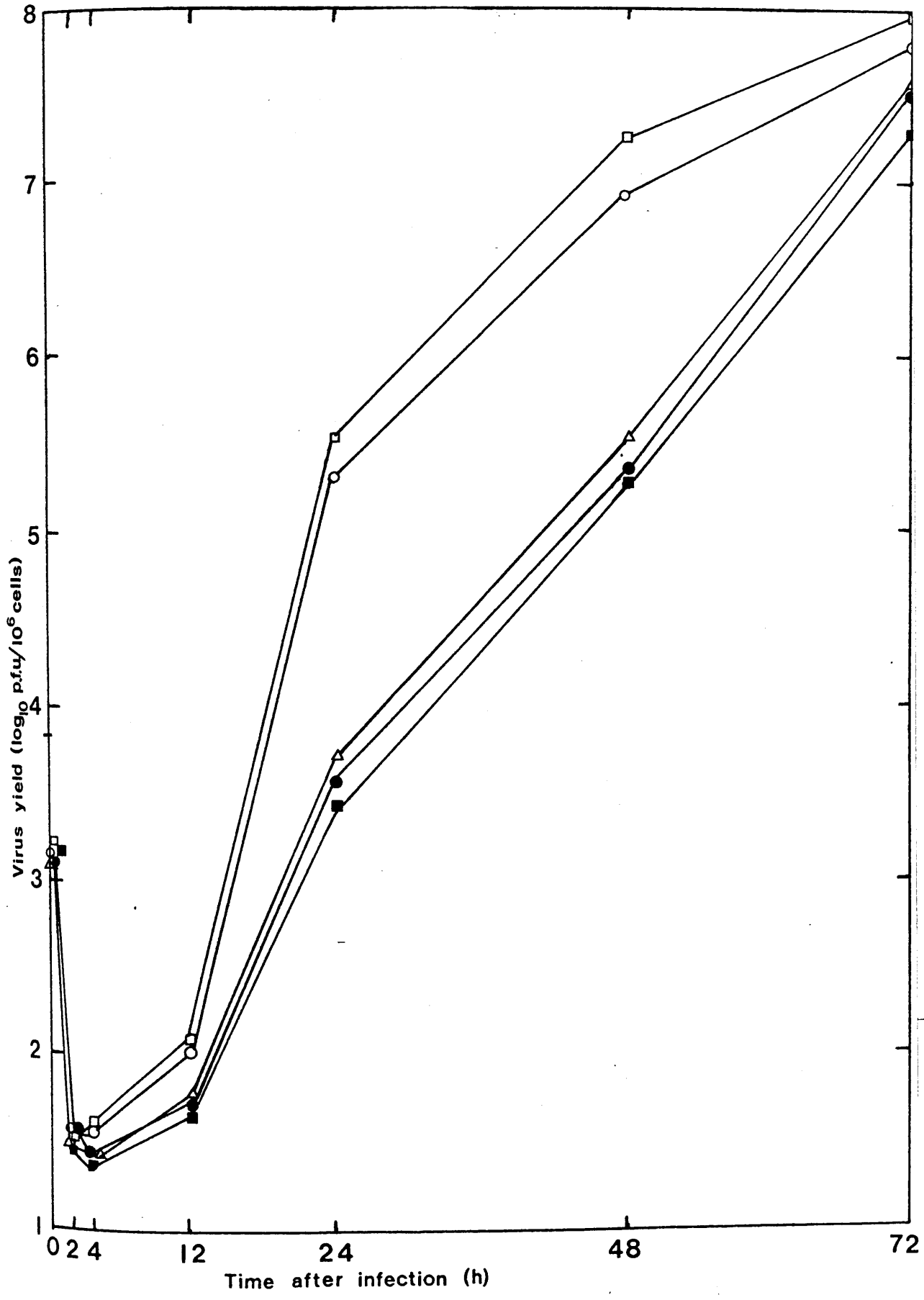

8
7
6
5
0
2
4
6
8
12
24
Virus yield (log_{10} p.f.u./10^6 cells)
Time after infection (h)

Figure 41. Long-term growth curves of 1704, 1705, 1706 and 1708.

Long-term growth curves of HSV-1 strain 17○, 1704●, 1705△, 1706■ and 1708□ were carried out in BHK21 C13 cells. Cells were infected at a multiplicity of 0.001 pfu/cell, the monolayers washed twice with PBS/calf serum, overlaid with ETC10 and incubated at 37°C. Plates were harvested at 0, 2, 4, 12, 24, 48 and 72 h post infection and titrated as normal.


8
7
6
5
4
3
2
1
Virus yield (log_{10} p.f.u/10^6 cells)
0 2 4
12
24
48
72
Time after infection (h)

DNA replication (Challberg, 1986; McGeoch, 1987; McGeoch *et al.*, 1988; Wu *et al.*, 1988) and therefore this *ts* mutant is DNA-negative at the non-permissive temperature. *Ts*1206, containing the wild-type 4 Xba I sites, and 1708, containing 5 Xba I sites, were recombined at 31°C, and single plaques were isolated and analyzed for temperature sensitivity. From one experiment, 200 plaques were isolated : 91 were *ts*, and of these 2 gave a restriction enzyme profile indicative of the presence of all 5 Xba I sites, ie. were identical to 1708 (data not shown). Of these isolates one was chosen as the prototype, and called *ts*1708.

To confirm that the correct *ts* lesion was present in *ts*1702 and *ts*1708, complementation experiments were carried out with the parental *ts* viruses, *ts*1201 and *ts*1206, and with each other (Table 8). *Ts*1702 and *ts*1201 failed to complement each other, as did *ts*1708 and *ts*1206, indicating that both viruses in each pair had a *ts* lesion in the same gene. However, *ts*1702 and *ts*1708 complemented each other, as did *ts*1201 and *ts*1206, indicating that the *ts* lesions were in different genes. Thus, as expected, *ts*1702 and *ts*1708 appear to contain the same *ts* lesions as those in the previously well characterized *ts*1201 and *ts*1206, respectively.

Due to unexpected results during recombination studies (III.2.c) marker rescue experiments were carried out, using cloned HSV fragments spanning the presumed position of the *ts* lesions (Table 9), to confirm the location of the *ts* lesions in *ts*1201 and *ts*1206. In the case of *ts*1708 and *ts*1206, the *ts* lesion was marker rescued by Kpn *o*, which spans the position of the 0.72 m.u. *ts* lesion. Therefore,

Table 8 : Complementation between ts mutants

	ts1201	ts1206	ts1702	ts1708
ts1201	1.3*	71.2	1.1	59.5
ts1206		0.9	67.6	1.2
ts1702			0.8	76.2
ts1708				1.4

* Complementation indices were calculated as described in section II.14.

Table 9 : Marker rescue of ts mutants

Fragment used	Recipient virus ts1201	ts1702	ts1206	ts1708
Eco RI f (0.312-0.414)	4.9*	1.05	nd	nd
Kpn I m (0.285-0.322)	nd	nd	nd	nd
Kpn I t (0.322-0.343)	1.5	nd	nd	nd
Kpn I n (0.343-0.373)	nd	nd	nd	nd
Kpn I p (0.373-0.401)	nd	nd	nd	nd
Kpn I v (0.401-0.413)	nd	nd	nd	nd
Kpn I a' (0.413-0.421)	nd	nd	nd	nd
Kpn I x (0.421-0.435)	nd	nd	nd	nd
Kpn I c (0.435-0.518)	nd	nd	nd	nd
Kpn I o (0.7-0.73)	nd	nd	2.2	1.4
Kpn I t + Kpn I v	1.2	nd	nd	nd
Kpn I t + Kpn I a'	1.7	0.11	nd	nd
Kpn I t + Kpn I x	1.9	nd	nd	nd
Kpn I t + Kpn I m	1.2	nd	nd	nd

* : Marker rescue frequency, expressed as $\frac{38.5^{\circ}C \text{ virus yield}}{31^{\circ}C \text{ virus yield}} \times 100$.

nd : No detectable virus at 38.5°C

these two viruses contain the expected ts lesion. However, in the case of tsl201 and tsl702, a different pattern was seen : tsl201 was rescued by both Eco RI f and Kpn I t (both of which span the 0.33 m.u. ts lesion); however, surprisingly, although Eco RI f rescued tsl702 at a low frequency, Kpn I t did not. Since Kpn I t is wholly contained within Eco RI f, and since the complementation data suggest that the same ts lesion was present in both tsl201 and tsl702, this result was somewhat surprising, and suggested the presence of a second ts lesion, elsewhere, in Eco RI f in tsl702. To confirm this, marker rescue experiments were carried out using two HSV fragments, one spanning the tsl201 lesion, Kpn I t, and one from another part of Eco RI f. When Kpn I a' was used in conjunction with Kpn I t, successful marker rescue was achieved, albeit at a low frequency, as would be expected for double marker rescue. Thus, tsl702 has two ts lesions, one at 0.33 m.u. in p40, and one between 0.413 and 0.414 m.u., probably in the DNA polymerase (Quinn and McGeoch, 1985). The location of this ts lesion is shown in Figure 42. This second ts lesion must have arisen spontaneously in the generation of tsl702, as 1702 is not temperature-sensitive (III.1.e). The position of the ts lesions relative to the Xba I sites in tsl702 and tsl708 is shown in Figure 43.

III.2.c Recombination between tsl702 and tsl708

A recombination experiment between tsl702 and tsl708 was carried out at 31°C and samples harvested at 0, 2, 4, 6, 8, 12 and 24 h post infection (see method section, II.15). The samples were titrated at 31°C (permissive temperature)

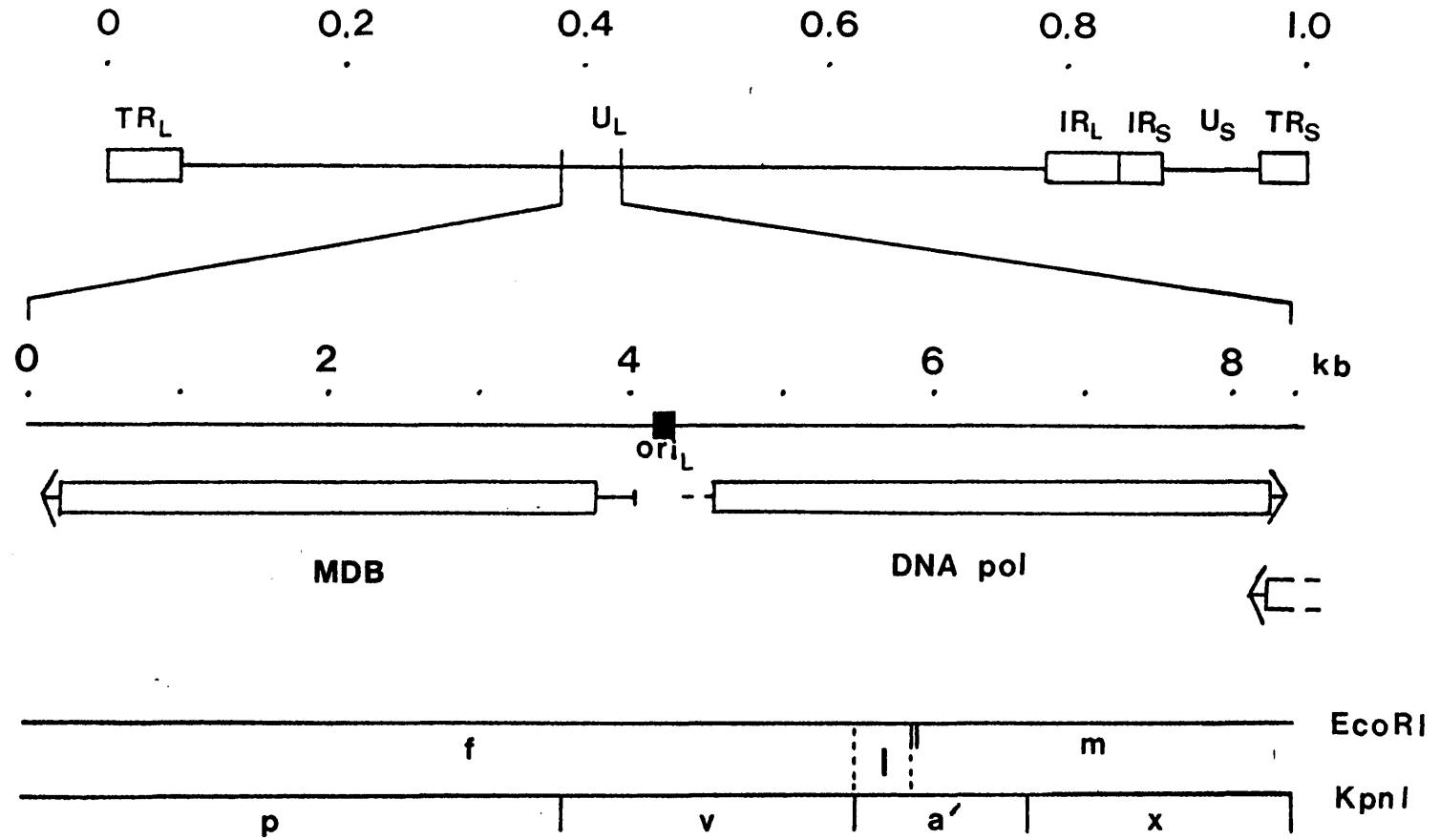
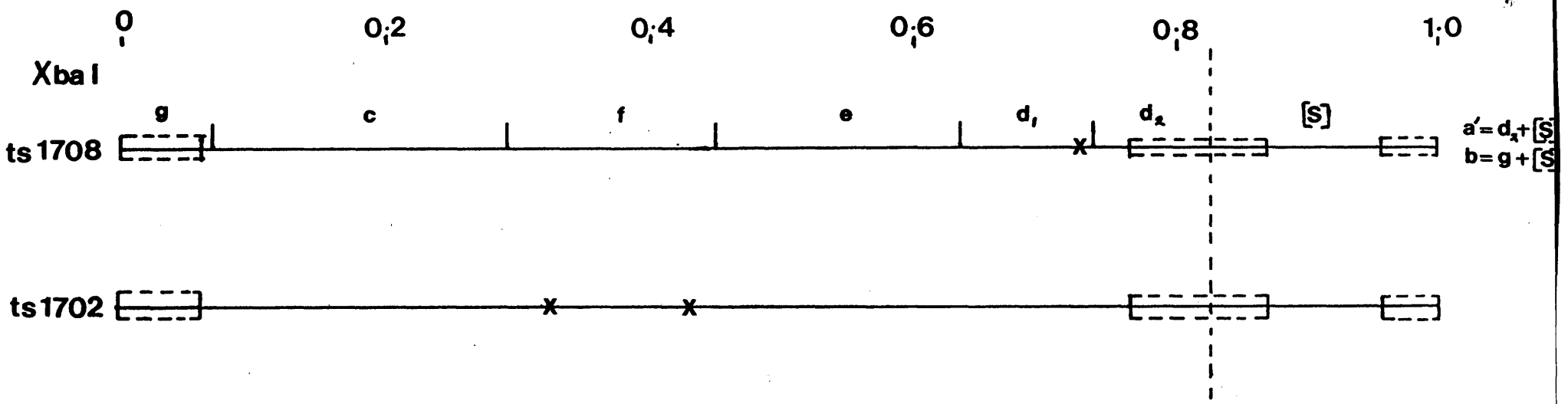

0
0.2
0.4
0.6
0.8
1.0
TR_L
U_L
IR_L
IR_S
U_S
TR_S
0
2
4
6
8
kb
ori_L
MDB
DNA pol
EcoRI
KpnI
f
l
m
p
v
a'
x

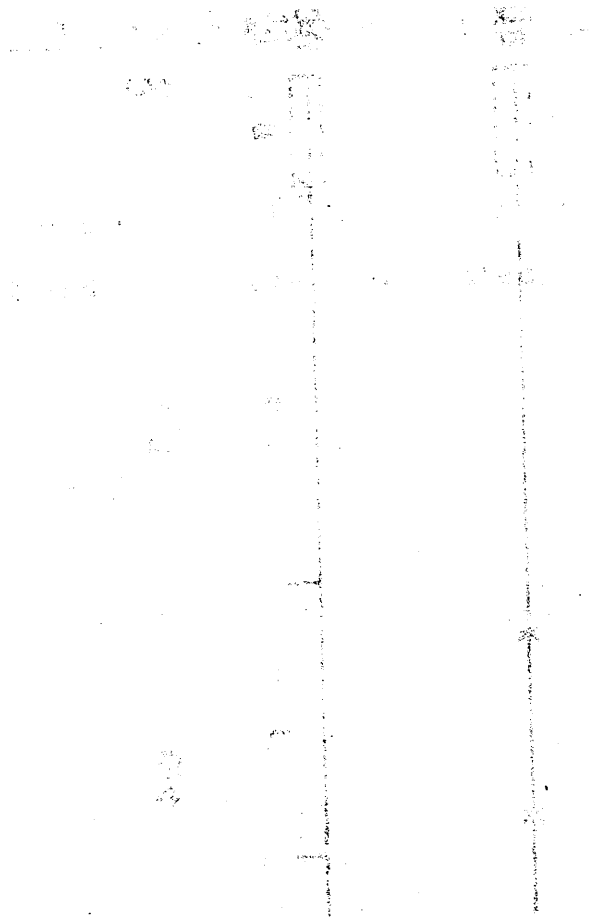
Figure 42. Position of the 0.414 m.u. ts lesion in ts1702.

The upper part of the diagram represents the intact HSV-1 genome. The lower part is an expansion of the region around 0.4 m.u. including the MDB, DNA polymerase and 3' end of a transcript overlapping the DNA polymerase. Coding regions are indicated by boxes and transcript boundaries by lines. The relevant Kpn I and Eco RI fragments are illustrated. The region common to Eco RI f and Kpn I a', boxed and labelled 1, is the location of the 0.414 m.u. ts lesion in ts1702 as both fragments rescue this lesion.

Figure 43. Xba I sites and ts lesions in ts1702 and ts1708.

The position of the Xba I sites and *ts* lesions in *ts*1702 and *ts*1708 are illustrated.


0
0·2
0·4
0·6
0·8
1·0
Xba I
g
c
f
e
d1
d2
[S]
a'= d2+[S]
b= g+[S]
ts 1708
ts 1702



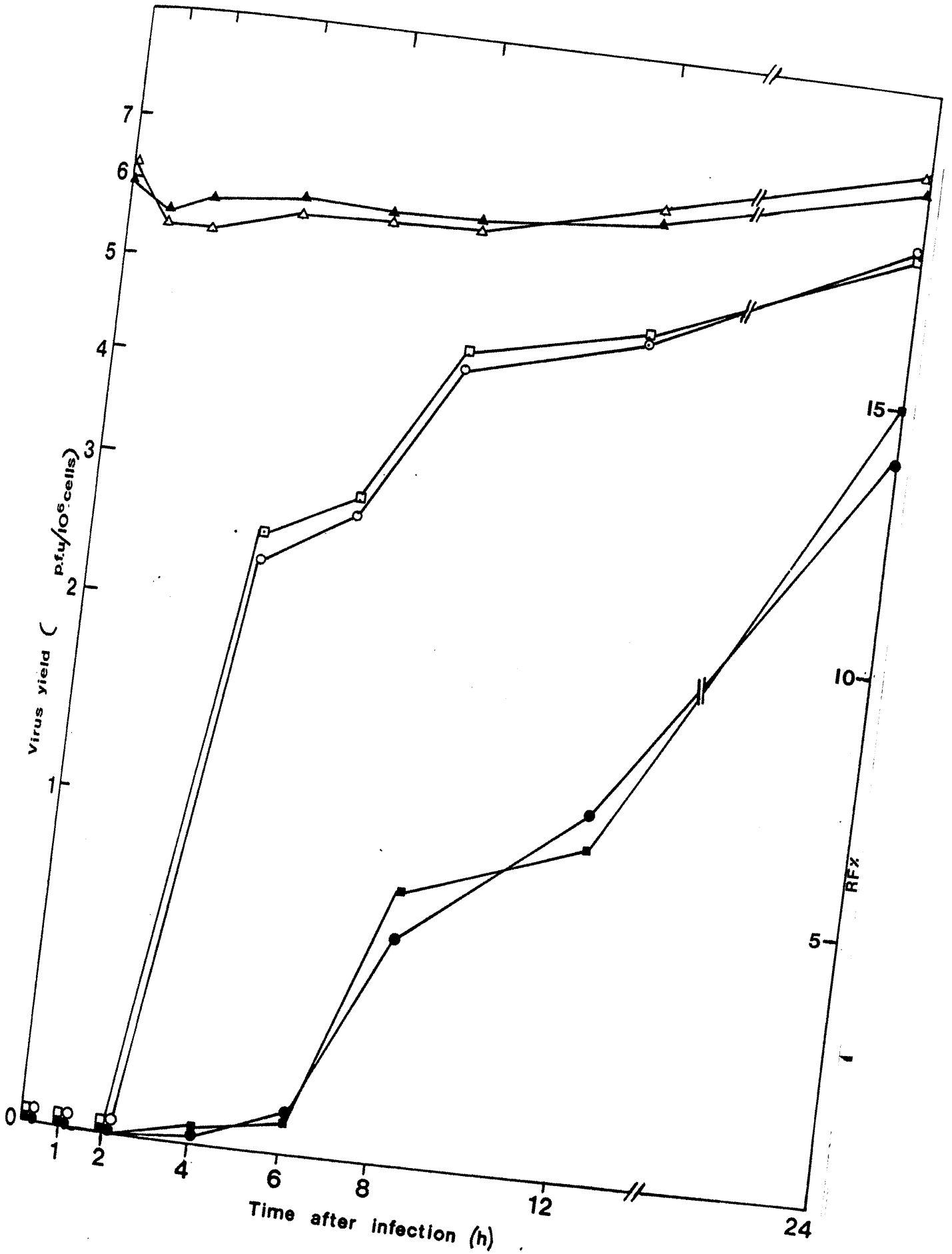
*It is possible, especially at early time points when recombination is low, that some of the viruses from each timepoint may be clonally related.

and 38.5°C (non-permissive temperature). The 31°C titre represents the total yield of progeny virus, and the 38.5°C titre the number of recombinant non-ts viruses. The recombination frequency was calculated for each time-point, as described in section II.15 (Figure 44). This represents recombination between the ts lesion in ts1708 at 0.72 m.u. and the right ts lesion in ts1702 at about 0.414 m.u. (Figure 43). Recombinant virus was first detected at 4 h post infection, increasing with time until 24 h post infection. Likewise, the recombination frequency increases from minimal levels at 4-6 h post infection to 15% by 24 h. A control recombination between ts1201 and ts1206, the ts parents, gave an almost identical pattern of growth and recombination, indicating that the difference in Xba I sites between ts1702 and ts1708 was not obviously affecting recombination (Figure 44). Control single infections with ts1702 and ts1708 gave identical patterns of growth at 31°C, but no ts^+ virus was produced, indicating that neither ts lesion was leaky or had a detectable reversion frequency (data not shown).

To determine the distribution of the non-selected markers (Xba I sites) in those viruses which had recombined between the selected markers (ts^+ recombinants), 100 single plaques were isolated from each timepoint (4, 6, 8, 12 and 24 h) at 38.5°C.* Plate stocks were grown from the isolates, and ^{32}P in vivo labelled DNA prepared and analyzed for the distribution of Xba I sites. Because the fragments generated by Xba I are large, and cleavage at some sites difficult to determine, Xba I/Bgl II double digests were carried out. The absence (when the Xba I site is present) or presence (when

Figure 44. Recombination frequency between ts1702 and ts1708.

Yield of progeny virus from a recombination between ts1702 and ts1708; and ts1201 and ts1206. Cells were infected at a multiplicity of 5 pfu/cell, each the monolayers washed twice with PBS/calf serum, overlaid with ETC10 and incubated at 31°C. Plates were harvested at 0, 1, 2, 4, 6, 8, 12 and 24 h post infection and titrated at 31°C and 38.5°C. The 31°C yields (log_{10} pfu/10^6 cells) are shown: ts1702 and ts1708 △ ; ts1201 and ts1206 ▲ ; as are the 38.5°C yields (log_{10} pfu/10^6 cells): ts1702 and ts1708 □ ; ts1201 and ts1206 ○ . The recombination frequency (%) (section III.15) for ts1702 and ts1708 ■ ,and ts1201 and ts1206 ● , are also illustrated.


Virus yield (p.f.u/10⁶ cells)
0
1
2
3
4
5
6
7
Time after infection (h)
1
2
4
6
8
12
24
5
10
15
RFX

the Xba I site is absent) of the Bgl II band spanning the Xba I sites was identified (section III.2.a, Figures 38 and 39).

As there was no evidence of leakiness or reversion of the ts lesions, all plaques isolated at 38.5°C must have recombined an odd number of times between the ts lesion at 0.414 m.u. in ts1702 and the ts lesion at 0.72 m.u. in ts1708, and contain the wild-type allele at these locations. Recombination between 0.414 and 0.72 m.u. can be divided into three regions : between the ts lesion at 0.414 m.u. and the Xba I site at 0.45 m.u.; between the 0.45 m.u. Xba I site and the 0.63 m.u. Xba I site; and between the 0.63 m.u. Xba I site and the ts lesion at 0.72 m.u. (see Figure 45 and Table 10). Recombination outwith the selected region can also be detected in three regions of the genome : between the 0.07 m.u. and the 0.29 m.u. Xba I sites; between the 0.29 m.u. Xba I site and the 0.33 m.u. ts lesion in ts1702; and between the ts lesion at 0.72 m.u. in ts1708 and the 0.74 m.u. Xba I site (Figure 45 and Table 10). No recombination between the ts lesions at 0.33 m.u. and 0.414 m.u. will be detected, as one of these ts lesions would still be present on each recombinant.

A sample of recombinants is shown in Figure 46 and the structure of their genomes and points of crossover are shown in Figure 45. An arbitrary crossover point between each set of markers has been shown, since it is not possible to further define the region of recombination. This does not imply that all recombinants within a given region have occurred at the same point. In the case of genomes such as number 8, which have the Xba I profile of ts1708, but which

Figure 45. Structures of recombinants between ts1702 and ts1708.

The position of the Xba I sites and ts lesions in ts1702 and ts1708 are illustrated at the top. (1) to (6) refer to the areas of recombination between the markers (see also Table 10). The dotted lines are the arbitrary point of recombination in these areas. Genomes 3 to 13 give the structures of the recombinant genomes shown in lanes 3 to 13 in Figure 46. The upper line refers to ts1708 and the lower line to ts1702.

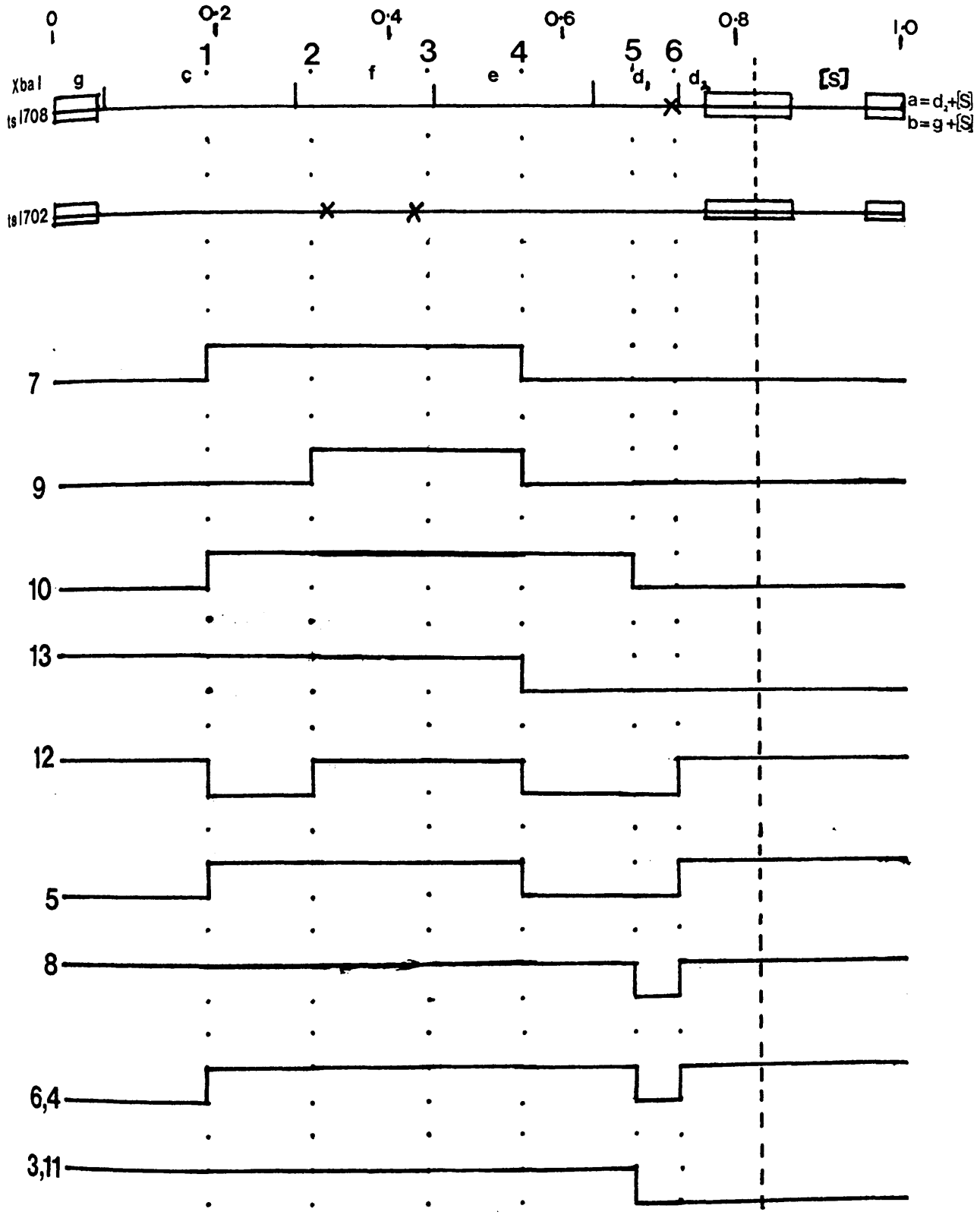

0
0·2
0·4
0·6
0·8
1·0
1
2
3
4
5
6
c
f
e
d_1
d_2
[S]
Xba I
g
ts 1708
ts 1702
a = d_2+[S]
b = g+[S]
7
9
10
13
12
5
8
6,4
3,11

Table 10 : Recombination frequency

	REGION OF RECOMBINATION$^{\phi}$					
Time (h)	1	2	3	4	5	6
4	2%* (0.06)†	2% (0.33)	0% (0)	54% (2.00)	46% (3.41)	4% (1.33)
6	16% (0.48)	4% (0.67)	0% (0)	55% (2.04)	45% (3.33)	3% (1.00)
8	17% (0.52)	6% (1.00)	0% (0)	45% (1.67)	55% (4.07)	7% (2.33)
12	36% (1.09)	7% (1.17)	0% (0)	42% (1.56)	58% (4.30)	14% (4.67)
24	49% (1.48)	11% (1.83)	0% (0)	49% (1.81)	51% (3.78)	17% (5.67)

ϕ Regions of recombination are shown in Figure 45 :

1. 0.07-0.29 m.u. (0.22 m.u. in length)
2. 0.29-0.33 m.u. (0.04 m.u. in length)
3. 0.414-0.45 m.u. (0.03 m.u. in length)
4. 0.45-0.63 m.u. (0.18 m.u. in length)
5. 0.63-0.72 m.u. (0.09 m.u. in length)
6. 0.72-0.74 m.u. (0.02 m.u. in length)

*† Recombination frequencies are expressed either as a percentage (*), or as recombination frequency per kilo base pair (†).

Figure 46. Xba I/Bgl II profile of recombinants between tsl702 and tsl708

Autoradiographs of HSV-1 DNA ^{32}P-labelled *in vivo* and digested with Xba I/Bgl II (0.5% agarose). Lane 1, *ts*l702; lane 2, *ts*l708; lanes 3 to 13, the ts^{+} recombinants between *ts*l702 and *ts*l708 illustrated in Figure 45. The nomenclature of the Bgl II bands is given in lane 1. In lanes 2 to 13, the *j*, *m*, *d*, *g* and *f* bands (containing Xba I sites) are marked if present; if absent their Xba I cleavage products are marked by the letter of the fragment from which they are derived plus a prime symbol ('). Only the easily recognizable Xba I cleavage products are marked; these allow unambiguous recognition of cleavage by Xba I and hence the presence or absence of the relevant Xba I site. The *j* and *f* containing L-S junction fragments *b* and *e*, and *a* and *c*, respectively, are not marked.

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

were established to be *ts*$^+$, recombination must have occurred within regions 5 and 6. It is possible that these could be revertants rather than recombinants, but this is unlikely, since no revertants have been found in the *ts*1708 stock to date (data not shown).

The number of genomes at each timepoint with crossovers in each region is shown in Table 10, while Table 11 shows the number of crossovers per genome at each timepoint. Tables 10 and 11 serve as a summary of the results of the recombination experiment. At 4 h post infection, most (>90%) of the recombinant viruses have only one crossover, whereas by 24 h post infection over 60% of recombinants have more than one crossover. As it seems unlikely that recombination later in infection will involve more crossovers than at early timepoints, this implies that the progeny of recombination at early timepoints, as well as the initial parent molecules, are involved in recombination at late time points.

Table 10 illustrates a number of points. Firstly, within the selected region, the frequency of recombination between the markers remains similar at different timepoints, namely around 50% each in areas 4 and 5, with no recombination at all being detected in area 3. Lack of recombination in area 3 (believed to extend from 0.33-0.45 m.u. at the time of this experiment) was what originally suggested the presence of an extra *ts* lesion in this region (III.2.b). Little conclusion can be drawn about the frequency of recombination in areas 4 and 5, as they are large (27 kbp and 13.5 kbp, respectively) and are approaching non-linkage, although recombination in area 5

Table 11 : Virus recombination - number of cross-over events as a function of time

	Number of cross-overs/genome					
Time (h)	1	2	3	4	5	6
4	92*	8	0	0	0	0
6	79	20	0	1	0	0
8	72	27	0	1	0	0
12	53	37	10	0	0	0
24	39	48	10	3	0	0

* Percentage of virus isolates with the specified number of detectable cross-over events at each time-point.

might be at a slightly higher level than expected. Because recombination in these areas is being selected, there is no increase with time.

Secondly, outwith the selected area of recombination, recombination frequency increases with time, as would be expected, reaching 49%, 11% and 17% by 24 h in areas 1, 2 and 6, respectively. The level of recombination in these regions is exceptionally high, given the frequency of recombination between the selected markers of only 15%, possibly implying a role for correct genome alignment in HSV recombination, ie. genomes recombining in one place are more likely than random to recombine elsewhere. Recombination reaches the theoretical maximum of 50% in area 1 (33 kbp long) by 24 h.

III.3 ISOLATION AND CHARACTERIZATION OF HSV-1 VARIANT 1703

During the course of experiments to isolate a virus lacking Xba I sites, over 5,000 single plaque isolates of HSV-1 were analyzed. Of these, only one gave a restriction profile on Xba I/Bgl II digestion which differed from that of X2, but had not lost either the 0.29 or 0.45 m.u. Xba I sites (data not shown). This was isolated from the same transfection which had given rise to 1701 (III.1.c). This variant was called 1703, or X2D.

III.3.a DNA profile of 1703

On Bgl II digestion of 1703 (Figure 47), the a band was absent and the f band had an altered migration (Figure 48, lane 2). In addition, a novel band of 8×10^6 mol. wt. was

Figure 47. Bgl II, Hpa I and Bam HI maps of HSV-1 strain 17.

Bgl II, Hpa I and Bam HI maps of the DNA of HSV-1 strain 17.

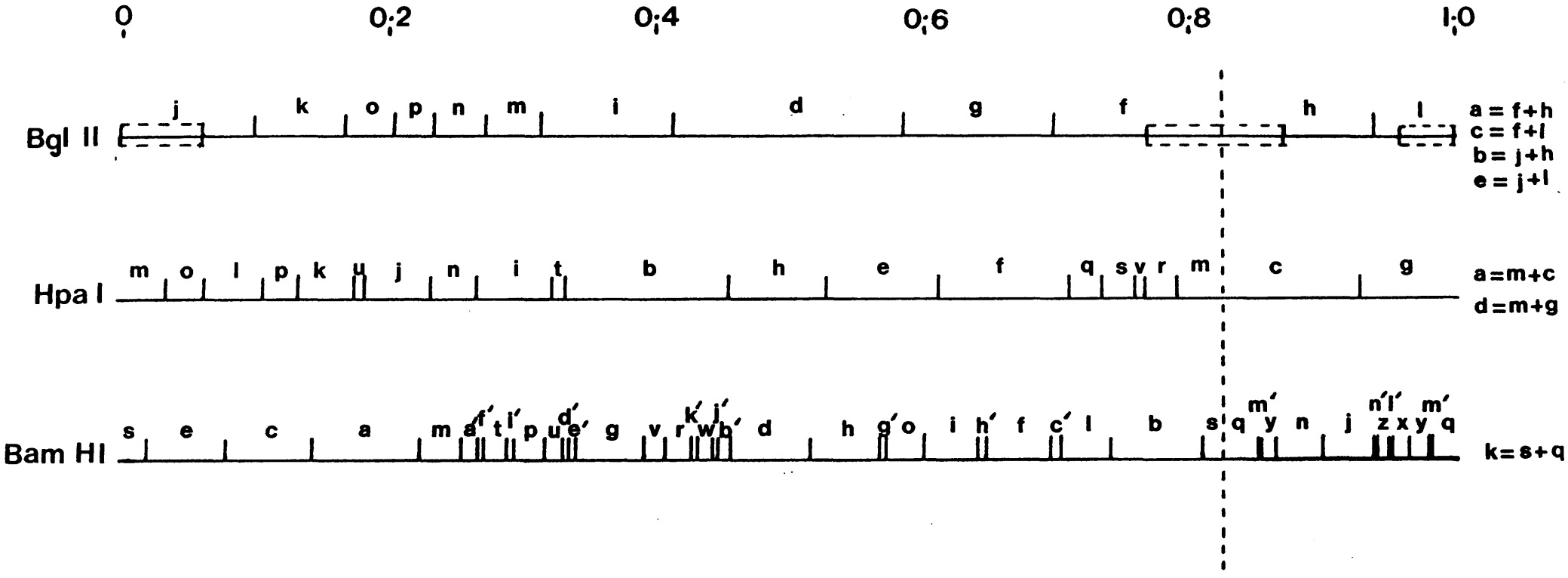
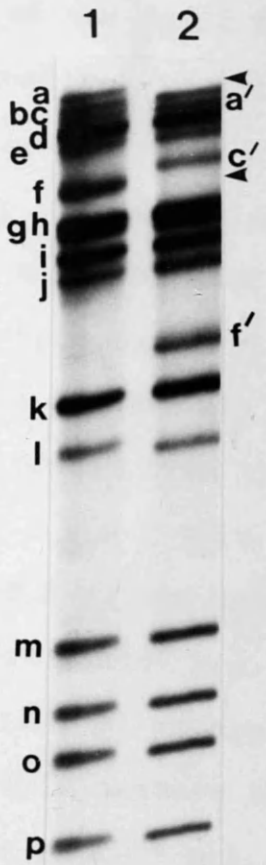

0
0;2
0;4
0;6
0;8
1;0
Bgl II
j k o p n m i d g f h l
a = f+h
c = f+l
b = j+h
e = j+l
Hpa I
m o l p k u j n i t b h e f q s v r m c g
a=m+c
d=m+g
Bam HI
s e c a m a' f' t l' p u d' e' g v r k' w j' b' d h g' o i h' f c' l b s q m' y n j n' l' z x y m' q
k=s+q

Figure 48. Bgl II profile of 1703.

Autoradiograph of a Bgl II restriction digest of HSV-1 DNA ^{32}P-labelled *in vivo* (0.5% agarose). Lane 1, strain 17; lane 2, 1703. Missing bands in 1703 are marked ▶ and novel bands are designated by the letter of the band from which they are derived plus a prime symbol (').

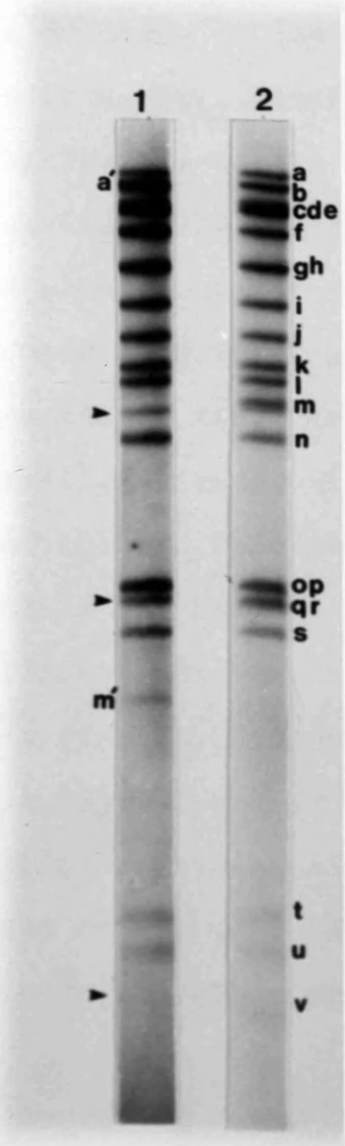

1
2
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
a′
c′
f′

present below i/j. These alterations could be explained by a deletion of around $5x10^6$ mol. wt. within IR_L/U_L, converting Bgl II f from a $13x10^6$ mol. wt. band to an f' band of $8x10^6$ mol. wt., running just below Bgl II i/j. This would result in altered migration of the joint fragments containing Bgl II f (a and c) such that Bgl II c ($19.5x10^6$ mol. wt.) would now be a c' band of $12.5x10^6$ mol. wt,. running just above the wild-type Bgl II f fragment, and Bgl II a ($24x10^6$ mol. wt.) would now run as an a' band of $19x10^6$ mol. wt., appearing in the wild-type Bgl II c position.

On Hpa I digestion (Figure 47), the profile of 1703 (Figure 49, lane 1) differed from that of wild-type strain 17 (Figure 49, lane 2) : Hpa I v is absent, Hpa I m is reduced in intensity (0.5 M) and two novel bands are present, one (0.25 M) of $11x10^6$ mol. wt., running with or just below b, and one (0.5 M) of approximately $1.7x10^6$ mol. wt., running below s. This pattern can be interpreted as follows : Hpa I m has a $1.8x10^6$ mol. wt. deletion located within IR_L sequences, therefore generating a novel 0.5 M band of $1.7x10^6$ mol. wt., whereas Hpa I m generated from TR_L is unaltered and migrates normally. Thus, joint fragments containing m, usually 0.5M, will now consist of undeleted 0.25 M copies, and deleted 0.25 M copies, a' and d', with a' running as the novel 0.25 M $11x10^6$ mol. wt. band, comigrating with b, while the d' band would run about $7.2x10^6$ mol. wt., comigrating with g/h and is therefore not detected. As Hpa I v is absent and Hpa I m deleted, the intervening fragment, r, would be expected to be absent; indeed the g/r band in 1703 is reduced in intensity in relation both to the parental X2 g/r band and to the

Figure 49. Hpa I profile of 1703.

Autoradiograph of a Hpa I restriction digest of HSV-1 DNA ^{32}P-labelled *in vivo* (0.8% agarose). Lane 1, 1703; lane 2, strain 17. Bands, in 1703, missing or reduced in intensity are marked ► and novel bands are designated by the letter of the band from which they are derived plus a prime symbol (').


1
2
a´
m´
a
b
cde
f
gh
i
j
k
l
m
n
op
qr
s
t
u
v

equivalent 2M o/p band in 1703. The data are therefore consistent with a deletion of approximately 4.9×10^6 mol. wt. at the U_L/IR_L junction.

On a Bam HI digest (Figure 47) the profile of 1703 again differed from that of X2 (Figure 50, lanes 1 and 2). The Bam HI b band (6.7×10^6 mol. wt.) is absent and a b' band now comigrates with the t band (1.9×10^6 mol. wt.), thus indicating a deletion in the order of 4.8×10^6 mol. wt. at the junction of U_L/IR_L. Southern blotting analysis confirmed that the deletion was in the order of 5×10^6 mol. wt. A Bam HI probe (position illustrated in Figure 51b) hybridized to a b' band of around 1.8×10^6 mol. wt. in 1703 compared to the wild-type b band at 6.7×10^6 mol. wt. (data not shown). To map the end-point of the deletion within U_L more precisely, a probe, pMC9(1), corresponding to the unique portion of Bam HI b (Figure 51b) was hybridized to X2 (Figure 52, lane 1) and 1703 (Figure 52, lane 2) DNA cleaved with Bam HI/Sma I/Hpa I. The Sma I fragments from Hpa I s (Perry, 1986) are all present, (861bp, 675bp, 344bp and 297bp) indicating that the deletion does not extend past the Hpa I s/v junction, but Hpa I v (1240 bp) is absent from 1703. As fragments below about 200 bp will run off the bottom of the gel, the deletion extends at least as far as 200 bp to the right of the Hpa I s/v junction (Figure 51).

III.3.b Immediate-early polypeptide profile of 1703

The deletion in 1703 removes the 3' portion of IE gene 1 located in IR_L and terminates at least 500 bp downstream from the 3' end of the mRNA for IE gene 2 (Figure 51a and 51c). Immediate-early polypeptides were prepared as

Figure 50. Bam HI profile of 1703.

Autoradiograph of a Bam HI restriction digest of HSV-1 DNA ^{32}P-labelled *in vivo* (0.8% agarose). Lane 1, strain 17; lane 2, 1703. Missing bands in 1703 are marked ► and novel bands are designated by the letter of the band from which they are derived plus a prime symbol (').

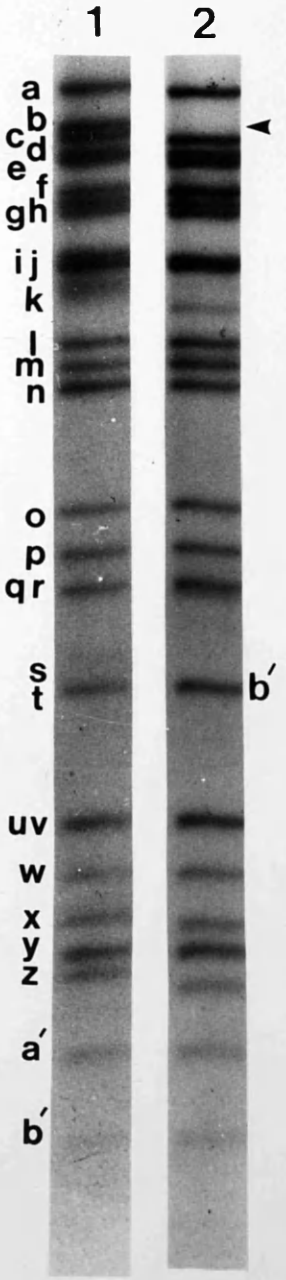

1
2
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
uv
w
x
y
z
a′
b′
b′

Figure 51. Map of deletion in 1703.

a) Map of the Bam HI (B) b and s fragments situated within U_L/IR_L up to the IR_L/IR_S joint, showing the Hpa I (H) sites. Hpa I s' is the part of Hpa I s contained within Bam HI b. The diagram also shows the positions of the genes encoding VmwIE110 and VmwIE63, and unidentified open reading frames U_L55 and U_L56 encoding predicted polypeptides of 20,000 and 22,000 mol. wt. and the extent of the $5x10^6$ mol. wt. deletion in 1703.

b) Position of the Bam HI b and pMC9(1) probes on the genome.

c) An enlarged diagram of the Hpa I v and s' regions showing the sizes in base pairs of fragments generated upon digestion with Bam HI (B)/Hpa I (H)/Sma I (S) which hybridize to pMC9(1) (see Figure 52).

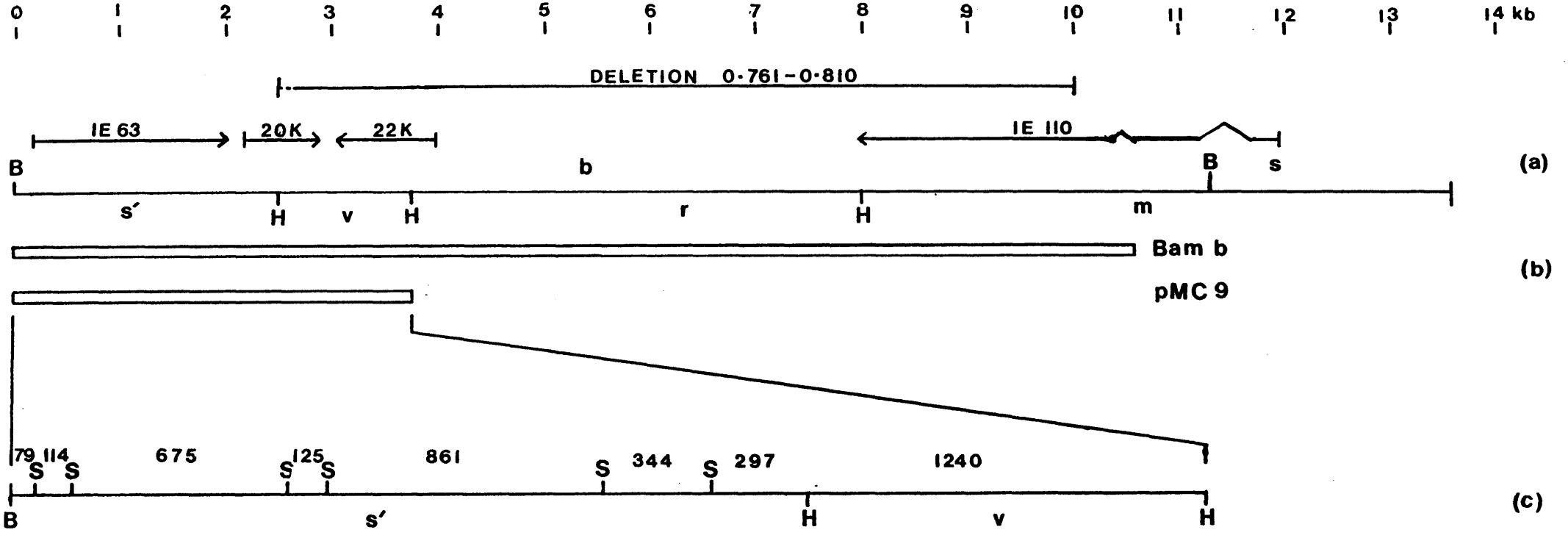
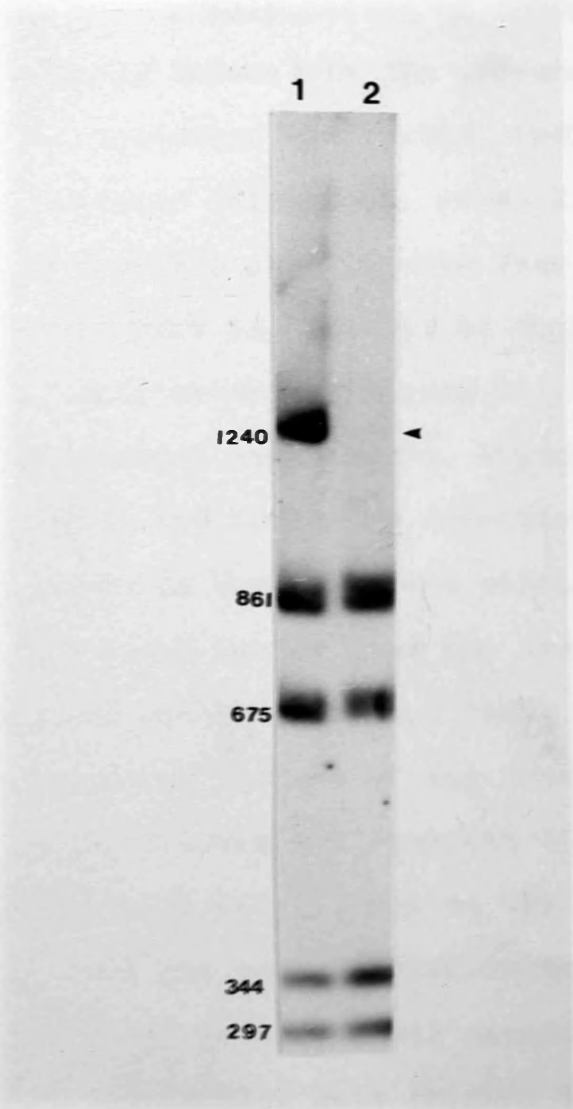

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 kb
DELETION 0·761–0·810
IE 63
20K
22K
IE 110
B
b
B
s
(a)
s′
H
v
H
r
H
m
Bam b
(b)
pMC 9
79 114
675
125
861
344
297
1240
S
S
S
S
S
S
B
s′
H
v
H
(c)

Figure 52. Southern blot of 1703 DNA using the pMC9(1) probe.

Autoradiograph of a Southern blot in which nick-translated pMC9(1) (see Figure 51b) was hybridized to X2 (lane 1) and 1703 (lane 2) DNA which had been digested with Bam HI/Hpa I/Sma I (1.5% agarose). For the location of the bands in the genome see Figure 51c. The missing band in 1703 is marked ► . Sizes of the bands are in base pairs on the left hand side.


1
2
1240
861
675
344
297

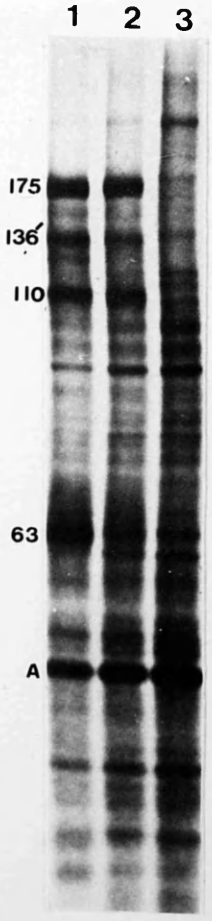
described in method section II.34, and analyzed by SDS-PAGE. VmwIE110 was detected in 1703 and X2 infected cells (Figure 53). Using an antiserum raised against a synthetic peptide corresponding to the carboxy-terminus of VmwIE110, a reduction in the level of VmwIE110 in 1703, as compared to X2, infected cells was seen (Figure 54). The antiserum specifically precipitated a polypeptide of apparent mol. wt. $110x10^3$ and several additional polypeptides (Figure 54, lanes 5, 6) - precipitation of these polypeptides was significantly reduced in the presence of the peptide against which the antiserum was raised, indicating the specificity of the reaction (Figure 54, lanes 2, 3). A reduction in the level of VmwIE110 precipitated from 1703 infected cell extracts (Figure 54, lane 5) as compared to control X2 infected cell extracts (Figure 54, lane 6) was consistently found in several experiments. Figures 53 and 54 show the results obtained following infection of HFL cells, but similar results were obtained using BHK21 C13 cells, although in the latter case the level of background host proteins tended to be higher (data not shown).

A striking feature of the immediate-early polypeptide profile of 1703 was the apparent lack of VmwIE63 (Figure 53, compare lanes 1 and 2). Due to the large number of host proteins and the poor resolution in this region on gradient gels, the same infected cell extracts were analyzed on 7.5% single concentration gels (Figure 55). Again, the results suggest that VmwIE63 is greatly reduced in amount following infection with 1703.

In Figures 53 and 55, VmwIE68 is apparently absent - this is due to the fact that it labels poorly with

Figure 53. Immediate-early polypeptide profile of 1703.

Immediate-early polypeptide extracts, labelled with [^{35}S]-methionine, of X2 infected (lane 1), 1703 infected (lane 2) and mock infected (lane 3) HFL cells, run on a 5-12.5% gradient polyacrylamide gel. Molecular weights (x 10^{-3}) are given on the left hand side. A is actin.


1
2
3
175
136
110
63
A

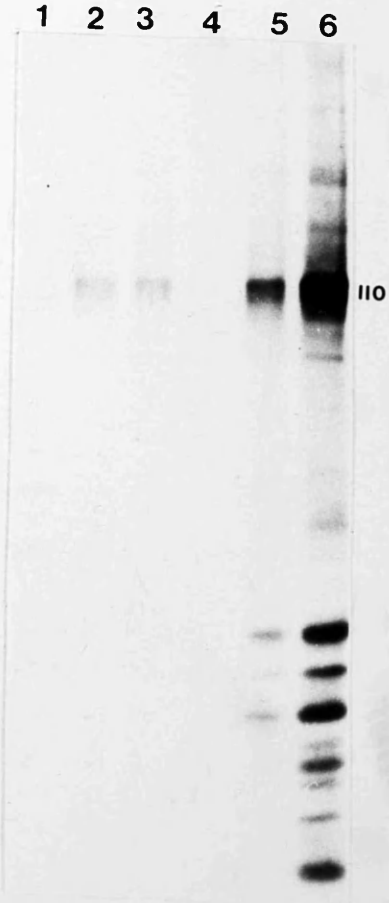
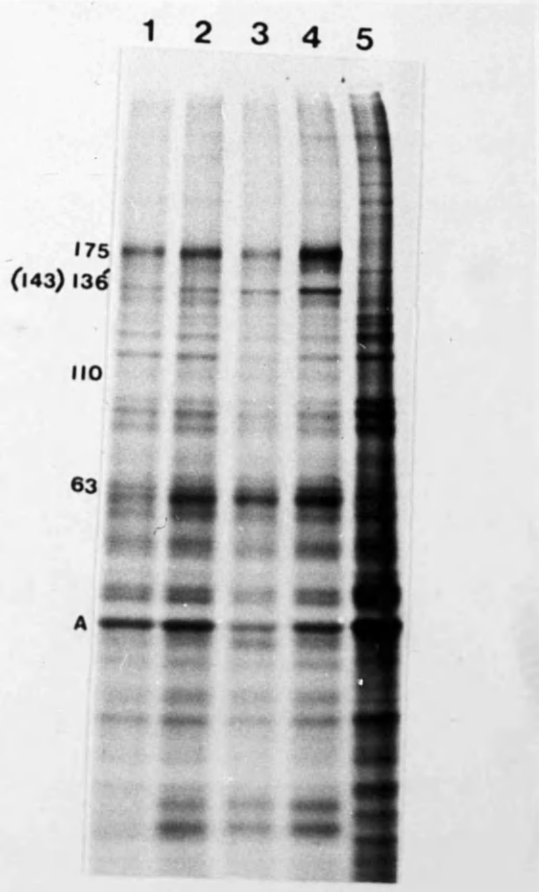

1 2 3 4 5 6
110

Figure 54. Immunoprecipitation of VmwIE110.

Immunoprecipitation using an antiserum raised against the carboxy-terminus of VmwIE110. Immediate-early polypeptides labelled with [^{35}S]-methionine were precipitated from mock infected (lanes 1 and 4), 1703 infected (lanes 2 and 5) and X2 infected (lanes 3 and 6) HFL cells. The immunoprecipitations were carried out in the presence (lanes 1, 2 and 3) or absence (lanes 4, 5 and 6) of the peptide against which the antiserum was raised. The specificity of the precipitation of VmwIE110 and lower molecular weight bands is demonstrated by inhibition by the peptide. The samples were analyzed on a 5-12.5% gradient polyacrylamide gel.

Figure 55. Immediate-early polypeptide profile of 1703.

Immediate-early polypeptide extracts, labelled with [^{35}S]-methionine, of 1703 infected (lane 1), X2 infected (lanes 2 and 4), strain 17 infected (lane 3) and mock infected (lane 5) HFL cells, run on a 7.5% polyacrylamide gel. Molecular weights (x 10^{-3}) are given on the left hand side. A is actin.


1
2
3
4
5
175
(143) 136
110
63
A

[^{35}S]-methionine, and that it is unstable under the long labelling conditions used (Dr. C. M. Preston, personal communication).

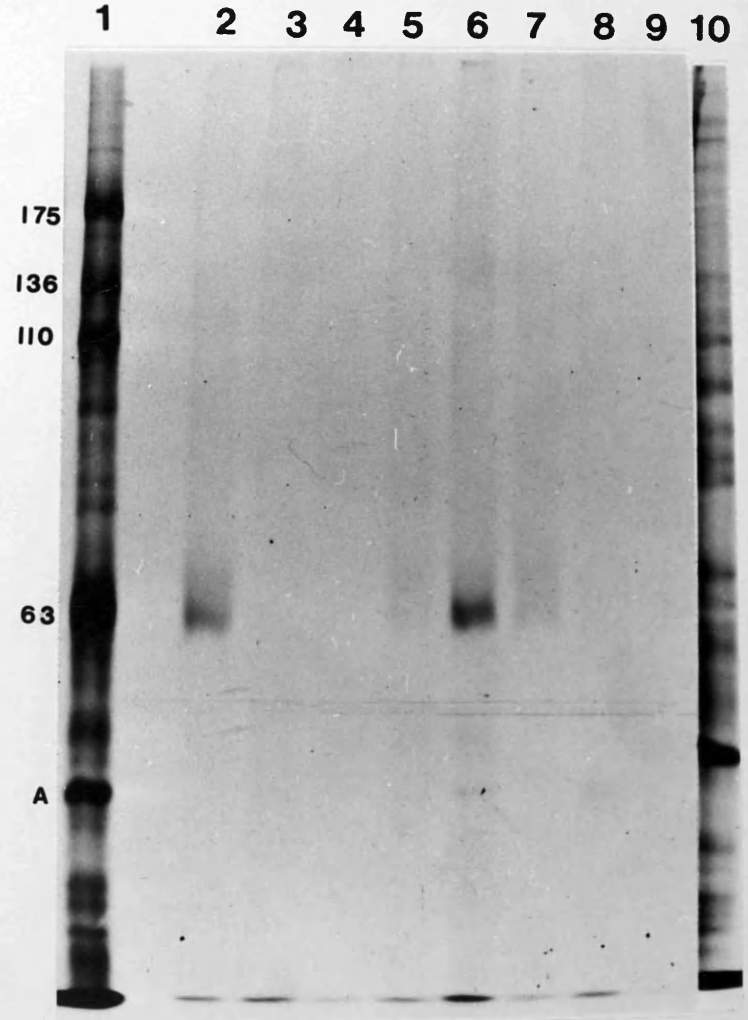
To further investigate the apparent reduction in the level of VmwIE63 following infection with 1703, an anti-peptide serum raised against the amino-terminus of VmwIE63 was used. This antiserum specifically precipitated a polypeptide of apparent mol. wt. $63x10^3$ (Figure 56, lane 2) - the presence of the relevant peptide significantly reducing the level of precipitation of this protein (Figure 56, lane 5). A wild-type strain 17 extract was serially diluted four fold to determine the sensitivity of this assay. VmwIE63 could still be detected at a 1:16 dilution (Figure 56, lane 7) and, on longer exposure of the gel, at a 1:64 dilution (Figure 56, lane 8, and data not shown). In contrast, no detectable VmwIE63 was precipitated from 1703 infected or mock infected cell extracts (Figure 56, lanes 3 and 4, respectively) even on longer exposures of the gel, suggesting that 1703 immediate-early extracts lack VmwIE63 (or contain less than 2% of wild-type levels).

III.3.c Immediate-early RNA profile of 1703

The level of virus-specific immediate-early RNA present in 1703 infected cells was examined. Radio-labelled, cytoplasmic immediate-early RNA was prepared and hybridized to Southern blots of Hpa I digested HSV DNA, as described in section II.11 (Figure 57). In the strain 17 control (Figure 57, lane 2) IE1 RNA should hybridize to Hpa I *m*, *a* and *d*, IE3 RNA hybridized to Hpa I *c*, *g*, *a* and *d*, IE4 RNA hybridized to Hpa I *c* and *a*, IE5 RNA hybridized to Hpa I *g* and *d* bands and IE2

Figure 56. Immunoprecipitation of VmwIE63.

Immunoprecipitation using an antiserum raised against the amino-terminus of VmwIE63. Polypeptides prepared under immediate-early conditions and labelled with [^{35}S]-methionine were precipitated from strain 17 infected (lanes 2 and 5), 1703 infected (lane 3) and mock infected (lane 4) HFL cells. The specificity of the precipitation of VmwIE63 is demonstrated by its inhibition in the presence of peptide (lane 5). Lanes 6 to 9 are dilutions of strain 17 extract with mock infected extract: lane 6, 1 in 4; lane 7, 1 in 16; lane 8, 1 in 64; and lane 9, 1 in 128. Lane 1 is strain 17 infected whole cell extract and lane 10 is mock infected whole cell extract. The samples were run on a 7.5% polyacrylamide gel. Molecular weights (x 10^{-3}) are given on the left hand side. A is actin.


1
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5
6
7
8
9
10
175
136
110
63
A

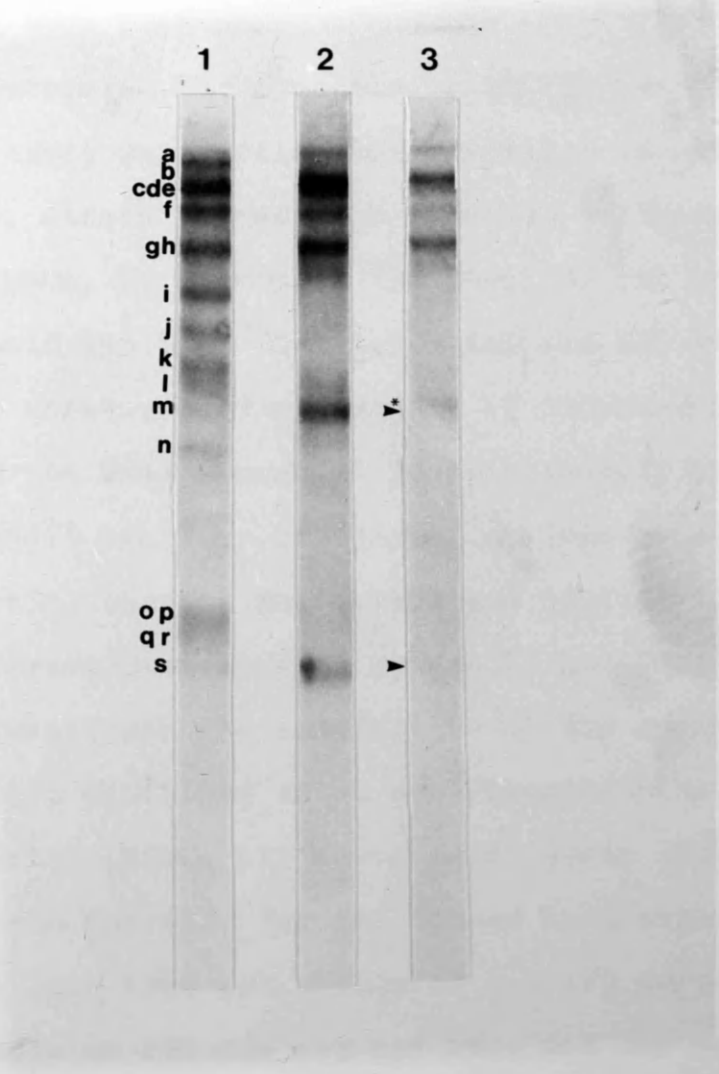

1
2
3
a
b
cde
f
gh
i
j
k
l
m
n
o p
q r
s

Figure 57. Immediate-early RNA profile of 1703.

Autoradiograph of a Southern blot of Hpa I digested HSV-1 strain 17 DNA (0.8% agarose) to which ^{32}P-labelled *in vivo* immediate-early RNA from strain 17 infected (lane 2) and 1703 infected (lane 3) cells and nick translated strain 17 DNA (lane 1) was hybridized. The band absent in 1703 is marked ► , and that reduced in intensity ➤* .

RNA hybridized to Hpa I s. This was the profile expected from the location of these IE genes (Figure 58; Clements et al., 1979). X2 IE RNA produced an identical profile (data not shown). In contrast, 1703 IE RNA (Figure 57, lane 3) failed to hybridize to Hpa I s. Thus, in 1703 extracts IE2 specified mRNA cannot be detected. Also, less 1703 RNA hybridized to Hpa I m than in strain 17, indicating a reduced amount of IE1 RNA, in agreement with the reduction in VmwIE110. Despite lower levels of hybridization to the 1703 lane, hybridization to Hpa I s would still be expected to be detected if IE mRNA levels comparable to the wild-type were present.

To determine the sensitivity of IE2 RNA detection, a dot blot assay was carried out. Labelled IE RNA ($2x10^6$ cpm) from 1703, strain 17 and mock infected cells was hybridized to : (i) pMC9, to determine the level of IE2 RNA present; (ii) plasmid Kpn I k, containing IE3 and IE5 genes, to determine whether 1703 and strain 17 infected cells contained the same amount of immediate-early RNA; and (iii) plasmid Kpn I m, containing no immediate-early genes, as a negative control for background hybridization (Figure 58). Different dilutions of strain 17 and 1703 IE RNA were used to quantitate the sensitivity of the assay, the input counts being equalized using mock infected RNA. The radioactivity (Table 12) above mock levels indicates the level of RNA specific for the cloned fragments. Table 12 indicates that 1703 and strain 17 specify approximately equal levels of IE3 RNA and IE5 RNA, and the sensitivity is such that 1703 specifies no more IE2 RNA than mock infected cells. At dilutions down to and including 1:16, strain 17

Figure 58. Immediate-early gene locations on a Hpa I map.

Hpa I map of HSV-1 strain 17 DNA, showing the locations of the immediate-early transcripts and the plasmids, Kpn I m, Kpn I k and pMC9, used in Table 12.

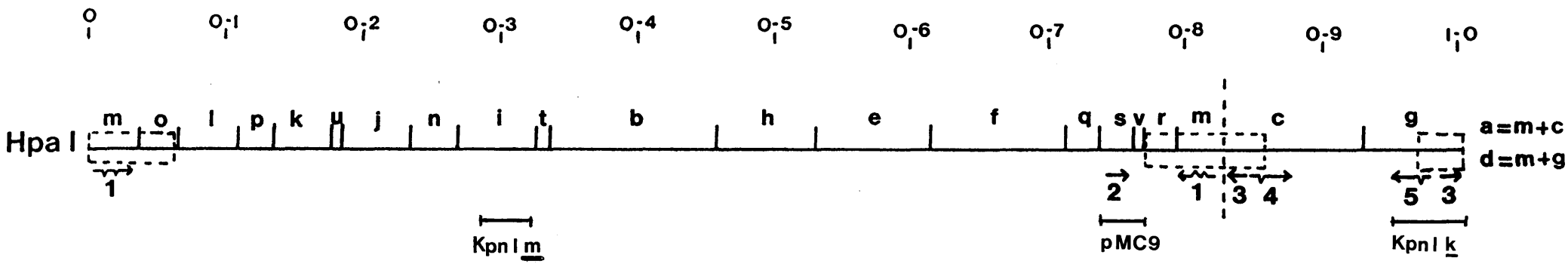

0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Hpa I
m o l p k u j n i t b h e f q s v r m c g
a=m+c
d=m+g
1
2
1
3 4
5 3
Kpn I m
pMC9
Kpn I k

Table 12 : Sensitivity of detection of IE2 RNA

	RNA														
	Mock	1703							strain 17						
Dilution (fold)	0	0	2	4	8	16	32	64	0	2	4	8	16	32	64
Plasmid used															
pMC9 (IE2)	41*	37	30	34	32	36	29	28	295	257	181	141	78	33	37
Kpn I k (IE3 + IE5)	36	309	320	341	230	190	109	65	321	301	310	271	202	125	71
Kpn I m (no IE)	26	39	25	31	38	30	33	29	33	39	31	42	31	34	38

* cpm bound to filter. Input virus (or mock infected cell) RNA was 2×10^6 cpm.

contained detectable levels of IE2 RNA, however, at a 1:32 dilution hybridization levels were comparable to those of 1703 and mock infected cell RNA. The conclusion of this experiment is that 1703 extracts contain at most 1:16 (6·25%) of the wild-type level of IE2 RNA.

III.3.d General polypeptide profile of 1703

The general infected cell polypeptide profile of 1703 was compared with that of strain 17 and X2 (Figure 59). The profile of 1703 infected cell extracts (lanes 3 and 4) differed from that of strain 17 (lane 5) and X2 (lanes 1 and 2) infected cell extracts, in that the 43K tk polypeptide was absent (see below). Apart from this, the polypeptide profile of 1703 was identical to that of strain 17 and X2.

The tk^- phenotype of 1703 was confirmed by BCdR yield experiments (method section II.13). Twenty-four hour yield experiments with strain 17, X2 and 1703 were carried out in the presence and absence of BCdR, and the progeny virus titrated. (see after p149) The results (Table 13) show that while the yield of strain 17 and X2 is markedly reduced by BCdR, there is little effect on the yield of 1703. Thus, 1703 is tk^-.

The mutation leading to the tk^- phenotype of 1703 was believed to be a secondary mutation, unrelated to the deletion in IR_L/U_L, and/or the failure to synthesize VmwIE63. This was confirmed by the construction of a tk^+ derivative of 1703, which still contains the deletion and does not synthesize VmwIE63 (data not shown).

III.3.e Growth properties of 1703

A one-step growth experiment in BHK21 C13 cells, over a

Figure 59. General polypeptide profile of 1703.

General infected cell polypeptide extracts labelled with [^{35}S]-methionine from 4 to 24 h post infection, run on a 5-12.5% gradient polyacrylamide gel. Molecular weights (x 10^{-3}) are given on the left hand side. A is actin. Lanes 1 and 2, X2 infected; lanes 3 and 4, 1703 infected; lane 5, strain 17 infected; and lane 6, mock infected cell extracts.

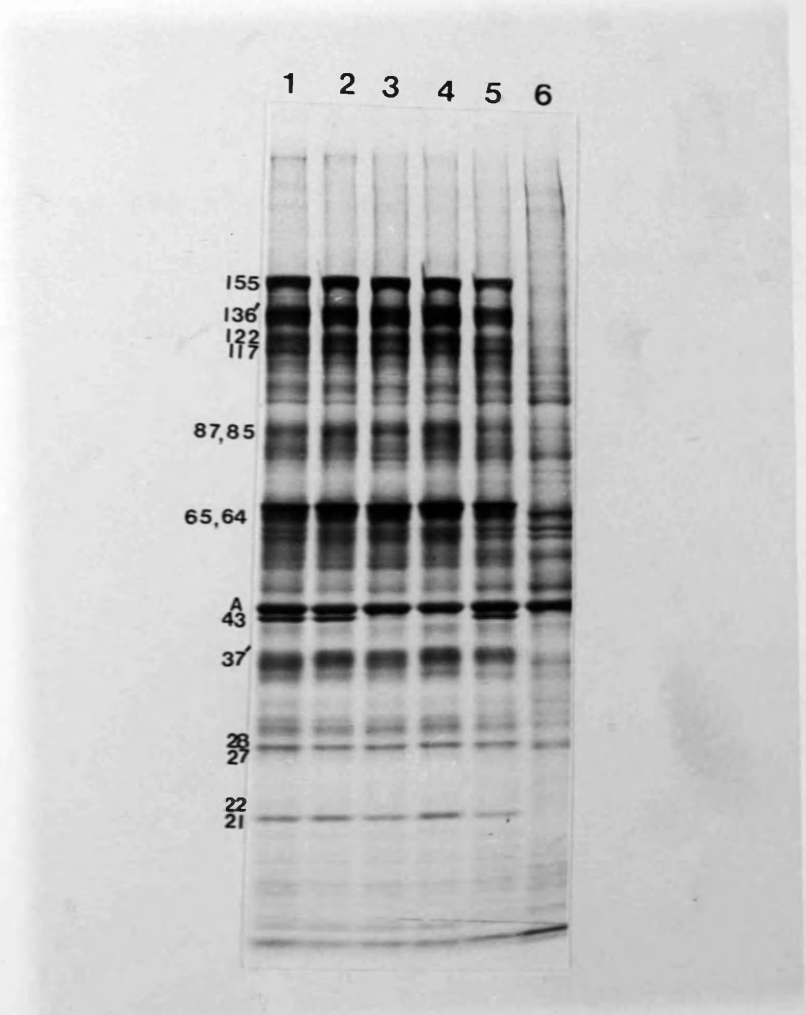

1
2
3
4
5
6
155
136
122
117
87,85
65,64
A
43
37
28
27
22
21

Table 13 : BCdR sensitivity of 1703

Virus	Yield* +BCdR	Yield* -BCdR
strain 17	1.9×10^5	3.2×10^8
X2	3.3×10^5	2.1×10^8
1703	3.2×10^8	3.6×10^8

* expressed as the virus titre per 4×10^6 BHK21 C13 cells, following infection for 24 h in the presence or absence of BCdR at 100ug/ml.

24 h period, showed no marked difference in growth properties between 1703, X2 and strain 17 (Figure 60). The lag period of 1703 appears slightly longer than that for X2 and strain 17, but as this is a very variable property, the significance is uncertain (Dr. D. Dargan, personal communication). 1703, X2 and strain 17 also show almost identical growth characteristics over several rounds of replication (Figure 61).

Sacks et al. (1985) have identified ts mutants in VmwIE63, suggesting that this protein is essential for growth in tissue culture. These authors used Vero cells, whereas here BHK21 C13 cells or HFL cells were used. To determine the viability of 1703 in Vero cells, 24 h yield experiments were carried out in both Vero and BHK21 C13 cells with strain 17, X2 and 1703, and the resultant progeny virus was titrated on both cell types. Table 14 shows that the yield of the three viruses obtained from the two cell types was very similar and the titres measured on the two cell lines were essentially identical.

III.4 HSV-1 VARIANTS CONTAINING ALTERATIONS WITHIN OR NEAR THE LONG REPEAT

From a recombination experiment designed to generate a HSV-1 genome containing 5 Xba I sites (see section III.2.a), 14 variants, out of a total of 80 plaque isolates isolated, gave an aberrant profile on restriction enzyme analysis. None of the 80 isolates appeared to be recombinants - 48 showing the Xba I profile of strain 17 and 32 showing the Xba I profile of 1707. However all of the 14 variants,

Figure 60. One-step growth curve of 1703.

One-step growth curves of HSV-1 strain 17■ , X2● and 1703▲ in BHK21 C13 cells. Cells were infected at a multiplicity of 5 pfu/cell. After absorption for 60 min at 37°C, the monolayers were washed twice with PBS/calf serum, overlaid with ETC10 and incubated at 37°C. Plates were harvested at 0, 2, 4, 6, 8, 12 and 24 h post infection and titrated as normal.

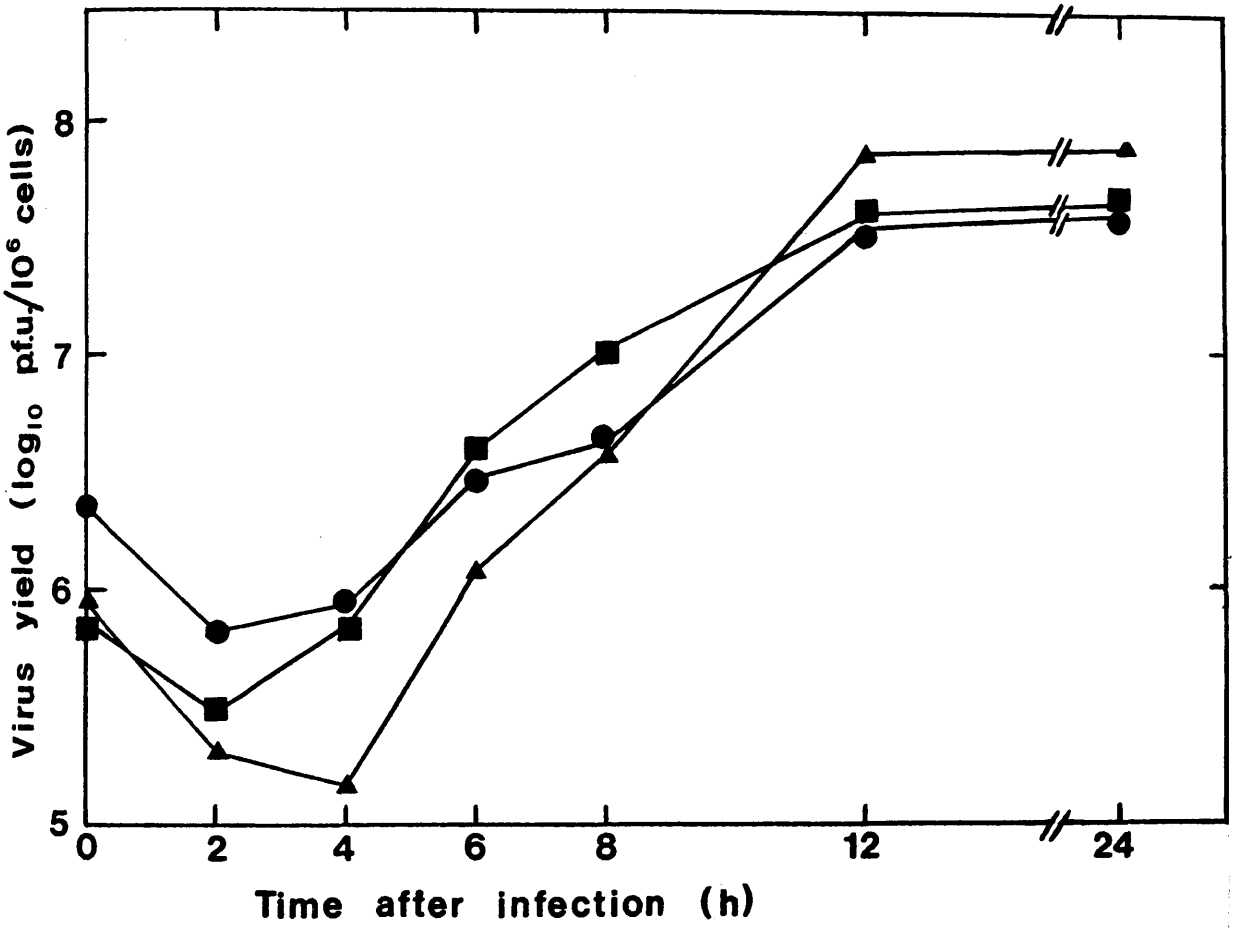

Virus yield (log_{10} p.f.u./10^6 cells)
8
7
6
5
0
2
4
6
8
12
24
Time after infection (h)

Figure 61. Long-term growth curve of 1703.

Long-term growth curves of HSV-1 strain 17■ , X2● and 1703▲ in BHK21 C13 cells. Cells were infected at a multiplicity of 0.001 pfu/cell. After absorption for 60 min at 37°C, the monolayers were washed twice with PBS/calf serum, overlaid with ETC10 and incubated at 37°C. Plates were harvested at 0, 2, 4, 12, 24, 48 and 72 h post infection and titrated as normal.

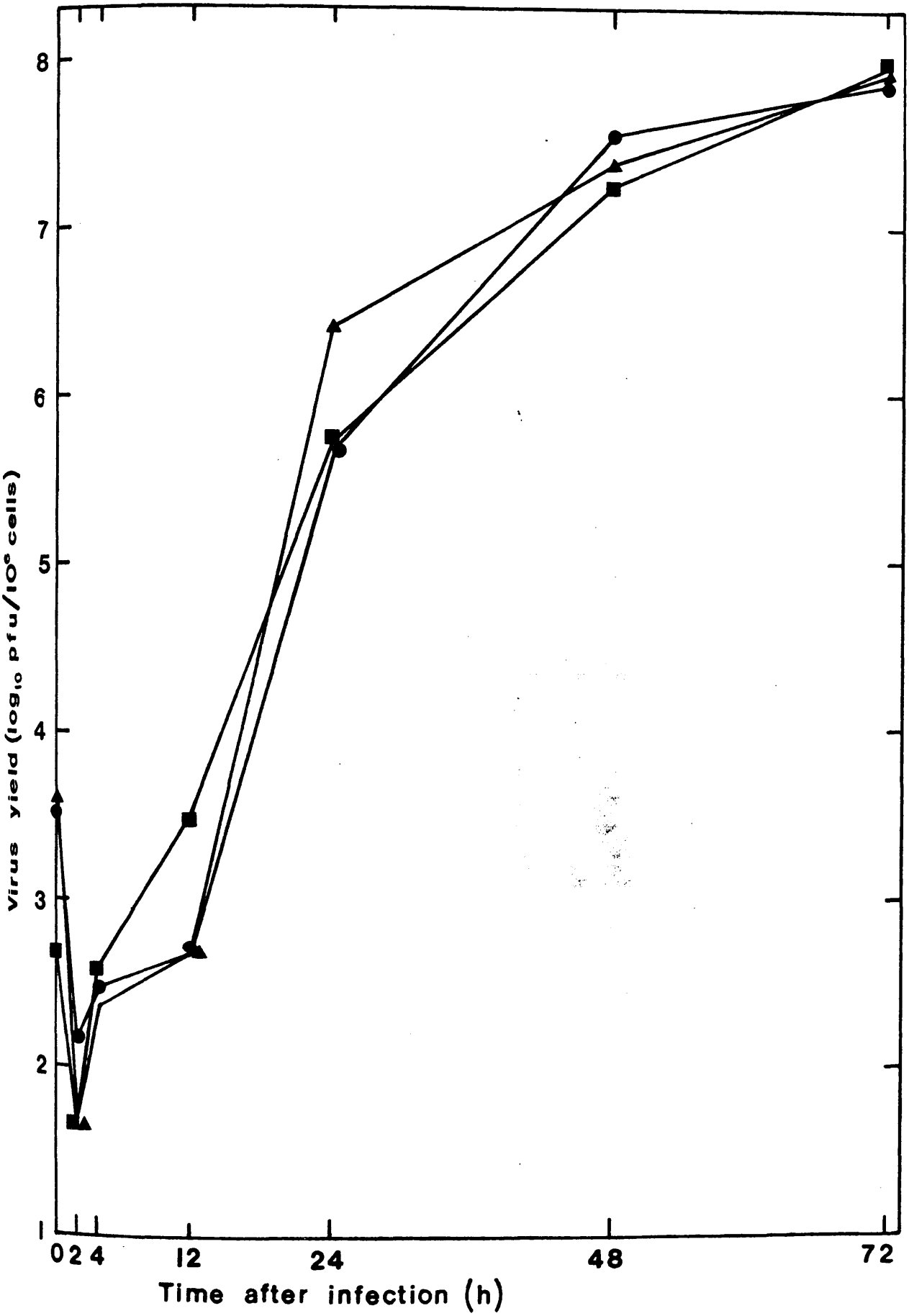

Virus yield (log_{10} pfu/10^5 cells)
8
7
6
5
4
3
2
1
0 2 4
12
24
48
72
Time after infection (h)

Table 14 : Virus yields in BHK21 C13 and Vero cells

Cells grown in	:	VERO	VERO	BHK21 C13	BHK21 C13
Cells titrated in	:	VERO	BHK21 C13	VERO	BHK21 C13
Virus - strain 17	:	1.8×10^{8}*	2.0×10^{8}	4.8×10^{8}	5.0×10^{8}
X2	:	2.1×10^{8}	1.9×10^{8}	4.3×10^{8}	4.2×10^{8}
1703	:	1.9×10^{8}	1.7×10^{8}	4.5×10^{8}	4.3×10^{8}

* Virus titre, expressed as yield per 4×10^{6} cells, following a 24 h infection.

although not containing identical deletions, had the Xba I profile of strain 17. This might suggest that these variants are clonally related. Furthermore, over the course of this study more than 5,000 single plaque isolates have been analyzed (including another 750 in similar recombination experiments), and only one other deletion variant (1703, section III.3) had previously been detected.

III.4.a Isolation of several variants in TR_L/IR_L

From the 80 plaques analyzed, three demonstrated large deletions on Xba I digestion : these variants were designated 1704, 1705 and 1706 (see III.4.b, III.4.c, III.4.d, respectively). Digestion with Hpa I indicated that in a further 11 plaque isolates Hpa I o and/or r fragments varied in size by up to 0.4×10^6 mol. wt. from the wild-type fragments (Figures 62 and 63). Hpa I p and q, located within U_L, comigrate, respectively, with Hpa I o and r, located almost entirely within the long repeats (see Figures 62 and 63). Thus, Hpa I digests were probed with cloned Hpa I r to confirm that the variable fragments were indeed Hpa I o and r (Figures 62 and 64). These differences represent, respectively, variations in regions of TR_L and IR_L distal from the 'a' sequence. Minor variation, in the order of 50 bp, is sometimes seen with these fragments (Lonsdale et al., 1980; own unpublished observations). However, here the variation is considerably more extensive, in some cases up to 600 bp (Figure 63, lane 4). Variation in Hpa I o and r occurs independently. For example, in one isolate, Hpa I o is increased in size while Hpa I r is decreased in size (Figure 63, lane 7). Thus, in these genomes TR_L and IR_L

Figure 62. Bgl II, Hpa I and Bam HI maps of HSV-1.

Bgl II, Hpa I and Bam HI maps of the DNA of HSV-1 strain 17.

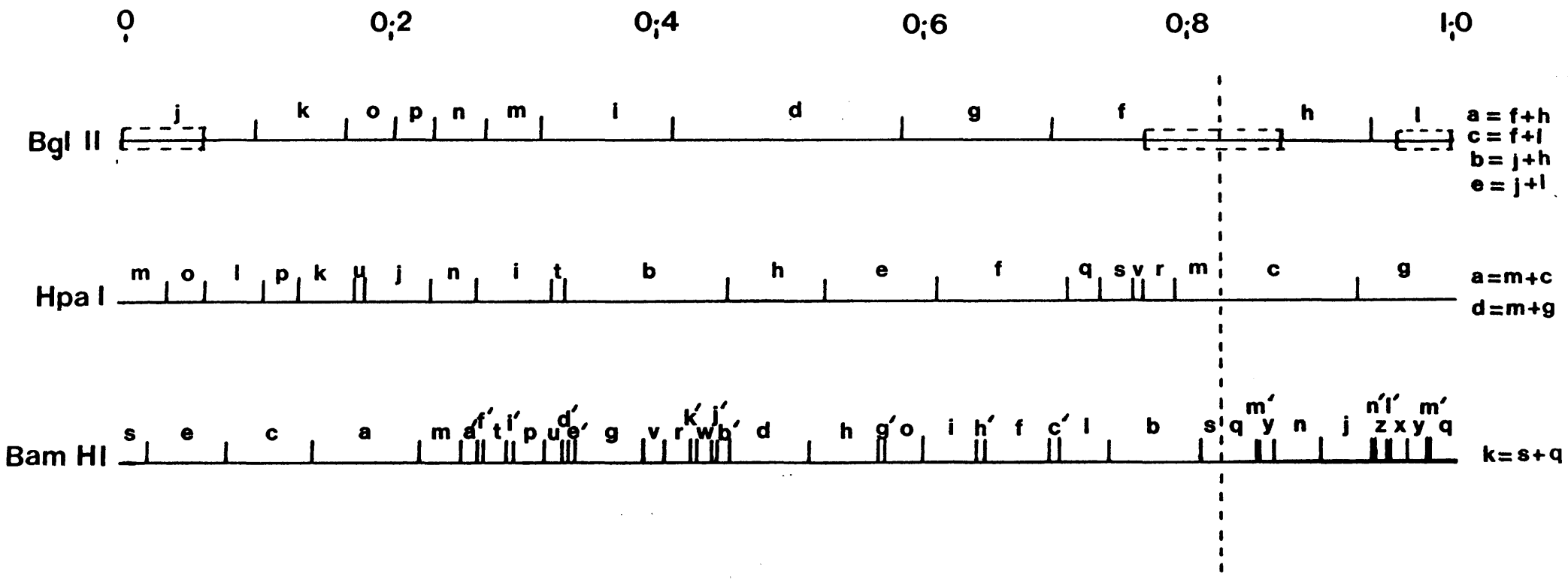
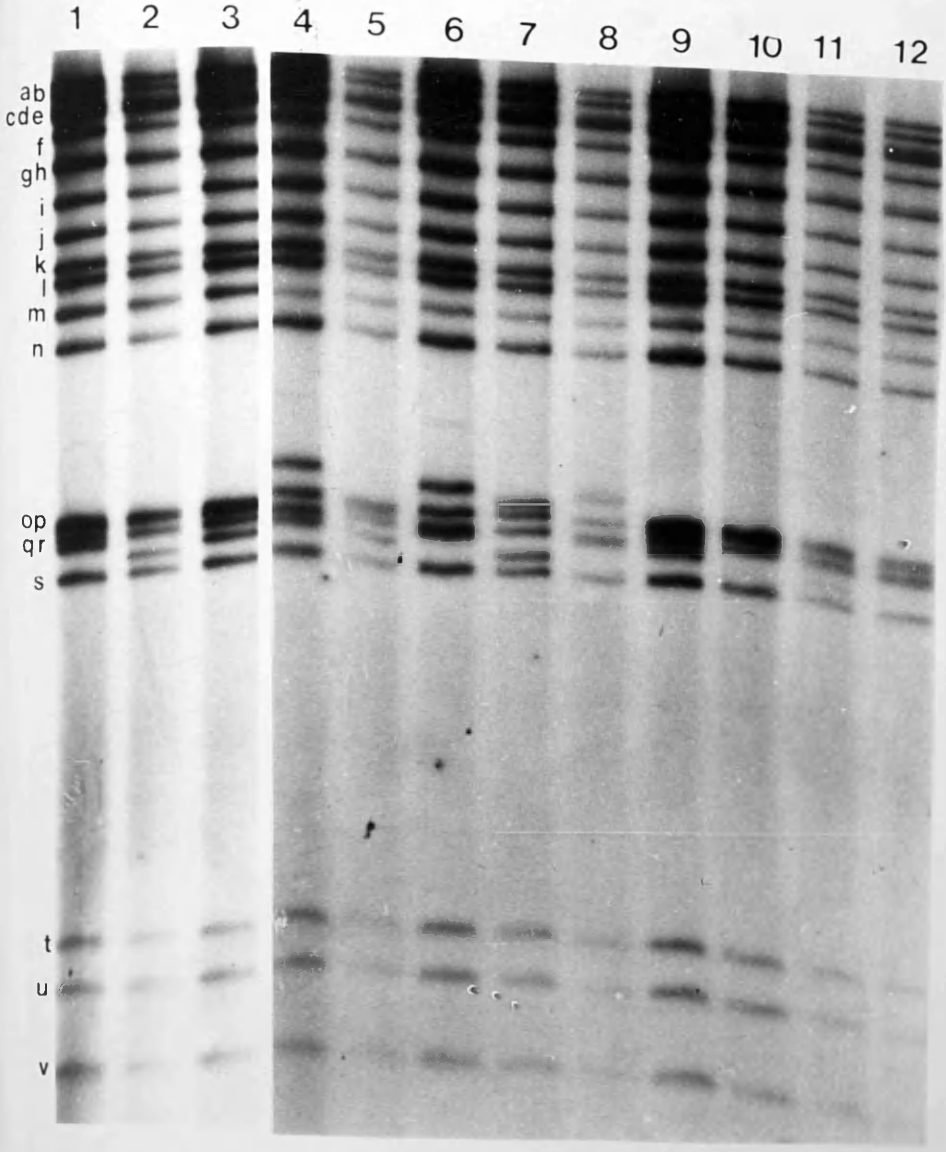

0
0·2
0·4
0·6
0·8
1·0
Bgl II
j k o p n m i d g f h l
a = f+h
c = f+l
b = j+h
e = j+l
Hpa I
m o l p k u j n i t b h e f q s v r m c g
a=m+c
d=m+g
Bam HI
s e c a m a f t l p u d e g v r k w j b d h g o i h f c l b s q m y n j n l m z x y q
k=s+q

Figure 63. Hpa I profile of variants in TR_L/IR_L.

Autoradiograph of Hpa I restriction digests of HSV-1 DNA ^{32}P-labelled *in vivo* (0.8% agarose). Lanes 1 to 11 are different plaque isolates of strain 17 exhibiting variation in Hpa I *o* and/or *r*; lane 12 is strain 17.


1
2
3
4
5
6
7
8
9
10
11
12
ab
cde
f
gh
i
j
k
l
m
n
op
qr
s
t
u
v

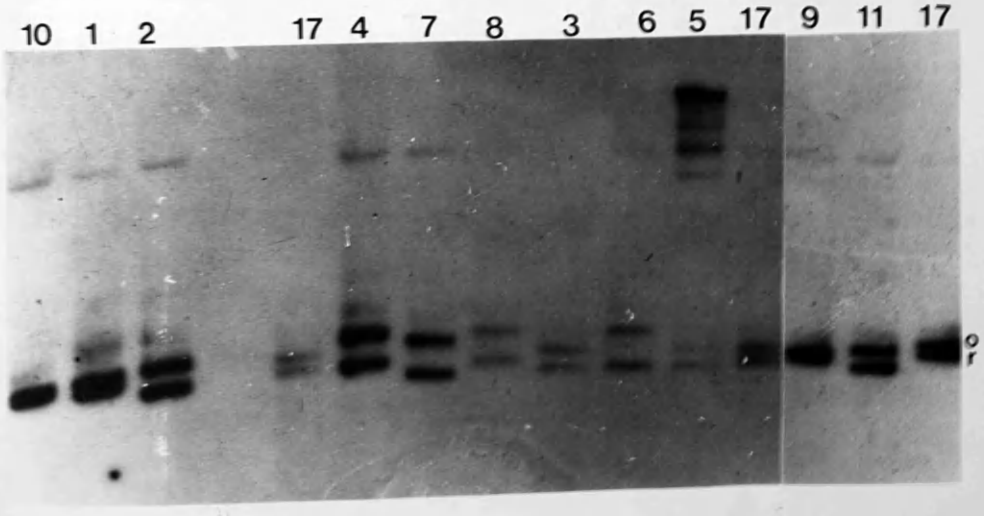

10 1 2 17 4 7 8 3 6 5 17 9 11 17
or

Figure 64. Southern blot of TR_L/IR_L variants.

Southern blot of Hpa I digested HSV-1 DNA probed with nick translated Hpa I r (see Figure 62) (0.8% agarose). Hpa I r contains sequences in R_L excluding the 'a' sequence and hybridizes to both Hpa I o and r (see Figure 62). Hybridization to other higher molecular weight bands is spurious, possibly as a result of hybridization to partial digestion products or adjacent bands on the genome, a phenomenon previously observed (Brown and Harland, 1987; and personal communication). Lanes 1 to 11 refer to the same isolates as in Figure 63. 17 is strain 17.

differ in size. The observed variations were stable over 3 rounds of plaque purification (data not shown).

III.4.b DNA profile of 1704

One HSV-1 variant, 1704, exhibited changes indicating an approximate 0.8×10^6 mol. wt. deletion in TR_L and an approximate 2.5×10^6 mol. wt. deletion in IR_L/U_L. On Xba I digestion (Figures 65 and 66, lane 2), the g band showed a 0.8×10^6 mol. wt. deletion and the d band a 2.5×10^6 mol. wt. deletion causing it to comigrate with the f band. The g and d containing joints b and a, respectively, were also reduced in size. In the gel shown in Figure 66 the g band appears as a doublet. This is often the case on long exposures of such gels (own unpublished observations, and Dr. S. M. Brown, personal communication), probably due to variation in the number of 'a' sequences at the L terminus (Davison and Wilkie, 1981).

Digestion with Bgl II (Figures 62 and 67, lane 2) revealed a 0.8×10^6 mol. wt. deletion in j and a 2.5×10^6 mol. wt. deletion in f causing it to comigrate with i. The j containing joints b and e are also deleted, as are the f containing joints a and c, the latter comigrating with d.

Digestion with Bam HI (Figure 62 and 68, lane 2) showed that the e band was deleted by 0.8×10^6 mol. wt., thereby comigrating with f, while the b band is deleted by 2.5×10^6 mol. wt. and comigrates with j. The variation in the mobility of the terminal s and joint k fragments seen in Figure 68, lane 2, is again probably due to variation in the number of 'a' sequences. The x band, situated within the short repeat has also been shown to exhibit variation

Figure 65. Xba I map of HSV-1 DNA.

Xba I map of the DNA of HSV-1 strain 17.

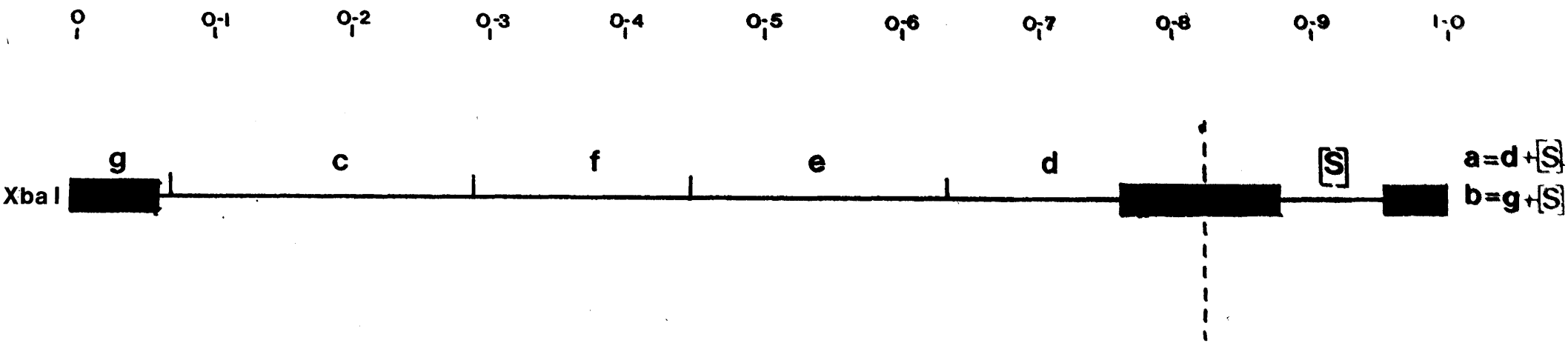
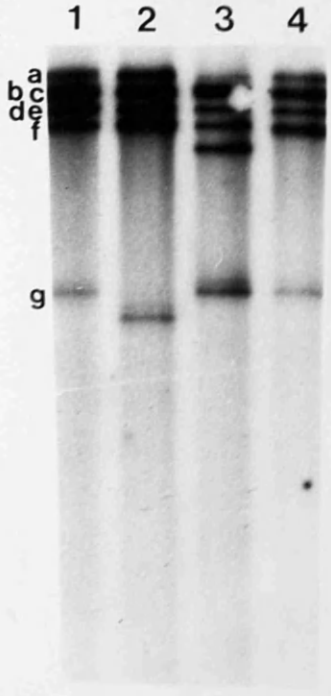

0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
g
c
f
e
d
[S]
a=d+[S]
b=g+[S]
Xba I

Figure 66. Xba I profile of 1704, 1705 and 1706.

Autoradiograph of Xba I restriction digests of HSV-1 DNA ^{32}P-labelled *in vivo* (0.5% agarose). Lane 1, strain 17; lane 2, 1704; lane 3, 1706; and lane 4, 1705.


1
2
3
4
a
b
c
d
e
f
g

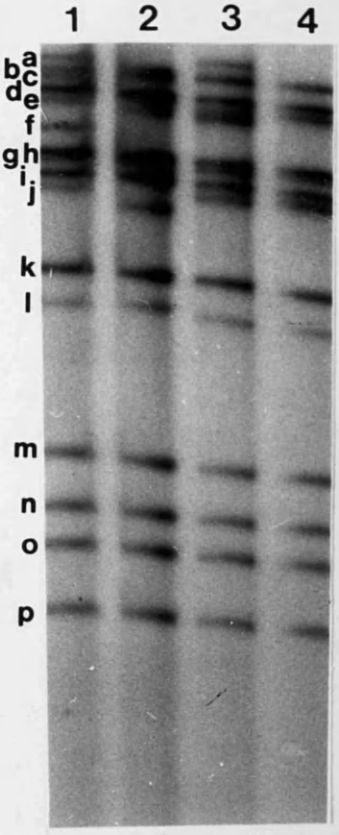
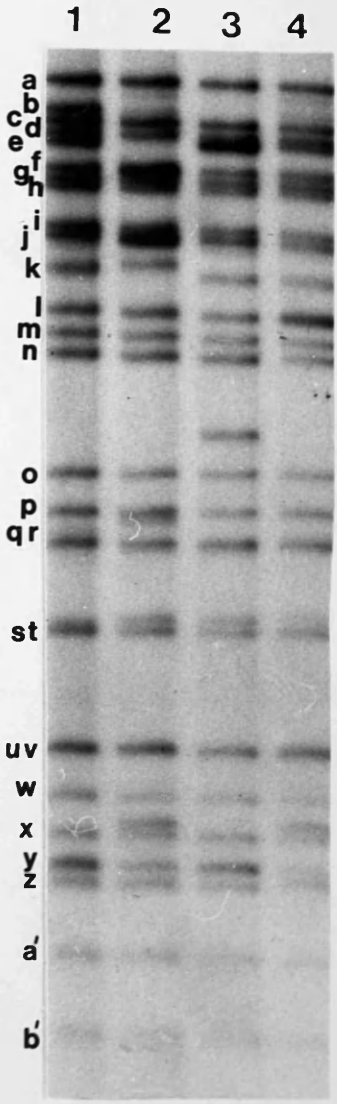

1
2
3
4
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

Figure 67. Bgl II profile of 1704, 1705 and 1706.

Autoradiograph of Bgl II restriction digests of HSV-1 DNA ^{32}P-labelled *in vivo* (0.5% agarose). Lane 1, strain 17; lane 2, 1704; lane 3, 1706; and lane 4, 1705.

Figure 68. Bam HI profile of 1704, 1705 and 1706.

Autoradiograph of Bam HI restriction digests of HSV-1 DNA ^{32}P-labelled *in vivo* (0.8% agarose). Lane 1, strain 17; lane 2, 1704; lane 3, 1706; and lane 4, 1705.


1
2
3
4
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
st
uv
w
x
y
z
a'
b'

between isolates (Davison and Wilkie, 1981). Within the stock of 1704 the x band appears to exist as 2 size classes (Figure 68, lane 2).

Digestion with Hpa I (Figures 62 and 69, lane 2) suggests that o is deleted by around 0.8×10^6 mol. wt. and is running below s, s is unaltered, v and r are absent while m is unaltered. These findings were confirmed by Southern blotting Hpa I digested DNA with a probe containing Hpa I r and one containing Hpa I v+s, pMC9(2) (Figures 70, 71 and 72). A 0.4×10^6 mol. wt. band was identified which hybridized to both probes, indicating that this was the fused remnants of Hpa I v and r.

Probing a Southern blot of Bam HI/Hpa I/Sma I digested DNA with pMC9(2) (Figures 70 and 73, lane 2) confirmed that the 1240 bp Hpa I v fragment was absent but all the Sma I fragments of Hpa I s were present. A novel band of around 750 bp was detected corresponding to the fused remnants of Hpa I v and r. This deletion terminates 400-1100 bp downstream of the 3' end of IE gene 2. The structure of 1704 is summarized in Figure 70a.

III.4.c DNA profile of 1705

Analysis of HSV-1 variant 1705 indicated that it was deleted in IR_L/U_L, by approximately 3.5×10^6 mol. wt.

On Xba I digestion, the d band was deleted by greater than 3×10^6 mol. wt., causing it to comigrate with the f band. The d containing joint a was also reduced in size (Figures 65 and 66, lane 4).

On Bgl II digestion, the f band was deleted by 3.5×10^6 mol. wt., causing it to comigrate with j. The f containing

Figure 69. Hpa I profile of 1704, 1705 and 1706.

Autoradiograph of Hpa I restriction digests of HSV-1 DNA ^{32}P-labelled *in vivo* (0.8% agarose). Lane 1, strain 17; lane 2, 1704; lane 3, 1706; and lane 4, 1705.

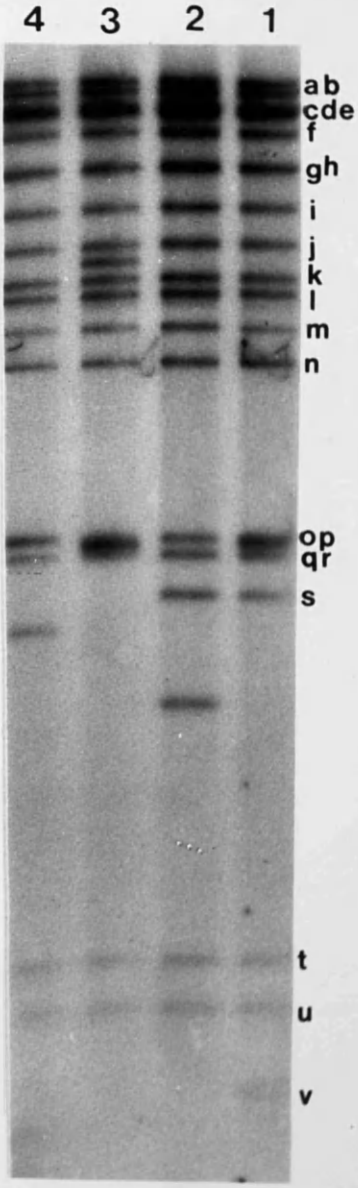

4
3
2
1
ab
cde
f
gh
i
j
k
l
m
n
op
qr
s
t
u
v

Figure 70. Detailed analysis of sequences deleted in 1704, 1705 and 1706.

a) Structure of the HSV-1 genome showing U_L and U_S flanked by IR_L/TR_L and IR_S/TR_S, respectively (above the line). The relevant restriction sites used for mapping the deletions/insertions are also shown - Xba I (X), Hpa I (H), Bam HI (B), Bgl II (Bg) and Kpn I (K). Below the line are shown the position of the Hpa I r and Bam HI c probes used in Figures 71 and 75 respectively; and (i) the deletions in 1704, 1705 and 1706; and (ii) the sequence repeated in 1706.

b) An expansion of the region from 0.73 m.u. to the IR_L/IR_S junction, showing the Kpn I (K), Bam HI (B) and Hpa I (H) restriction sites. Bam HI b and s, and Hpa I m, r, v and s' (the part of s illustrated) are labelled. The transcripts and polypeptide products in this region are shown above the line. Probes Bam HI b and pMC9(2) are shown below the line.

c) An expansion of pMC9(2) (Hpa I v and s') showing the sizes in base pairs of fragments generated upon digestion with Bam HI (B)/Hpa I (H)/Sma I (S) which hybridize to pMC9(2) (Figure 73).

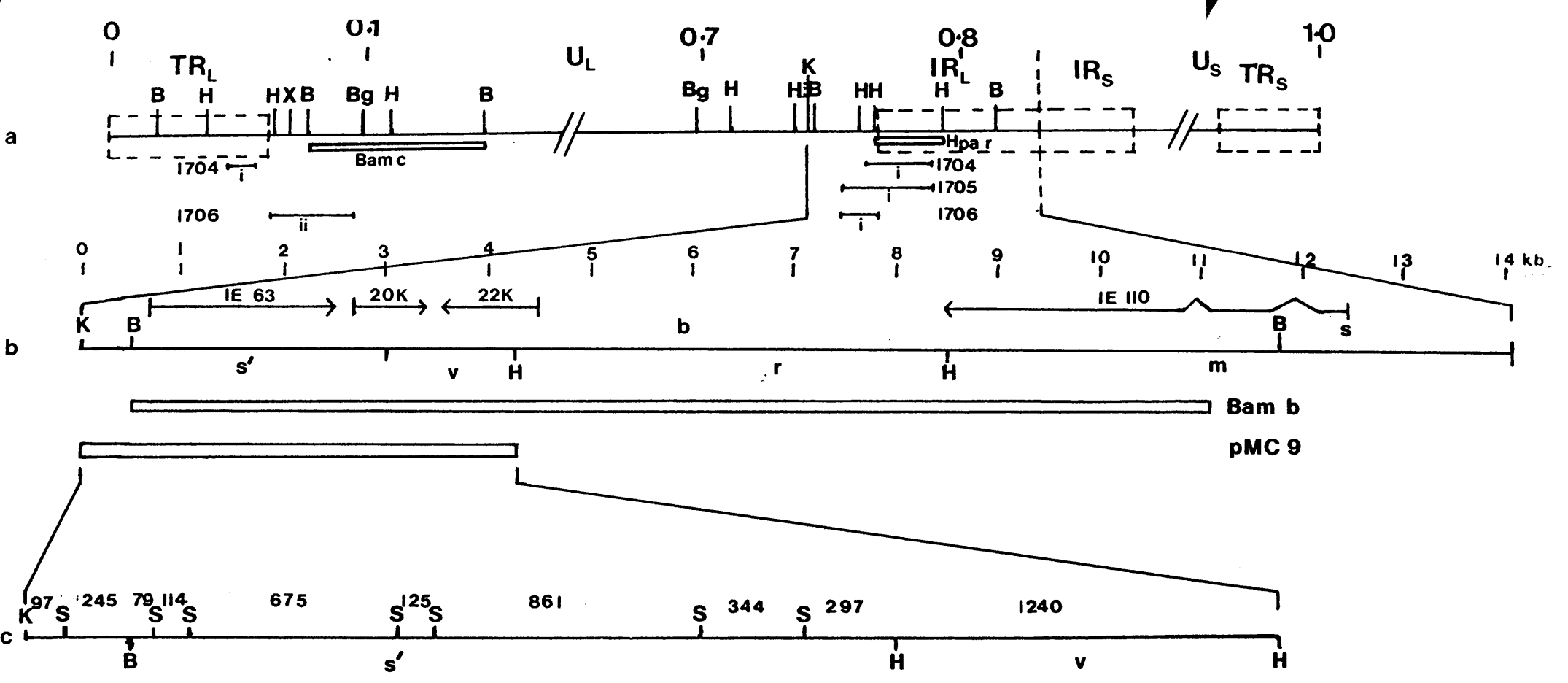
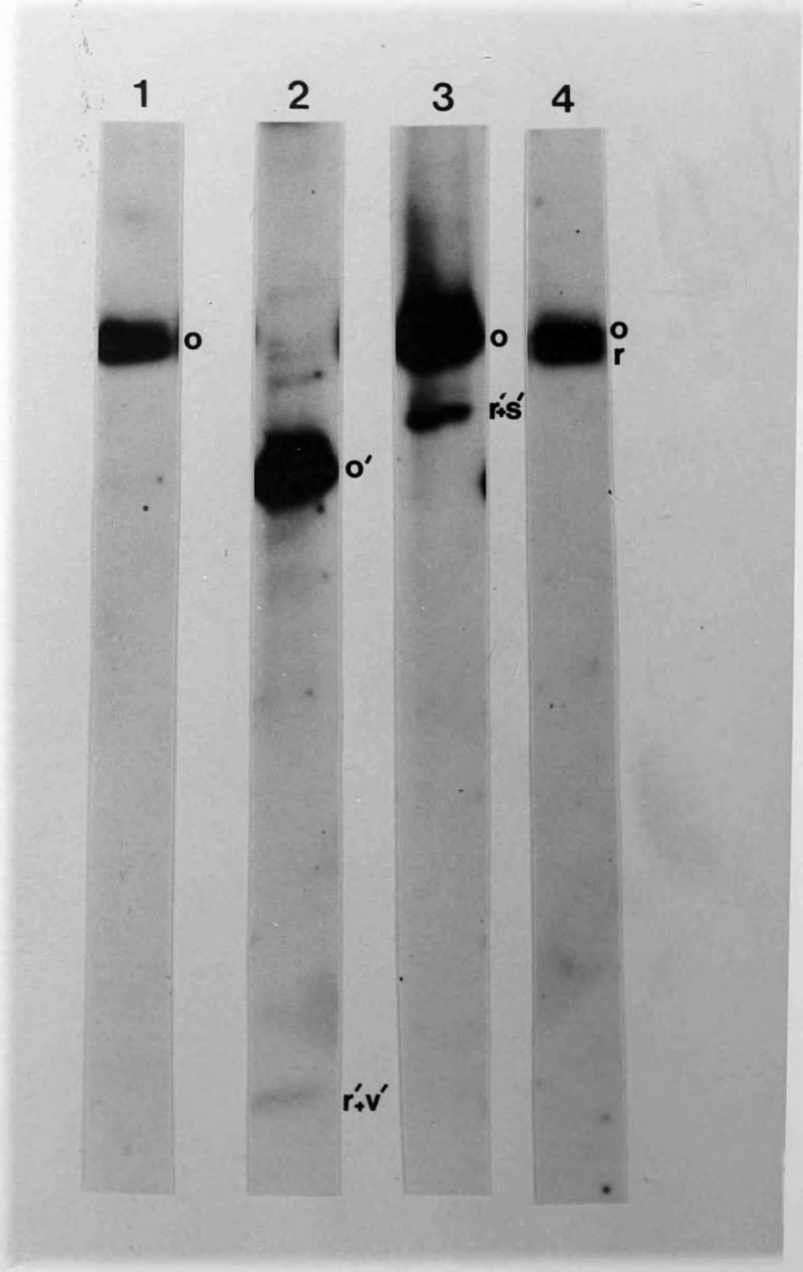

0
0·1
0·7
0·8
1·0
TR_L
U_L
IR_L
IR_S
U_S
TR_S
a
B H HXB Bg H B
Bg H HB HH H B
K
Bam c
Hpa r
1704
i
1705
1706
ii
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 kb
IE 63
20K
22K
IE 110
K B
b
B s
s′ v H r H m
Bam b
pMC 9
c
K 97 S 245 79 S 114 S 675 S 125 S 861 S 344 S 297 1240
B
s′
H
v
H

Figure 71. Southern blot of 1704, 1705 and 1706 using Hpa I r.

Autoradiograph of a Southern blot in which nick translated Hpa I r (Figure 70) was hybridized to HSV-1 DNA digested with Hpa I. Lane 1, 1706; lane 2, 1704; lane 3, 1705; and lane 4, strain 17. The bands in each lane hybridizing to the probe are labelled as the appropriate Hpa I fragments. Deleted fragments are labelled with the letter of the fragment from which they are derived plus a prime symbol ('). Assignment of fused bands was carried out in conjunction with Figure 72.


1
2
3
4
o
o′
r′+v′
o
r′+s′
o
r

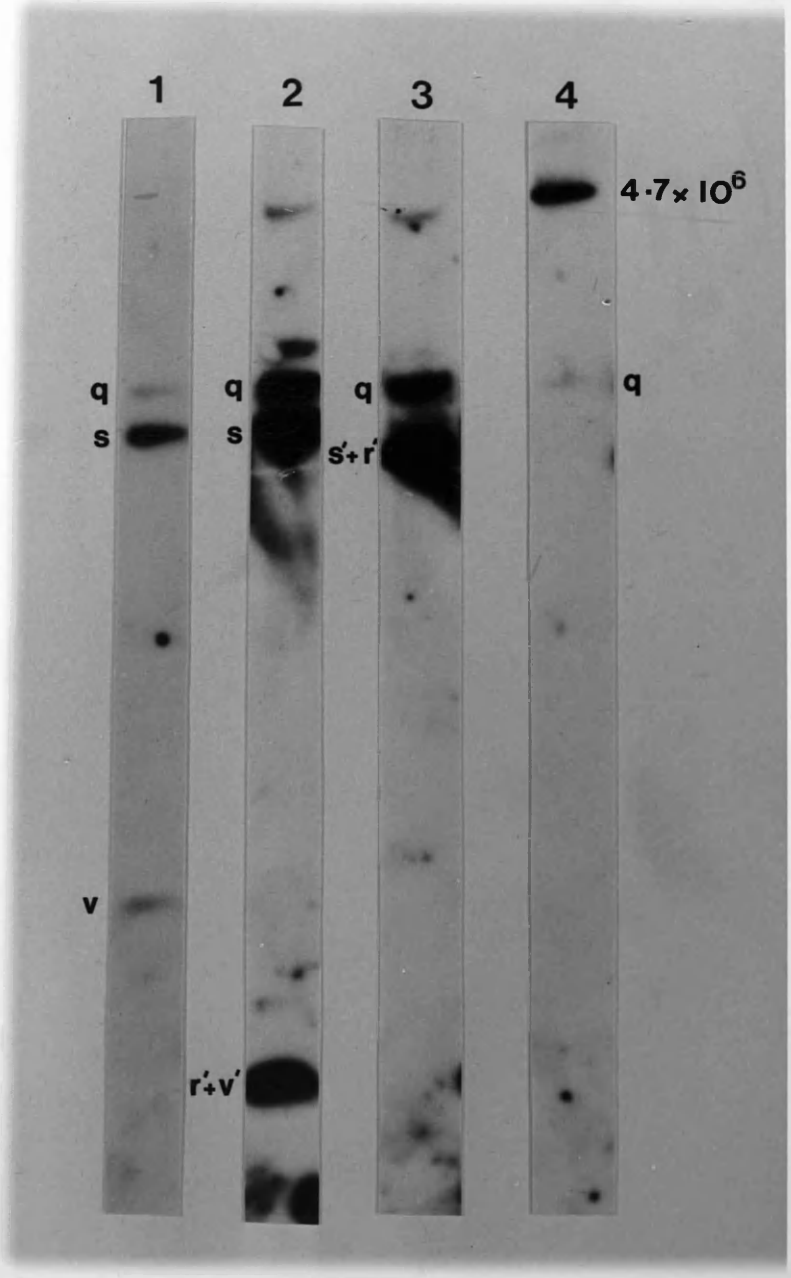
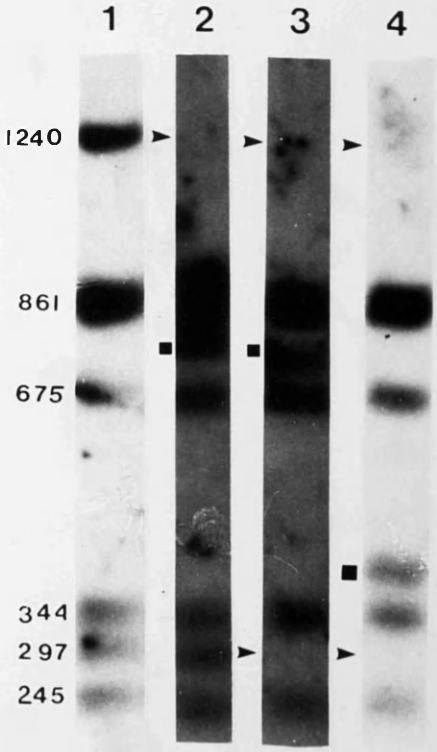

1
2
3
4
4·7× 10^6
q
s
q
s
q
s'+r'
q
v
r'+v'

Figure 72. Southern blot of 1704, 1705 and 1706 using Hpa I v and s.

Autoradiograph of a Southern blot in which nick translated Hpa I v and s [pMC9(2)] (Figure 70) was hybridized to HSV-1 DNA digested with Hpa I. Lane 1, strain 17; lane 2, 1704; lane 3, 1705; and lane 4, 1706. The bands in each track hybridizing to the probe are labelled as the appropriate Hpa I fragment. Deleted fragments are labelled with the letter of the fragment from which they are derived plus a prime symbol ('). Assignment of fused bands was carried out in conjunction with Figure 71. Hybridization to Hpa I q is spurious, possibly due to hybridization to the fragment adjacent to the probe (Brown and Harland, 1987; and personal communication).

Figure 73. Southern blot of 1704, 1705 and 1706 using pMC9(2).

Autoradiograph of a Southern blot in which nick-translated pMC9(2) (see Figure 70c) was hybridized to HSV-1 DNA which had been digested with Bam HI/Hpa I/Sma I. Lane 1, strain 17; lane 2, 1704; lane 3, 1705; and lane 4, 1706. For the location of the bands on the genome see Figure 70c. The missing bands in 1704, 1705 and 1706 are labelled ► and novel bands in 1704, 1705 and 1706 ■. Lanes 1 and 4 were exposed at room temperature, while lanes 2 and 3 were exposed at -70°C with an image intensifying screen.


1
2
3
4
1240
861
675
344
297
245

joints a and c were also reduced in size, now comigrating with b and d, respectively (Figures 62 and 67, lane 4).

On Bam HI digestion, the b band was deleted, comigrating with l, indicating a deletion of 3.5×10^6 mol. wt. (Figures 62 and 68, lane 4).

On Hpa I digestion, s, v and r are absent, m is unaltered while a novel band of around 1.8×10^6 mol. wt., running below s, is present (Figures 62 and 69, lane 4). Southern blotting Hpa I digested DNA with a probe containing Hpa I r and another containing Hpa I v+s, pMC9(2), confirmed these findings and indicated that the novel band is a fusion between remnants of Hpa I s and r (Figures 70, 71 and 72).

The endpoint of the deletion in Hpa I s was mapped by Southern blotting of a Bam HI/Hpa I/Sma I digest with pMC9(2) (Figure 73, lane 3). The location of the fragments are illustrated in Figure 70. The 1240 bp Hpa I v fragment is absent, as is the 297 bp Sma I fragment, but the 344 bp Sma I fragment is present. A novel band of approximately 700 bp was detected, corresponding to the fused product of the 297 bp Sma I fragment and Hpa I r. Therefore, the deletion extends from within Hpa I r into the 297 bp Sma I fragment and terminates around the 3' end of IE gene 2. The structure of 1705 is summarized in Figure 70a.

III.4.d DNA profile of 1706

The HSV-1 variant 1706 has a 1×10^6 mol. wt. deletion in the right end of U_L adjacent to IR_L, which has been replaced by 3×10^6 mol. wt. from the opposite end of U_L, resulting in the extension of the long repeat by 3×10^6 mol. wt., and consequently the extension of the overall genome size by

$2x10^6$ mol. wt.

On Xba I digestion (Figures 65 and 66, lane 3) the g band, which is usually 0.5 M, becomes 1 M. The 0.5 M d band is absent and is replaced by a novel 1 M band of $14x10^6$ mol. wt. running below f. The d containing joint a is absent and the g containing joint b is increased in intensity. This suggests that there is a deletion in the right end of U_L, while the left end of U_L is now also present at the right end; as a result there are two copies of g and also of its joint b, both now being 1 M. The d band is absent in this model and is replaced by the 1 M band of $14x10^6$ mol. wt., which consists of the fused d remnants with the duplicated c sequences (Figure 74).

On Bgl II digestion (Figures 62 and 67, lane 3), the j band remains 0.5 M, indicating that the inserted sequences do not extend as far as the j/k site. The f band is increased in size by $2.3x10^6$ mol. wt. and is running just below e. The f containing joints, a and c, are also increased in size, c comigrating with b.

On Bam HI digestion (Figures 66 and 68, lane 3), the b band is absent and the e band is now 2 M. A novel band of $2.7x10^6$ mol. wt., running above o, is now present. This is a fusion between part of Bam HI c and part of Bam HI b, as confirmed by blotting with Bam HI b and c, which both hybridized to this band (Figures 70, 74 and 75).

On Hpa I digestion (Figures 62 and 69, lane 3) the o band is 2 M, the s, r and v bands are absent and a novel band of $4.7x10^6$ mol. wt., running above k, is present. This was confirmed to be a fusion between s and l bands by its ability to hybridize to Bam HI b and c (Figures 70, 74 and

Figure 74. Structure and derivation of 1706.

Map of appropriate restriction enzyme sites around TR_L/IR_L of HSV-1 strain 17 - Bam HI (B), Hpa I (H), Xba I (X) and Bgl II (Bg). The top genome is drawn in the prototype (P) orientation and the bottom genome with the long unique inverted (I_L). Illegitimate recombination between these two genomes in the region marked 1 would result in a genome of the structure of 1706, again showing the appropriate restriction enzyme sites. Recombination between 1705 in the P orientation (above the line) and 17 (or 1705) in the I_L orientation would also result in 1706. Affected restriction enzyme fragments are shown below the line. The unlabelled fragments correspond to the novel Bam HI and Hpa I fragments which hybridize to both Bam HI b and c (see Figure 75).

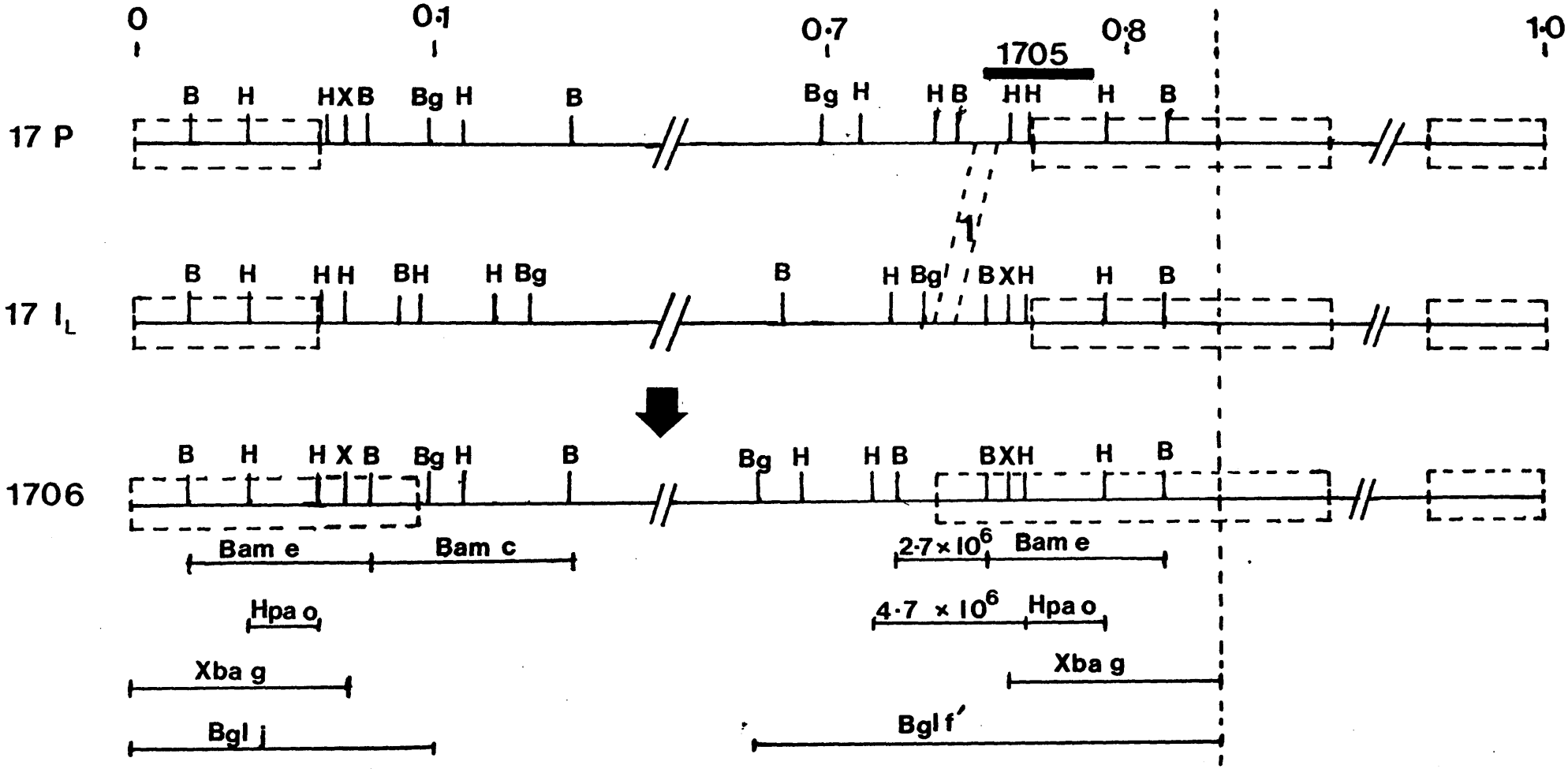

0
0·1
0·7
0·8
1·0
1705
17 P
17 I_L
1706
B H HXB Bg H B
Bg H HB HH H B
B H HH BH H Bg
B H Bg BXH H B
B H HXB Bg H B
Bg H HB BXH H B
Bam e
Bam c
Hpa o
Xba g
Bgl j
$2·7 \times 10^6$
Bam e
$4·7 \times 10^6$
Hpa o
Xba g
Bgl f′

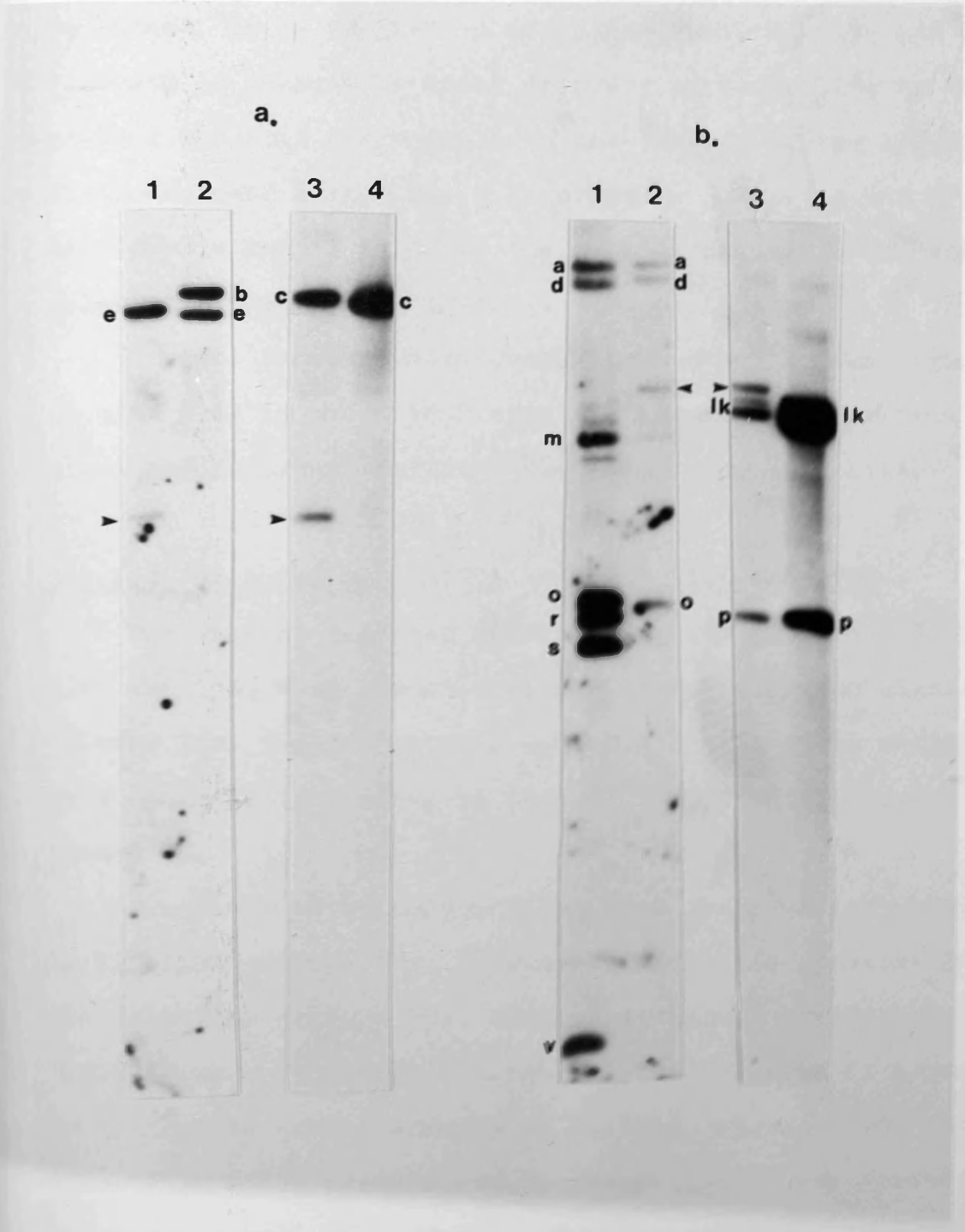
Figure 75. Southern blot of 1706 using Bam HI b and c.

Autoradiograph of a Southern blot in which nick-translated Bam HI b and c (Figures 70a and 74) were hybridized to :

a) Bam HI digested strain 17 and 1706 DNA. Lane 1, 1706 probed with Bam HI b; lane 2, strain 17 probed with Bam HI b; lane 3, 1706 probed with Bam HI c; and lane 4, strain 17 probed with Bam HI c.

b) Hpa I digested strain 17 and 1706 DNA. Lane 1, strain 17 probed with Bam HI b; lane 2, 1706 probed with Bam HI b, lane 3, 1706 probed with Bam HI c; and lane 4, strain 17 probed with Bam HI c.

The novel Bam HI and Hpa I bands which hybridize to both Bam HI b and c are marked ► (see Figure 74).


a.
1 2 3 4
e
b
e
c
c
b.
1 2 3 4
a
d
a
d
lk
lk
lk
m
o
r
s
o
p
p
v

75). Probing with Hpa I v+s, pMC9(2), and Hpa I r, showed that this band (4.7×10^6 mol. wt.) hybridized to the Hpa I v+s probe but not to the Hpa I r probe, as would be expected (Figures 70, 71, 72 and 75).

A Southern blot of a Bam HI/Hpa I/Sma I digest with pMC9(2) (Figures 70 and 73, lane 4) mapped the left end of the deletion in Hpa I s. Again the 1240 bp Hpa I v fragment is absent, as is the 297 bp Sma I fragment, but the 344 bp fragment is present. A novel fragment of around 400 bp is present and this corresponds to the remnant of the 297 bp fragment fused to the Sma I fragment at the right end of the deletion in Bam HI c. As in the case of variant 1705, the deletion extends close to the 3' end of IE gene 2.

A model showing where recombination would have occurred to give 1706 is shown in Figure 74, as are the restriction sites and fragments defining the long repeats in 1706.

III.4.e Polypeptide profile of 1704, 1705 and 1706

The general infected cell polypeptide profiles of 1704, 1705 and 1706 were compared with that of wild-type strain 17 (Figure 76). The polypeptide profiles of all three variants were essentially similar to that of the parental virus, strain 17.

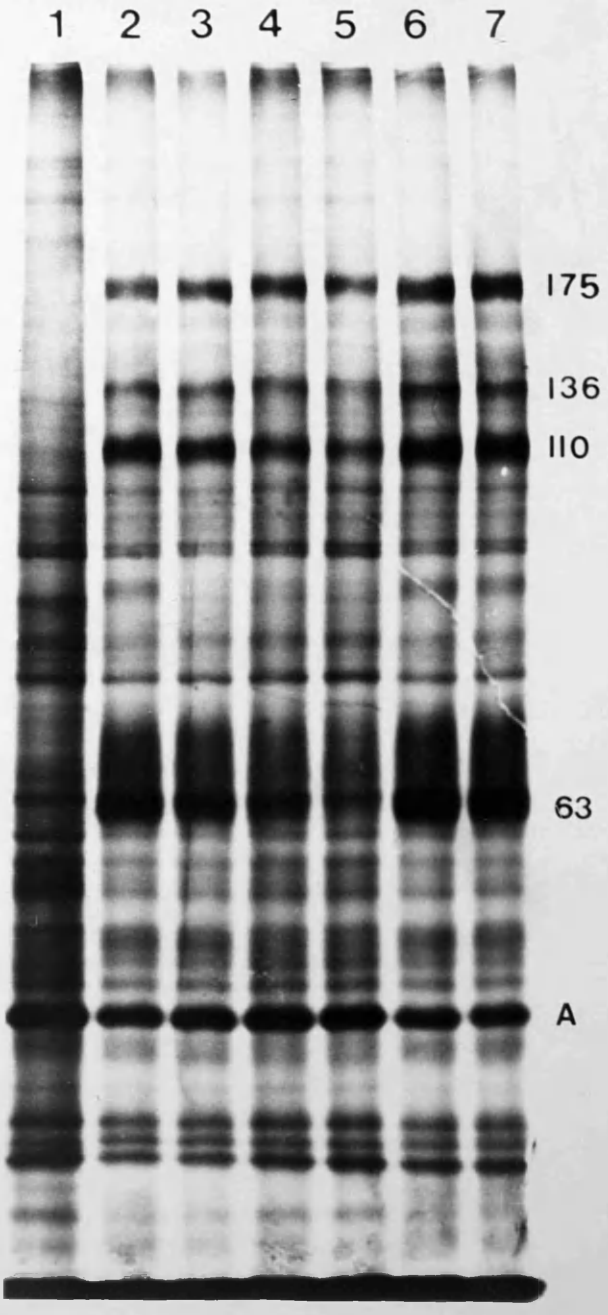
Immediate-early polypeptides were prepared and analyzed by SDS-PAGE (Figure 77). As expected from the location of the deletions (Figure 70), apparently normal amounts of VmwIE110 were synthesized. 1704 (Figure 77, lane 2) appeared to synthesize normal amounts of VmwIE63, whereas 1705 and 1706 (Figure 77, lanes 3 and 4, respectively) may synthesize reduced amounts. This finding was confirmed by

Figure 76. General polypeptide profile of 1704, 1705 and 1706.

Whole cell polypeptide extracts, labelled with [^{35}S]-methionine from 4-24 h post infection, were analyzed on a 5-12.5% gradient polyacrylamide gel. Molecular weights (x 10^{-3}) of predominant HSV-1 polypeptides are given on the left hand side. A is actin. Lane 1, strain 17; lane 2, 1703 (tk^-, III.3.4); lane 3, 1704; lane 4, 1705; and lane 5, 1706.

Figure 77. Immediate-early polypeptide profile of 1704, 1705 and 1706.

Immediate-early polypeptides, labelled with [^{35}S]-methionine, of mock infected (lane 1), 1704 infected (lane 2), 1705 infected (lane 3), 1706 infected (lane 4), 1703 infected (III.3.2) (lane 5) and strain 17 infected (lane 6) HFL cells were analyzed on a 7.5% polyacrylamide gel. Molecular weights (x 10^{-3}) are given on the left hand side. A is actin.


1
2
3
4
5
6
7
175
136
110
63
A

immunoprecipitation experiments using an antiserum raised against the amino-terminus of VmwIE63 (section III.3.b). In strain 17, a band of $63x10^3$ mol. wt. was precipitated (Figure 78, lane 3), which was not precipitated in the presence of the relevant peptide (Figure 78, lane 7). In 1704, a 63K band of roughly equal intensity to that in strain 17 was precipitated (Figure 78, lane 4). However, in 1705 and 1706 the intensity of the 63K band was reduced, possibly to less than a half the wild-type level (Figure 78, lanes 5 and 6, respectively). Over several experiments, this finding was confirmed and it appeared that 1705 and 1706 synthesized similar amounts of VmwIE63. Again, the immediate-early extracts shown were prepared in HFL cells, but similar findings were obtained in BHK21 C13 cells (data not shown).

III.4.f Growth properties of 1704, 1705 and 1706

One-step growth experiments were carried out over a 24 h period in BHK21 C13 cells (Figure 79). All three deletion variants, 1704, 1705 and 1706, grow at a slower rate than the wild-type virus, and produce a lower 24 h yield. Similar 72h yields results were obtained in growth experiments carried out at low multiplicity of infection and allowing multiple rounds of replication (Figure 80). High titre stocks (>10^9 pfu/ml) have been obtained from the variants, indicating that the growth impairment is overcome with time. All three variants gave similar titres at 38.5°C and 31°C, indicating that they are not temperature-sensitive (data not shown).

Figure 78. Immunoprecipitation of VmwIE63 in 1704, 1705 and 1706.

Immunoprecipitation of immediate-early extracts using an antiserum raised against an amino-terminal peptide of VmwIE63. Lane 1, strain 17 infected whole cell extract; lane 2, mock infected whole cell extract; lanes 3 to 7 immunoprecipitations; lane 3, strain 17; lane 4, 1704; lane 5, 1705; lane 6, 1706; and lane 7, strain 17 in the presence of the peptide against which the antiserum was raised, showing the specificity of the immunoprecipitation.

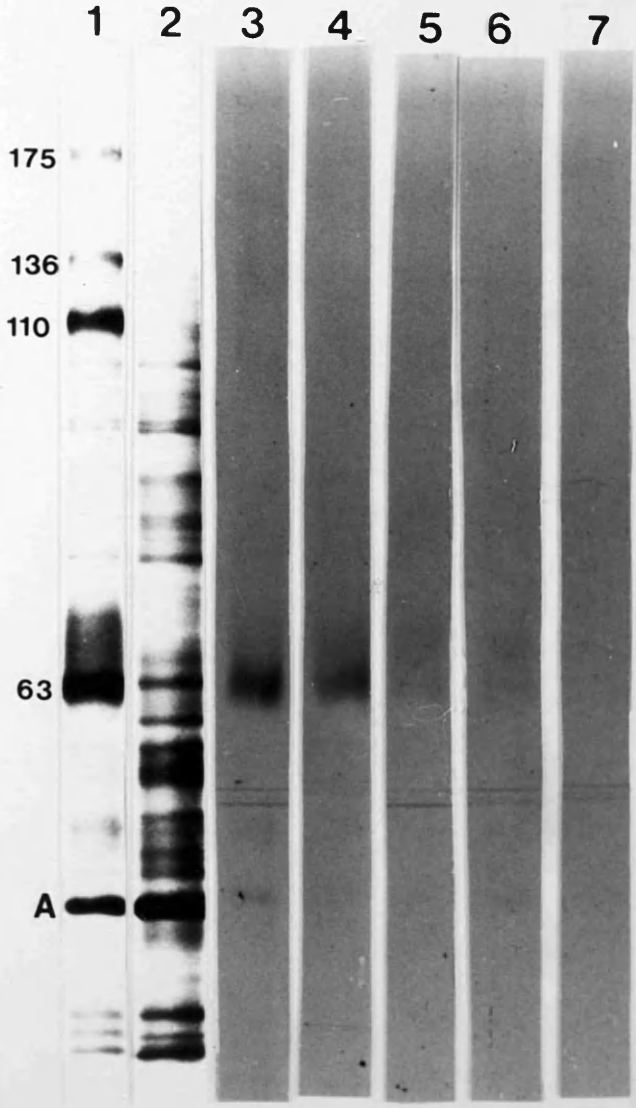

1
2
3
4
5
6
7
175
136
110
63
A

Figure 79. One-step growth curves of 1704, 1705 and 1706.

One-step growth curves of HSV-1 strain 17 ○ , 1704 ● , 1705 △ , 1706 ■ and 1708 □ in BHK21 C13 cells. Cells were infected at a multiplicity of 5 pfu/cell, the monolayers washed twice with PBS/calf serum, overlaid with ETC10 and incubated at 37°C. Plates were harvested at 0, 2, 4, 6, 8, 12 and 24 h post infection and titrated as normal.

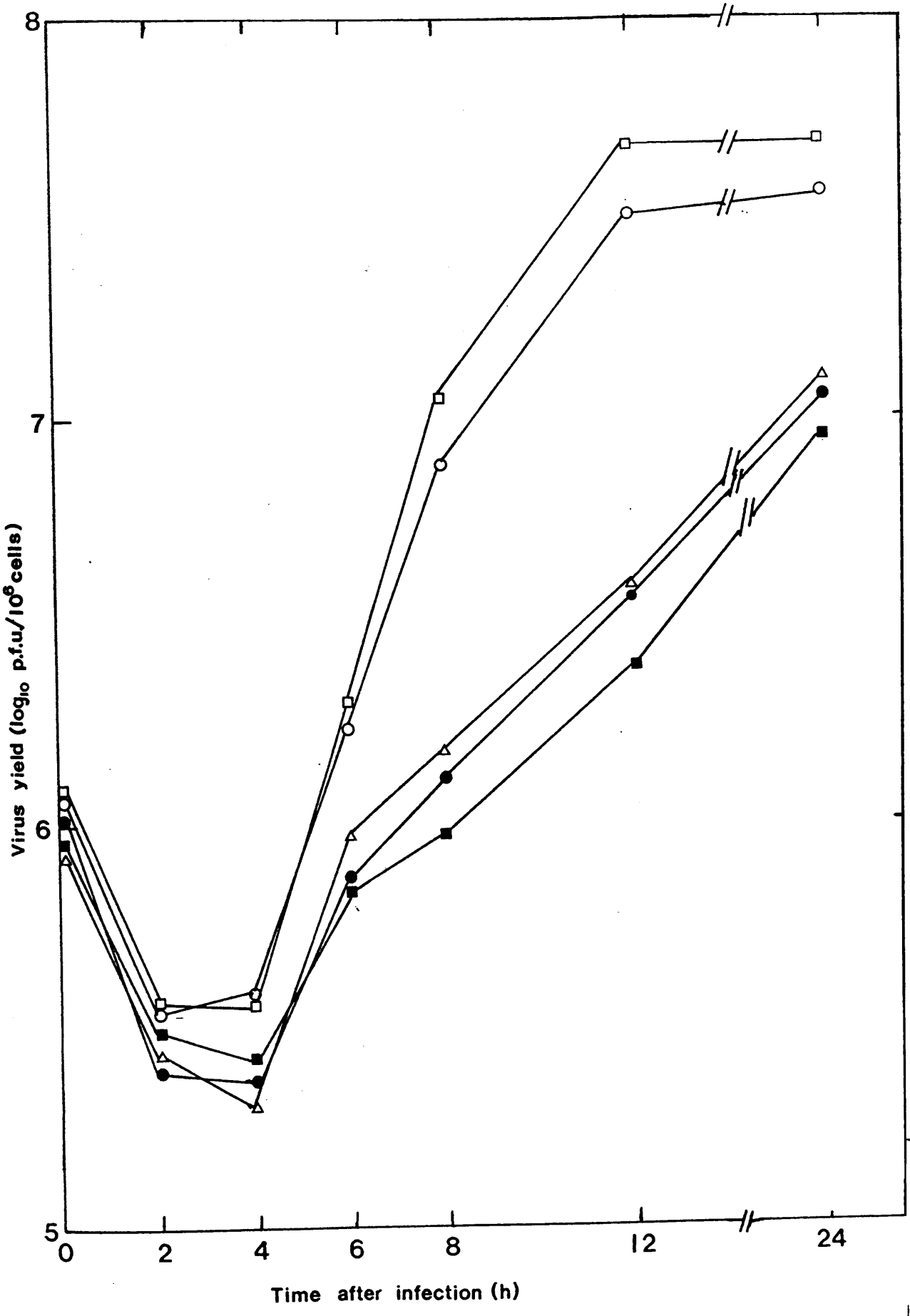
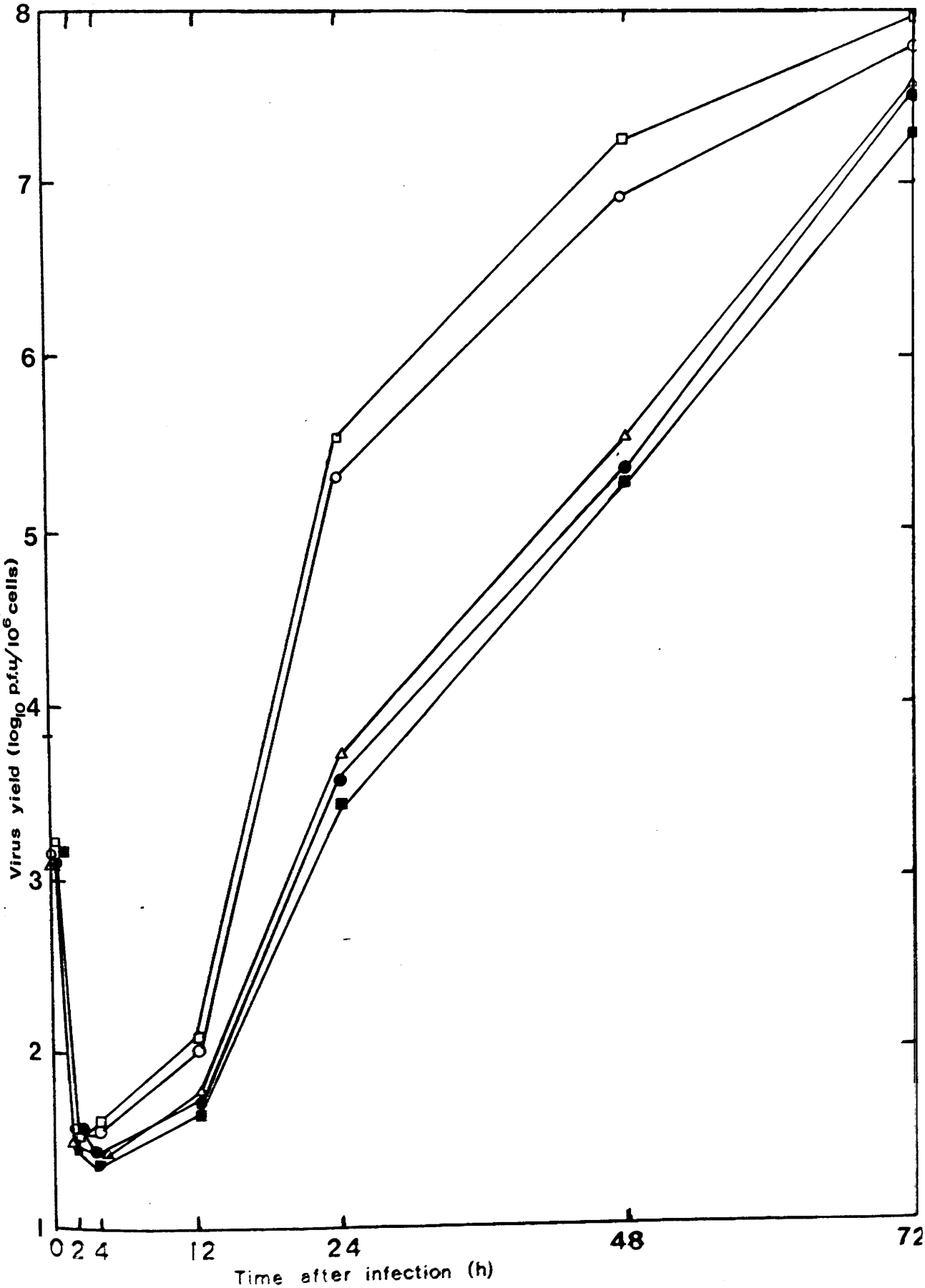

8
7
6
5
0
2
4
6
8
12
24
Virus yield (log_{10} p.f.u/10^6 cells)
Time after infection (h)

Figure 80. Long-term growth curves of 1704, 1705 and 1706.

Long-term growth curves of HSV-1 strain 17 ○ , 1704 ● , 1705 △ , 1706 ■ and 1708 □ in BHK21 C13 cells. Cells were infected at a multiplicity of 0.001 pfu/cell, the monolayers washed twice in PBS/calf serum, overlaid with ETC10 and incubated at 37°C. Plates were harvested at 0, 2, 4, 12, 24, 48 and 72 h post infection and titrated as normal.


8
7
6
5
4
3
2
1
Virus yield (log_{10} p.f.u/10^6 cells)
0 2 4
12
24
48
72
Time after infection (h)

III.5 ISOLATION OF HSV-1 VARIANT 1709

Towards the end of the project a variant of 1703, 1709, was isolated, which had a deletion in both short repeats. Only preliminary characterization was carried out.

On the Bgl II digestion (Figures 81 and 82, lane 1), the h and l bands are deleted by approximately 0.6×10^6 mol. wt. This alteration is too small for an effect on the joints to be detected.

On Hind III digestion (Figures 81 and 83, lane 1) the g and m bands are deleted by approximately 0.5×10^6 mol. wt. Again, the effect on the joint is not distinguishable.

Overall, this variant appears to have a deletion of around 0.5×10^6 mol. wt. in both short repeats. It is not known if these deletions overlap each other. It would not be unreasonable to assume that they are identical, having arisen in one short repeat and being transferred to the other short repeat by recombination.

Figure 81. Bgl II and Hind III maps of HSV-1.

Bgl II and Hind III maps of HSV-1 strain 17 DNA.

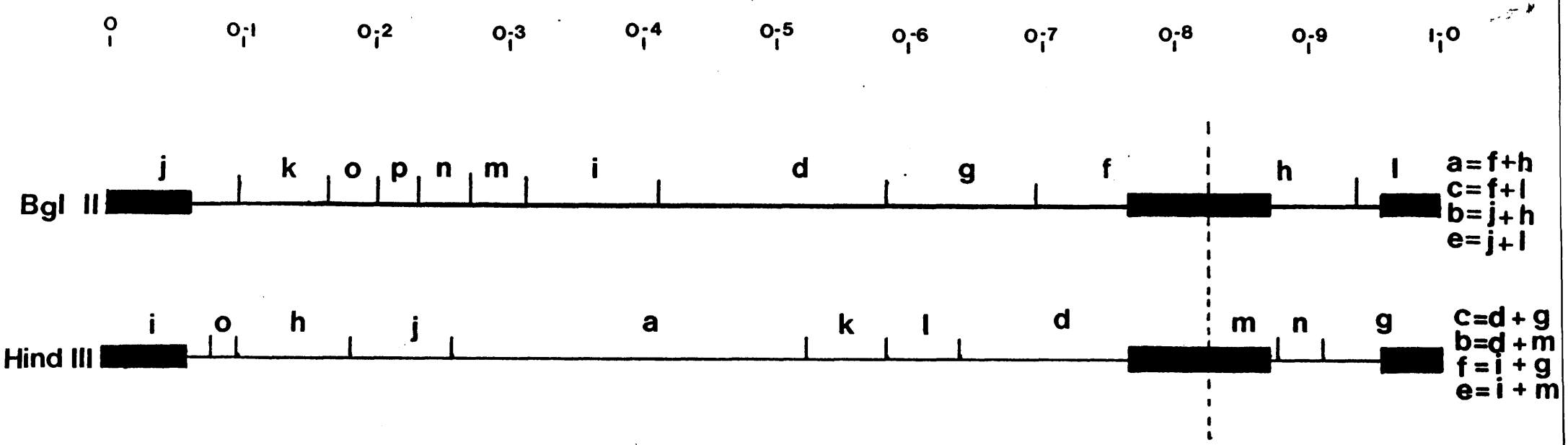

0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Bgl II
j
k
o
p
n
m
i
d
g
f
h
l
a=f+h
c=f+l
b=j+h
e=j+l
Hind III
i
o
h
j
a
k
l
d
m
n
g
c=d+g
b=d+m
f=i+g
e=i+m

Figure 82. Bgl II profile of 1709.

Autoradiograph of a Bgl II restriction digest of HSV-1 DNA ^{32}P-labelled *in vivo* (0.5% agarose). Lane 1, 1709; and lane 2, 1703. Altered bands in 1703 compared to its parent, strain 17, are marked with the letter of the fragment from which they are derived plus a prime symbol ('), as are bands in 1709 compared to its parent, 1703.


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

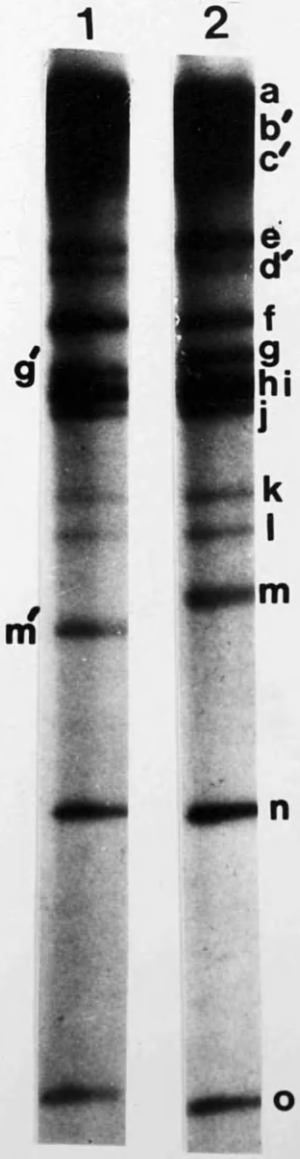

1
2
a
b'
c'
e
d'
f
g
h
i
j
k
l
m
g'
m'
n
o

Figure 83. Hind III profile of 1709.

Autoradiograph of a Hind III restriction digest of HSV-1 DNA ^{32}P-labelled *in vivo* (0.5% agarose). Lane 1, 1709; and lane 2, 1703. Altered bands in 1703 compared to its parent, strain 17, are marked with the letter of the fragment from which they are derived plus a prime symbol ('), as are bands in 1709 compared to its parent, 1703.

DISCUSSION

DISCUSSION

The original objective of this project was to investigate herpes simplex virus recombination through the use of constructed HSV-1 genomes carrying both non-selectable (restriction enzyme site) and selectable (ts) markers. Therefore, over the course of the project a HSV-1 genome was constructed which contains no Xba I restriction enzyme sites (1702), and hence differs detectably from wild-type virus which contains 4 Xba I sites (located at 0.07, 0.29, 0.45 and 0.63 m.u.). A fifth Xba I site (at 0.74 m.u.) was introduced into the wild-type HSV-1 genome, to generate the virus 1708. Temperature-sensitive lesions were introduced into 1702 and 1708, to generate ts1702 and ts1708 which were used in preliminary recombination experiments.

During the isolation of the HSV-1 genomes lacking Xba I sites, a number of variant genomes showing rearrangements or deletions were detected. The isolation and characterization of these variants was carried out.

IV.1 ISOLATION AND USE IN RECOMBINATION OF HSV-1 VARIANTS LACKING XBA I SITES

The HSV-1 strain 17 variant X2, which lacks 2 Xba I sites, had previously been isolated (Brown et al., 1984) by a process of selection enrichment. This procedure involves cleavage of viral DNA with the appropriate restriction enzyme (in this case Xba I) prior to transfection onto BHK21 C13 cell monolayers. Natural variants within the population which lack one or more Xba I sites compared with wild-type

virus should have a greater probability of surviving Xba I digestion and hence of establishing infection. It is also possible that Xba I cleavage may lead in some cases to mutation of the Xba I site.

Wild-type HSV-1 strain 17 contains 4 Xba I sites at 0.07, 0.29, 0.45 and 0.63 m.u. (Figure 25). X2 contains only the 0.29 and 0.45 m.u. sites. At the time of isolation of X2 and at the start of this project, the DNA sequences surrounding the Xba I sites had not been established, and nothing was known about the polypeptides (if any) encoded by the DNA in which the sites were located. This information is now available : the 0.07 m.u. site is situated within an open reading frame (U_L2) predicted to encode a polypeptide of approximately 36K (Perry, 1986); the 0.29 m.u. site lies within gH, an essential glycoprotein (Weller et al., 1983; Buckmaster et al., 1984; McGeoch and Davison, 1986b; Gompels and Minson, 1986); the 0.45 m.u. site lies within an open reading frame (U_L33) predicted to encode a polypeptide of 14K (Dr. D. J. McGeoch, personal communication) to which a ts lesion has recently been mapped (Dr. V. G. Preston, personal communication); and the 0.63 m.u. site lies at the 3' end of gC (Dr. D. J. McGeoch, personal communication), a non-essential glycoprotein (Manservigi et al., 1977).

In X2, the 0.07 m.u. site was removed with no detectable (ie. less than 150 bp) deletion or insertion, while the 0.63 m.u. site was removed with a deletion of approximately 150 bp. The precise alteration at these two sites has not been determined, although preliminary evidence suggests that a truncated form of gC is synthesized (data not shown). Other than the alteration to gC, the polypeptide

profile of X2 is apparently normal. The growth characteristics of X2 over both one-step and long term growth experiments are very similar to wild-type virus. The latter experiment should detect any defect in cell-to-cell spread and amplify any small growth defect not apparent in a one-step growth experiment. High titre stocks of X2 ($>10^9$ pfu/ml) were routinely obtained.

Virus genomes lacking the 0.07 and 0.63 m.u. sites were obtained fairly easily : ie. the loss of each site was detected following Xba I analysis of the DNA of only 200 plaques. Further attempts to remove the remaining two Xba I sites by selection enrichment were unsuccessful, despite the analysis of several hundred plaques (Dr. S. M. Brown, personal communication).

In this project, the initial approach used to remove the remaining two Xba I sites was to obtain cloned HSV-1 genomic fragments spanning the 0.29 and 0.45 m.u. Xba I sites, remove the unique Xba I sites within the plasmids and marker rescue the resultant plasmids back into X2 DNA. All the manipulations carried out would change any polypeptides encoded by the DNA around the Xba I sites and in some cases would lead to a frameshift mutation. This approach was unsuccessful, despite the analysis of several thousand plaques. Control marker rescue experiments of a lesion in a ts mutant were successful, suggesting that the failure to select variants lacking Xba I sites was not due to technical problems, but was probably due to the introduction of lethal mutations into essential viral polypeptides. This possibility was strengthened when it became apparent that the 0.29 and 0.45 m.u. Xba I sites were contained within

known and apparently essential open reading frames (see above).

A second approach to generate a HSV-1 genome lacking Xba I sites was that of serial selection enrichment (described fully in sections II.8 and III.1.c). Following seven rounds of serial selection enrichment, 14 out of 196 plaques isolated had lost the 0.45 m.u. Xba I site with no detectable (ie. less than 150 bp) deletion or insertion. The 14 isolates were assumed to be clonally related, and one was chosen as the prototype and called 1701.

Attempts to remove the 0.29 m.u. Xba I site by serial selection enrichment were unsuccessful, despite the analysis of a further 2,000 plaques. At this time the coding sequence of gH became available and was kindly supplied by Drs. A. C. Minson and D. J. McGeoch. An oligonucleotide was synthesized which contained a single base alteration which would remove the Xba I site without changing the amino acid coding potential of the DNA. Site-directed mutagenesis of a cloned HSV-1 genomic fragment resulted in the isolation of plasmid molecules lacking the 0.29 m.u. Xba I site. One such isolate was then used in marker rescue experiments with 1701 DNA. Out of 96 virus plaques analyzed, 8 lacked the 0.29 m.u. Xba I site, with no detectable (less than 150 bp) deletion or insertion. These 8 isolates were assumed to be clonally related and one was chosen as the prototype and called 1702. Thus, 1702 lacks all 4 of the wild-type Xba I sites.

The infected cell polypeptide profiles of 1701 and 1702 suggested that, like their parent, X2, these viruses synthesize a truncated form of gC (data not shown). However, 1701 and 1702 differ from both wild-type strain 17 and X2,

in that they are tk^-; a defect presumably having arisen spontaneously during the generation of 1701. The cloned HSV-1 genomic fragment used to remove the 0.29 m.u. Xba I site from 1701 spans the tk locus and therefore has the potential to rescue the tk^- phenotype. However, since recombination is proportional to distance (see section I.14.a), and since the distance between the 0.29 m.u. Xba I site and the tk gene is greater than that between the tk gene and the end of the fragment, it is probably not surprising that 1702 is also tk^-. Indeed, all 8 isolates lacking the 0.29 m.u. Xba I site were tk^-, strengthening the assumption that they are clonally related. The tk lesion in 1702 was shown to be in the tk gene, since the tk lesion alone could be rescued by a fragment spanning tk (Dr. F. J. Rixon, personal communication).

With the exception of gC and tk, the infected cell polypeptide profiles of 1701 and 1702 are apparently identical to wild-type strain 17. As the 0.29 m.u. Xba I site in the gene encoding gH was lost by site-directed mutagenesis, it is assumed that gH synthesis is normal. Unfortunately, our preliminary attempts at immunoprecipitation using a monoclonal antibody against gH (kindly supplied by Dr. A. C. Minson) were unsuccessful with either wild-type or 1702 infected cell extracts. The growth characteristics of both 1701 and 1702 were very similar to those of wild-type and X2 over both one-step and long term growth experiments, and high titre stocks were obtained.

Thus, a HSV-1 strain 17 genome lacking Xba I sites has now been obtained.

* An alternative explanation could be that DNA replication in HG52 is more error prone giving rise to a higher frequency of viruses lacking Xba I sites in the population. This could be due to a more error prone DNA polymerase or poorer proof-reading function when compared to strain 17.

During the process of selection enrichment, it became apparent that it was almost impossible to totally destroy the infectivity of HSV-1 strain 17 DNA by Xba I digestion. A low level of infectivity (around 2-3 $\log_{10}$ pfu/ml lower than untreated DNA) nearly always remained after 16 h digestion, despite apparently complete digestion by 4 h, as assessed by gel electrophoresis. However, this was not indicative of deleted Xba I sites. Two explanations could account for this retention of infectivity. Firstly, as detection of DNA by ethidium bromide staining is relatively insensitive, a low level (<5-10%) of undigested DNA would probably not be detected. It is possible that a small amount of DNA could be in a restriction enzyme resistant configuration. Secondly, it is possible that digestion was complete but that religation was occurring within the cells. Dr. F. J. Rixon (personal communication) has recently shown that religation of Xba I digested HSV DNA, generating viable genomes, does occur within infected cells.

Selection enrichment has been successfully used to remove all 4 Xba I sites from HSV-2 strain HG52 (Harland and Brown, 1985, 1988; Brown and Harland, 1987). In contrast to this thesis the frequency of isolation of viruses lacking Xba I sites became higher as sites were progressively removed, suggesting that, in HG52, the Xba I sites reside within non-essential regions of the genome.* In HSV-1, the 0.63 m.u. site within the gene encoding the non-essential glycoprotein, gC, was readily removed, with a concomitant small (approximately 150 bp) deletion. Similarly, the 0.07 m.u. site was readily lost, suggesting that gene U_L2 may

also be non-essential. Alternatively, a fortuitous alteration resulted in loss of this site without a significant change in the amino acid sequence of the encoded polypeptide. The 0.45 m.u. site proved difficult to remove, implying that the apparently essential open reading frame within which it is contained (U_L33) (Dr. V. G. Preston, personal communication) is essential and would tolerate only a limited change. We were unable to remove the 0.29 m.u. Xba I site by selection enrichment. Site-directed mutagenesis was required to introduce a conservative change which did not alter the polypeptide coding region. Thus, the Xba I site may lie within an important functional domain of gH. Inability to marker rescue plasmids lacking either the 0.45 or 0.29 m.u. Xba I site due to a non-conservative change would tend to strengthen the above hypotheses.

Identification of the removal of the four Xba I sites in HSV-1 strain 17 involved the analysis of over 5,000 single plaque isolates. In contrast, in HSV-2 strain HG52, just over 300 single plaque isolates were analyzed after selection enrichment to identify loss of the four Xba I sites (Harland and Brown, 1985, 1988; Brown and Harland, 1987). Within HG52, two of the Xba I sites (0.70 (in HSV-2 strain 333) and 0.94 m.u.) lie within an intergenic region, one (0.91 m.u.) is in the coding region of the gene for the non-essential glycoprotein gG-2 (gG-2 is either not synthesized, or is present in an aberrant form, following infection with the variants lacking the 0.91 m.u. Xba I site), and one (0.45 m.u.) is in an unsequenced region, apparently colinear with the HSV-1 0.45 m.u. Xba I site, and thus may be in a gene analogous to the HSV-1 U_L33. The HSV-2

strain HG52 genome is much more variable than HSV-1 strain 17 (see section IV.2), suggesting that altered restriction enzyme sites within a population may be more common.

It will be of interest to determine the precise nature of the alterations removing the Xba I sites by DNA sequence analysis.

Originally, it was intended to carry out recombination experiments using the constructed genome, containing no Xba I sites, and strain 17, containing 4 Xba I sites. However, during *in vitro* latency reactivation experiments, an additional Xba I site at 0.74 m.u. had arisen spontaneously in X2 (Cook and Brown, 1987). This site did not arise by insertion of one of the existing Xba I sites or by a detectable (>150 bp) deletion or insertion. It may have been a spontaneous base change [there are two 5 out of 6 correct base Xba I sites in this region (Dr. D. J. McGeoch, personal communication)]. Alternatively, a small undetectable host insert containing an Xba I site may have occurred. The virus with the extra Xba I site was recombined with strain 17, to create a genome with 5 Xba I sites called 1708. The exact position of the 0.74 m.u. Xba I site with respect to known or predicted open reading frames is unknown. The infected cell polypeptide profile of 1708 is identical to strain 17. Its growth characteristics are very similar to those of wild-type virus over both a one-step and long term growth experiment, and high titre stocks were obtained ($>10^9$ pfu/ml).

Now that genomes containing multiple unselected markers (the presence or absence of Xba I sites) had been

constructed, a different selectable marker (ts lesion) was inserted into each genome. The ts lesion from ts1206 (a DNA negative mutant), which maps at 0.72 m.u. (Dr. V. Preston, personal communication) was inserted into 1708 to create ts1708. This ts lesion is situated in a gene (U_L52) which is essential for DNA replication in a plasmid-based system (Challberg, 1986; McGeoch, 1987; McGeoch et al., 1988; Wu et al., 1988). Complementation and marker rescue experiments confirmed that the ts lesion from ts1206 had been inserted into ts1708.

The ts lesion from ts1201, which maps at 0.33 m.u. (Preston et al., 1983), was inserted into 1702 to create ts1702. Ts1201 is DNA-positive, and the ts lesion is situated in U_L26, the gene encoding p40, a polypeptide involved in DNA packaging. Complementation experiments confirmed that the ts lesion from ts1201 had been inserted into ts1702. However, following recombination (see below), when some unexpected results occurred, marker rescue was carried out. This revealed that ts1702 had a second ts lesion situated at 0.414 m.u., probably in the DNA polymerase. Since it is unlikely that a spontaneous ts lesion would have arisen in the 1702 genome which had gained the ts1201 lesion, and as the 0.414 m.u. lesion is contained within Eco RI f, the fragment used to reverse marker rescue the ts1201 lesion, it is possible that the second ts lesion arose during the cloning and passaging of the plasmid. Almost the whole of Eco RI f must have been inserted, as the ts lesions are located at opposite ends of the fragment. Alternatively, two recombination events may have occurred from the one fragment. The double ts virus may have been

"tighter" at 38.5°C, and therefore more likely to be assigned ts (although ts1201 appears "tight" at this temperature). As the recombination experiments were carried out at the end of this project, time did not permit the marker rescue of the 0.414 m.u. ts lesion in ts1702 to create a genome with only the 0.33 m.u. ts lesion.

In order to further understand the role of progeny as well as parental molecules in recombination and the possible presence of recombinational hotspots in the HSV-1 genome, a time course of recombination was carried out between ts1702 and ts1708 at the permissive temperature (31°C). Samples were harvested at 0, 2, 4, 6, 8, 12 and 24 h post infection, titrated at the permissive and non-permissive (38.5°C) temperature, and the recombination frequency (RF%) between the ts lesions calculated. A control experiment was carried out between the ts parents ts1201 and ts1206, to establish that the difference in Xba I sites was not affecting recombination to a measurable extent. Similar recombination frequencies were found in each case. Originally this was taken to mean that the difference in Xba I sites was not affecting recombination. This is still true, but since in the ts1702/ts1708 experiment recombination between 0.414-0.72 m.u. was measured, whereas in the ts1201/ts1206 experiment recombination between 0.33-0.72 m.u. was measured, it probably means that recombination frequency between these markers is unlinked and at its maximum under the experimental conditions used.

Analysis of the ts1702/ts1708 results indicated that recombination frequency was increasing with time,

recombinants being first detected at 4 h post infection [close to the start of DNA replication (Wilkie, 1973)] and increasing rapidly to reach a maximum of 15% by 24 h post infection. This represents a 1000 fold increase during the course of the experiment.

Single plaques were isolated at the non-permissive temperature. By definition, these isolates must all have recombined an odd number of times between the ts lesions at 0.414 and 0.72 m.u. and contain the wild-type alleles at these regions, since no virus was detected at 38.5°C when single control infections with the ts mutants were carried out. The restriction enzyme profiles of the recombinants allowed determination of the regions of the genome within which the crossover had occurred for each virus. The number of apparent crossover events between each pair of markers (selected and non-selected) was calculated for each time point from the restriction enzyme analysis of the DNA of 100 recombinant plaque isolates. Since plaques were isolated at the non-permissive temperature, there was selection for recombination between the 0.414 and 0.72 m.u. ts lesions. For this reason, there is no increase with time in the recombination frequency between markers

in regions 4 and 5. No recombination was detected in region 3 (0.414-0.450 m.u.). The reason for this is unclear.

Recombination between the markers within the selected area is so high and the distance between the markers so large, that it can not be determined if recombination between them is above or below expectation.

The most surprising finding was the very high, time increasing, recombination frequency detected outwith the selected markers. Despite the fact that the overall recombination frequency was only 15%, between 0.07 and 0.29 m.u. the recombination frequency reached almost 50%, ie. similar to the frequency of recombination between the selected markers. This may suggest that molecules which have correctly aligned and complexed for recombination (entered the "mating pool" - section I.14a) will recombine at a very high frequency (here the theoretical maximum) compared to the frequency determined from the total pool (15% here). In other small non-selected regions (0.29-0.33 m.u.; 0.72-0.74 m.u.) the recombination frequency at 24 h was also higher than might be expected. This was especially true for the region between 0.72-0.74 m.u. It has previously been shown that the region between 0.7-0.74 m.u. of HSV-1 MP, if placed in inverted orientation elsewhere in the genome, can mediate inversion of the intervening sequences (Pogue-Geile et al., 1985; Pogue-Geile and Spear, 1986). Therefore, it is possible that a sequence lying between the 0.72 m.u. ts lesion and the 0.74 m.u. Xba I site may mediate high frequency recombination and serve as a recombination hotspot.

The average number of crossover events per virus genome increases with time. At an early timepoint (4 h post infection), 90% of genomes had only one crossover, whereas by 24 h post infection, 60% of isolates had more than one

crossover.

As previously observed in HSV-1 Glasgow strain 17 (Ritchie et al., 1977), recombination increased significantly with time, and was first detected near the onset of viral DNA replication. However, the relationship between viral DNA replication and recombination is unclear, and no experiments designed to investigate this question have been attempted. The use of inhibitors of viral DNA synthesis might help to clarify this point.

As proposed previously for HSV-1, the increasing complexity of recombinants with time would suggest that the progeny of recombination as well as parental virus are involved in recombination events (Ritchie et al., 1977). A conclusion differing from that of Ben-Porat et al. (1982) on recombination with PRV which suggested that only parental molecules take part in recombination.*

In conclusion, recombination experiments using both selected and unselected markers have shown that : (i) both progeny and parental molecules are involved in HSV-1 recombination; (ii) recombinant molecules recombine outwith selected regions at the theoretical maximum frequency, indicating that proper alignment of genomes plays an important role in determining the overall but not relative rates of recombination; (iii) the region between 0.72-0.74 m.u. displays a higher than expected frequency of recombination : 10% compared to the expected 2.1%. This may be due to a recombinogenic sequence found in this region (Pogue-Geile et al., 1985; Pogue-Geile and Spear, 1986); and (iv) nothing can be determined about hotspots of

recombination elsewhere, due to the large distance between the Xba I sites.

To determine the overall recombination frequency outwith the selected region , and also to see if the ts marker affects recombination between 0.414-0.45 m.u., it would be interesting to analyze plaques isolated at the permissive temperature, ie. those viruses which had recombined either outwith the ts markers, or an even number of times between the ts markers, or those viruses which need not necessarily have recombined at all.

IV.2 ISOLATION AND ANALYSIS OF HSV-1 GENOMES CONTAINING REARRANGEMENTS

During the generation of HSV-1 genomes for use in recombination studies, a number of HSV-1 variants with genomic rearrangements were isolated. Some of the variants displayed altered immediate-early polypeptide synthesis. These variants will be discussed at two levels : firstly, in terms of their genomic structure and factors which might have caused the altered structures, and secondly, in terms of their immediate-early polypeptide synthesis.

IV.2.a Genomic structure of rearranged HSV-1 genomes

The DNA analysis of single plaque isolates allows the elucidation of genomic rearrangements within a virus population; something which is not normally identifiable in studies of pooled virus populations. Such studies may lead to the identification of (i) regions of the genome exhibiting instability and, in the long term, sequences

involved in genome rearrangements; (ii) viable genome structures, especially with regard to the length and necessity of the repeat regions of the genome; and (iii) regions of the genome (and genes contained therein) that are non-essential for growth in tissue culture.

To detect viruses lacking Xba I sites DNA analysis of over 5,000 plaques derived from transfection experiments was carried out. From such screening, one variant (1703) which had a deletion of about $5x10^6$ mol. wt. in the long internal repeat (IR_L) and adjacent unique sequences was isolated. The deletion starts just downstream from the second intron of IE1, extends across the IR_L/U_L junction and terminates approximately 500 bp downstream of IE2. Thus, the deletion removes the 3' end of the IR_L copy of IE1 and two unique open reading frames (U_L55 and U_L56) predicted to encode polypeptides of approximately 20K and 22K (Perry, 1986). The predicted 20K polypeptide is conserved in VZV (Davison and Scott, 1986; Dr. A. J. Davison, personal communication).

The origin of 1703 is open to speculation. It arose following transfection of BHK21 C13 cells with Xba I digested HSV DNA. Interestingly, this was the same transfection that gave rise to 1701, which lacks the 0.45 m.u. Xba I site. Both 1701 and 1703 were tk^-, presumably as a result of a secondary mutation unrelated to the site loss or deletion. The tk^- mutation in 1703 was rescued without affecting the size of its deletion or its apparent lack of synthesis of VmwIE63 (see II.3); this is also the case for 1701 and its derivative 1702 (see IV.1) (Dr. F. J. Rixon, personal communication). It is possible that some event occurred to cause mutagenesis during this transfection. It

has previously been suggested that transfection induces mutations (Calos et al., 1983), although the findings presented in this thesis, and similar findings with wild-type HSV-2 strain HG52 (Harland and Brown, 1985; personal communication), would tend to disagree with this suggestion.

Determination of the exact endpoints of the deletion in 1703 requires DNA sequence analysis. However, restriction enzyme analysis indicates that the deletion endpoints are not located at any of the short tandem reiterations present in R_L, although the deletion itself removes the 3 sets of reiterations situated downstream of IE1 (I.6). It would be interesting to determine if there is any sequence homology at the endpoints of the deletion, as has previously been found with a HSV-1 virus deleted in U_S (Umene, 1986). Small regions of sequence homology have previously been suggested to play a role in illegitimate recombination (section I.14.b). No other variants with detectable genome rearrangements were isolated from these particular transfections, although small deletions (<150 bp) would not be detected within the limits of the restriction enzyme analysis carried out.

The DNA of single plaque progeny of recombination experiments was also subjected to restriction enzyme analysis. From one recombination experiment, the genomes of 11 out of 80 plaques analyzed showed extensive size variation (up to several hundred base pairs) in the long repeats of the genome (excluding the 'a' sequence). The variation displayed is much more extensive than that previously identified in the long repeat (Lonsdale et al.,

*However, in 1704 the latency associated transcripts (LATs) situated downstream of and overlapping the 3'end of IE1 are absent from both long repeats as there is no evidence of their transcription during latency. However this virus becomes latent, is maintained in a latent state and reactivates (possibly somewhat slower) in an apparently normal manner (Dr. N. Fraser, personal communication).

VmwIE110 (Stow and Stow, 1986; Sacks and Schaffer, 1987), have been totally lost. Harland and Brown (1985) have previously identified a variant of HSV-2 strain HG52 which has short overlapping deletions in both long repeats.

Variant 1705 has a deletion of $3.5x10^6$ mol. wt. in IR_L/U_L. The deletion again starts downstream from IE1, removes the open reading frames for the putative 20K and 22K polypeptides (U_L55 and U_L56), and terminates just downstream from the 3' end of IE2. Thus, again IE2 should be intact.

Variant 1706 is considerably more complex. It has a similar deletion in U_L to that of 1705, but the deleted sequences have been replaced by sequences from the left end of U_L in the opposite orientation, thus generating an extended long repeat. Approximately $1.0x10^6$ mol. wt. has been deleted from the right end of U_L and this has been replaced by $3.0x10^6$ mol. wt. from the left end of U_L. Thus, the long repeat has been extended by $3.0x10^6$ mol. wt. and the overall genome length by $2.0x10^6$ mol. wt. Again, IE1 is unaffected, U_L55 and U_L56 have been deleted, and the deletion terminates just downstream from the 3' end of IE2. Genes in the left terminal portion of U_L are therefore in diploid amounts. No known polypeptides are coded for in the left terminal portion of U_L, but two open reading frames (U_L1 and U_L2) have been identified (Perry, 1986).

The relationship, if any, between these 3 variants, and the mechanism by which they arose, are unclear. Variants 1704, 1705 and 1706 arose in the same recombination experiment, along with the 11 variants with minor alterations described above. Considering that previously only one other HSV-1 strain 17 variant had been isolated

1980; and personal observations); it could be due to variation in the copy number of the blocks of tandemly reiterated short sequences found in R_L (Rixon et al., 1984; Perry, 1986); much smaller variation in their copy number is found between different plasmid clones (Davison and Wilkie, 1981; Rixon and McGeoch, 1984; Dr. F. J. Rixon, personal communication).

The size variation in TR_L and IR_L occurred independently, and thus in some of the variants the long terminal repeat differs in size from the long internal repeat, a finding supporting that previously described by Lonsdale et al. (1980). The variation remained stable over three rounds of plaque purification; thus, HSV-1 isolates with different sizes of R_L appear to be stable.

A further three plaques exhibited extensive rearrangements in IR_L/TR_L. These three deletion variants were designated 1704, 1705 and 1706, respectively.

Variant 1704 has a 0.8×10^6 mol. wt. deletion in TR_L and a 2.5×10^6 mol. wt. deletion in IR_L/U_L, extending up to 0.6×10^6 mol. wt. into U_L. No coding sequences are known to be removed from the long repeat, as the deletions start downstream from the 3' end of IE1.* The IR_L/U_L deletion removes the open reading frames for the putative 20K and 22K polypeptides (U_L55 and U_L56), and terminates about 500-1200 bp downstream from IE2. Thus, IE2 appears to be intact. The size of the deleted fragments suggests that there must be an overlap between the TR_L and IR_L deletions, with $0.2\text{-}0.8 \times 10^6$ mol. wt. of DNA missing from both long repeats. To our knowledge, this is the first time that sequences from part of the long repeat of HSV-1, outwith that coding for

from a total of over 5,000 plaque isolates analyzed, it seems likely that these variants have a common origin. As previously stated, it is possible that the variation in R_L is due to variation in the copy number of the tandemly repeated sequences. Three sets of repeat sequences are located downstream from IE1 and it is possible that one of these repeat sequences forms the right end of the deletions in 1704 and 1705, and that recombination with short homologous sequences in U_L was responsible for the generation of the deletions. It is suggested that the right end of the IR_L deletions in 1704 and 1705 is identical and that the variants arose from the same progenitor molecule, with the deletion extending further to the left in 1705. The TR_L deletion in 1704 could have arisen in the process of recombination between a wild-type and a deleted repeat.

How did the extended repeat in 1706 arise? The model previously proposed for the expansion or contraction of the repeats (McGeoch, 1984; Whitton and Clements, 1984b; Davison and McGeoch, 1986) could explain the origin of 1706. Recombination between two wild-type viruses with U_L in opposite orientations would occur homologously in the repeat regions and illegitimately, possibly through short partially-homologous sequences, in the unique portion of the two genomes, to give an extended repeat arising from one end of U_L with the other end of U_L becoming deleted (Figure 74). If it is assumed that the endpoint of the deletion in 1705 and 1706 is the same, then recombination between IR_L of 1705 and TR_L of wild-type virus in the opposite orientation could have resulted in 1706. The novel unique/repeat junction in 1705 may be less stable than the wild-type junction,

facilitating such an event. The other potential product of this recombination event, in which the left end of U_L is deleted and the right end duplicated, has not been isolated, either because such a genome is inviable or because of the low number of plaque isolates analyzed.

This is the first reported occurrence of an extended long repeat in HSV, although Umene (1986) and Brown and Harland (1987) have postulated a similar mechanism in the derivation of genomes with an extended short repeat. From the same transfection, Brown and Harland (1987) also isolated a variant genome with a deletion at one end of U_S (similar to that in the viruses with an extended repeat) but with no duplication of sequences from the left of U_S. A situation analogous to that with 1705 and 1706. Brown and Harland (1987) speculated that the variant with a deletion in U_S might have been involved in the formation of the two variants with extended short repeats.

The occurrence of three deletion variants, as well as genomes with extremely variable repeat lengths, in a HSV-1 strain not previously known to be prone to rearrangements, raises the question of why these occurred. Possibly they were mediated by high multiplicity of infection in a recombination experiment. However, previous and subsequent recombination experiments have not yielded any variants. It is possible that the particular cells used affected the outcome. It is known that HSV recombination frequencies vary with different batches of BHK21 C13 cells (Taylor, 1978). If the particular cell batch used in this experiment was highly recombinogenic, this might have led to a high frequency of HSV recombination, both homologous and illegitimate. The

deletion variants were isolated from a cross to generate a recombinant with five Xba I sites; from the 80 plaques analyzed the desired recombinant was not isolated, which would appear to run counter to the postulate that the cells were highly recombinogenic.

A more favoured explanation is that one variant genome arose which, because of instability, went through several rounds of rearrangement and recombination prior to the generation of stable genomes; thus, one initial event could potentially lead to the isolation of several variants in a population.

The relationship between these variants and the sequences involved in such rearrangements could be determined by DNA sequence analysis of the mutants and comparison with the wild-type sequence.

A variant (1709) with an approximate 0.5×10^6 mol. wt. deletion in both TR_S and IR_S has also been identified from a later recombination experiment. This was isolated towards the end of this project and therefore neither the characteristics of the variant nor the exact boundaries of the deletion have been determined. It is not known if the same sequences are deleted from both repeats. The short reiterated sequences in R_S might have had a role to play in the generation of the deletions in 1709.

All the deletions reported in this thesis involve one of the repeat regions (mainly R_L) and adjacent unique sequences, suggesting that the repeat elements may act as hotspots for illegitimate recombination. This is similar to the findings of other workers, who have isolated virus

genomes with rearrangements around the repeats (Brown et al., 1984; Harland and Brown, 1985; Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987). While some of the latter deletion variants have pre-existed in wild-type virus stocks, most noticeably in the case of Harland and Brown (1985), where 24% of single plaque isolates from one HSV-2 strain HG52 wild-type stock contained a deletion in R_L, most have been found in "manipulated" virus populations. "Manipulations", such as transfection and restriction enzyme treatment (Brown et al., 1984; Harland and Brown, 1987), genome disruption (Longnecker and Roizman, 1986) or high multiplicity of infection used in recombination experiments (Umene, 1986), may serve to increase the instability of virus genomes, leading to an increased rate of generation of rearrangements in naturally unstable regions of the genome.

Although the described rearrangements are present in similar regions of the genome in different strains of HSV, the frequency of deletions varies considerably. Thus, HSV-2 strain HG52 is highly unstable (Harland and Brown, 1985; Brown and Harland, 1987; Miss J. Harland, personal communication), while HSV-1 strain 17 is highly stable (Brown et al., 1984; this thesis). However, this is not a general property of HSV-1 compared to HSV-2, but appears to be due to particular strain differences (Harland and Brown, 1985). The reason for the difference in genome stability is unknown, but it is interesting to note that in the HSV-1 strain 17/HSV-2 strain HG52 intertypic recombinant R12-5, which has one long repeat region from HSV-1 and the other from HSV-2, deletions were found in both the HSV-1 and HSV-2 sequences at roughly similar frequencies (Brown et al.,

1984), in contrast to the situation found in intact strain 17 or HG52. One possible reason might be the presence of more frequent and larger banks of tandem repeats in some strains. Alternatively, some virus strains may encode more mutagenic replication/recombination enzymes. The findings with R12-5 tend to support the latter suggestion.

It is interesting to note that as well as exhibiting large scale genomic rearrangements, the virulence in mice of single plaque isolates of HSV-2 strain HG52 is extremely variable; in contrast, HSV-1 strain 17 single plaque isolates do not differ significantly in virulence (Mr. M. Taha, personal communication; Dr. G. B. Clements, personal communication). This is perhaps due to minor, undetectable sequence alterations affecting virus phenotype.

The isolation of HSV variants with extended and deleted long repeats provides experimental evidence for the previously proposed models for extension and contraction of the repeats (McGeoch, 1984; Whitton and Clements, 1984b; Davison and McGeoch, 1986; I.12.d).

IV.2.b Immediate-early polypeptide synthesis of rearranged HSV-1 genomes

Variant 1703 is deleted in one copy of IE1 and hence underproduces VmwIE110. This is similar to the findings of Harland and Brown (1985) for HSV-2 genomes deleted in one copy of IE1. Since the deletions in 1704, 1705 and 1706 do not extend into IE1, they synthesize VmwIE110 apparently normally.

Surprisingly, despite the fact that the deletion in 1703 terminates at least 500 bp downstream from the 3'

terminus of IE2, VmwIE63 could not be detected at either the polypeptide or RNA level under immediate-early conditions. The sensitivity of the assays used was at least 6.7%.

It seems unlikely that the detected deletion in 1703 is affecting IE2 synthesis, as 1704, whose deletion terminates at a similar position, synthesizes VmwIE63 normally. In 1705 and 1706, where the deletion extends to around the 3' terminus of IE2, the synthesis of VmwIE63 is reduced to approximately half the wild-type level, possibly because of an effect on the termination signals, such as the 'TGTGTTYY' box (McLauchlan et al., 1985). All recognized sequences affecting termination are situated within 50-150 bp of the polyadenylation signals (McLauchlan, 1986). DNA sequence analysis of the endpoints of the deletions in 1705 and 1706 will be required to confirm this hypothesis.

The lack of synthesis of VmwIE63 by 1703 is extremely puzzling. There is no apparent deletion within either the coding, or the 5' or 3' regulatory sequences. Furthermore, Sacks et al. (1985) have identified 4 ts mutants of HSV-1 strain KOS which map in IE2, suggesting that VmwIE63 is essential for virus growth in tissue culture. Although it is possible that the 4 ts mutants of Sacks et al. are in fact host range ts mutants, like the IE4 mutants (Ackerman et al., 1985; Sears et al., 1985), it was found that 1703 grew equally well on BHK21 C13 cells and Vero cells, the cells used by Sacks et al. (1985). A number of possible explanations exist for the apparent discrepancy between the two sets of results. (i) There is a strain difference between strain 17 and KOS regarding the requirement for VmwIE63. (ii) A non-functional ts VmwIE63 may hinder

protein-protein or protein-DNA interactions, rendering the virus inviable, whereas the complete lack of production of VmwIE63 may be tolerated. A similar suggestion has been made to explain ts IE175 trans-dominance (DeLuca et al., 1985). (iii) 1703 produces a very low, but undetectable, level of VmwIE63 under immediate-early conditions which is adequate for normal lytic growth. (iv) In 1703, VmwIE63 is not synthesized as an immediate-early polypeptide, but is synthesized under non-immediate-early, probably early, conditions. In this respect it is interesting that the ts mutants of Sacks et al. (1985) produce normal early polypeptides and DNA synthesis, but do not synthesize late polypeptides. Everett (1986) has shown that VmwIE63, in conjunction with VmwIE110 and VmwIE175, serves to transactivate late, but not early, gene transcription. Thus, VmwIE63 may be required at later times in infection.

Unfortunately, due to high background levels, it has not been possible to investigate IE2 expression under non-immediate-early conditions. Immunoblotting with the anti-peptide sera failed to detect VmwIE63 in wild-type virus, while the background in immunoprecipitation experiments carried out under non-immediate-early conditions prevented the specific identification of VmwIE63. Use of a sensitive and specific S1 assay might allow the detection of IE2 RNA expression at later times in infection. Of the explanations given above, it would seem likely that either (iii) and/or, more probably, (iv) is the reason for the apparent anomaly between our results and those of Sacks et al. (1985).

The nature of the defect leading to altered IE2

expression has not been elucidated. It is not likely to be associated with the $5x10^6$ mol. wt. deletion, and is probably due to a secondary, undetected alteration. It is unlikely that the defect lies outwith IE2, in a gene responsible for the transactivation of this gene, as there is at present no evidence for a polypeptide being specifically responsible for transactivation of IE2, and there are no other obvious alterations in the polypeptide profile of the virus. It would also seem unlikely that the alteration lies within the coding sequences for VmwIE63, since, in that case, we would expect to detect IE2 RNA. More probably, the lesion lies within either the 3' or 5' regulatory sequences of the gene. For example, a mutation within the immediate-early regulatory sequence 'TAATGARAT' (Gaffney et al., 1985), could lead to IE2 becoming an early gene.

IV.2.c Growth properties of the deletion/rearrangement variants

Variant 1703 grows normally in tissue culture. Variants 1704, 1705 and 1706 show similar growth properties to each other, but are impaired in their rate of growth when compared to wild-type virus. However, they produce a similar 24 h yield compared to wild-type virus, and high titre stocks ($>10^9$ pfu/ml) have been obtained, indicating that the growth impairment is overcome in time. It seems possible that the growth impairment is not due to the observed deletion (as the deletions in these viruses differ slightly, and are similar to 1703) but to a secondary unrelated defect elsewhere in the genome. An alternative explanation is that different plaque isolates in a stock exhibit different

growth properties. As 1704, 1705 and 1706 exhibit similar growth properties this would favour the suggestion that they arose from the one original plaque isolate.

The variants have not been tested *in vivo* for either neurovirulence or latency.

FUTURE PROSPECTS

As well as their use in recombination experiments, HSV-1 genomes lacking Xba I restriction enzyme sites have been useful for intratypic superinfection to rescue viral DNA from latent cultures (Cook and Brown, 1987). A virus lacking Xba I restriction enzyme sites allows the possibility of introducing new Xba I sites at desired positions for use as a eukaryotic vector, or as a recipient for mutagenized cloned viral fragments at desired positions (Dr. F. J. Rixon, personal communication), in a manner analogous to the adenovirus mutant dl309 (Jones and Shenk, 1979; Stow, 1981).

The virus lacking Xba I sites has been used in preliminary recombination experiments. Several other experiments could now be carried out. In a manner analogous to adenovirus recombination, superinfection could be carried out at different times following initial infection with one virus, to determine how late into the virus replication cycle recombination will occur, and especially to determine whether the onset of DNA replication prior to superinfection would block recombination. The use of DNA synthesis inhibitors, such as phosphonoacetic acid, would allow the determination of whether replication (or the replication

machinery) is essential for recombination, and what role DNA synthesis plays in the increase in recombination frequency with time. Use of parent molecules containing the same ts lesion might allow the role of certain HSV gene products in DNA recombination to be determined. Markers in U_L and U_S could be used to determine the role of different genome isomers and the replicative forms involved in recombination. To this end, a HSV-2 genome lacking Xba I sites (wild-type sites at 0.45, 0.7, 0.91 and 0.94 m.u.) has been constructed and ts lesions inserted for recombination experiments (Harland and Brown, 1985, 1988; Dr. S. M. Brown and Miss J. Harland, personal communication).

To determine the relative frequency of recombination in different areas of the genome much more closely spaced markers are required. Thus, the sites of more frequent cutting restriction enzymes, Hind III in L and S and Bam HI in S, are currently being removed in our laboratory. As the whole of the HSV-1 genome has now been sequenced, the approach of site-directed mutagenesis is being used. Thus, the protein coding potential is not altered and minimal changes are made to sequence homology. Study of recombination in the short region will be of interest, as it might be expected that the tandem repeats scattered throughout this region may affect recombination frequency. One problem, however, will be identifying the small Bam HI fragments in the short region.

To determine the precise genomic alterations which have led to the loss of the Xba I restriction enzyme sites following selection enrichment, these should be sequenced at the DNA level and compared to wild-type virus.

One of the objects of studying deletion variants is to determine whether specific sequences tend to be involved in illegitimate recombination and the role of short sequence homologies. Thus, DNA sequence analysis of both the HSV-1 deletion variants generated in this study, and the HSV-2 strain HG52 deletion variants generated by Miss J. Harland and Dr. S. M. Brown, will be important to determine the precise endpoints of the respective deletions, and would also help to establish the proposed relationship between the variants 1704, 1705 and 1706.

Regarding the apparent lack of expression of VmwIE63, it is important to determine whether this polypeptide is being expressed under non-immediate-early conditions. To determine if there is a secondary deletion in either the coding or 5' or 3' regulatory regions, these should be sequenced and compared to wild-type. Finally, to fully resolve the question concerning the essential nature of VmwIE63, a deletion mutant in this gene could be constructed.

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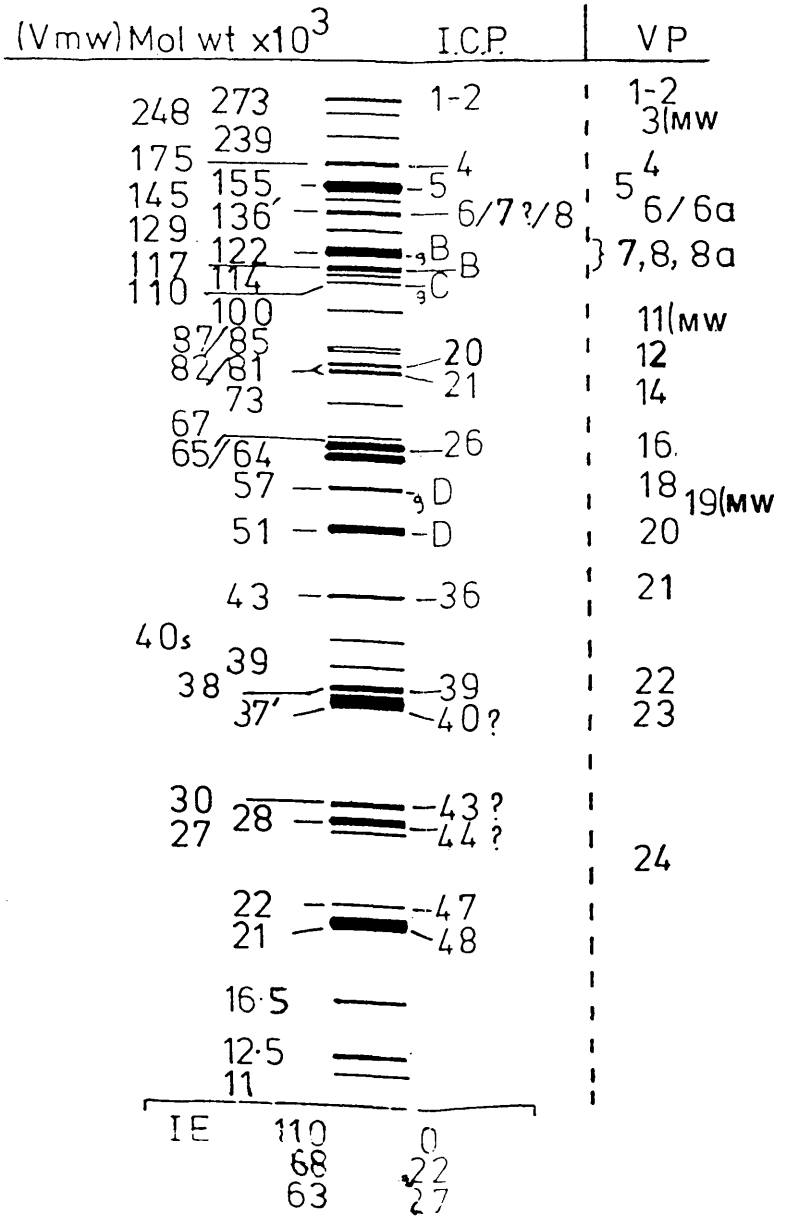
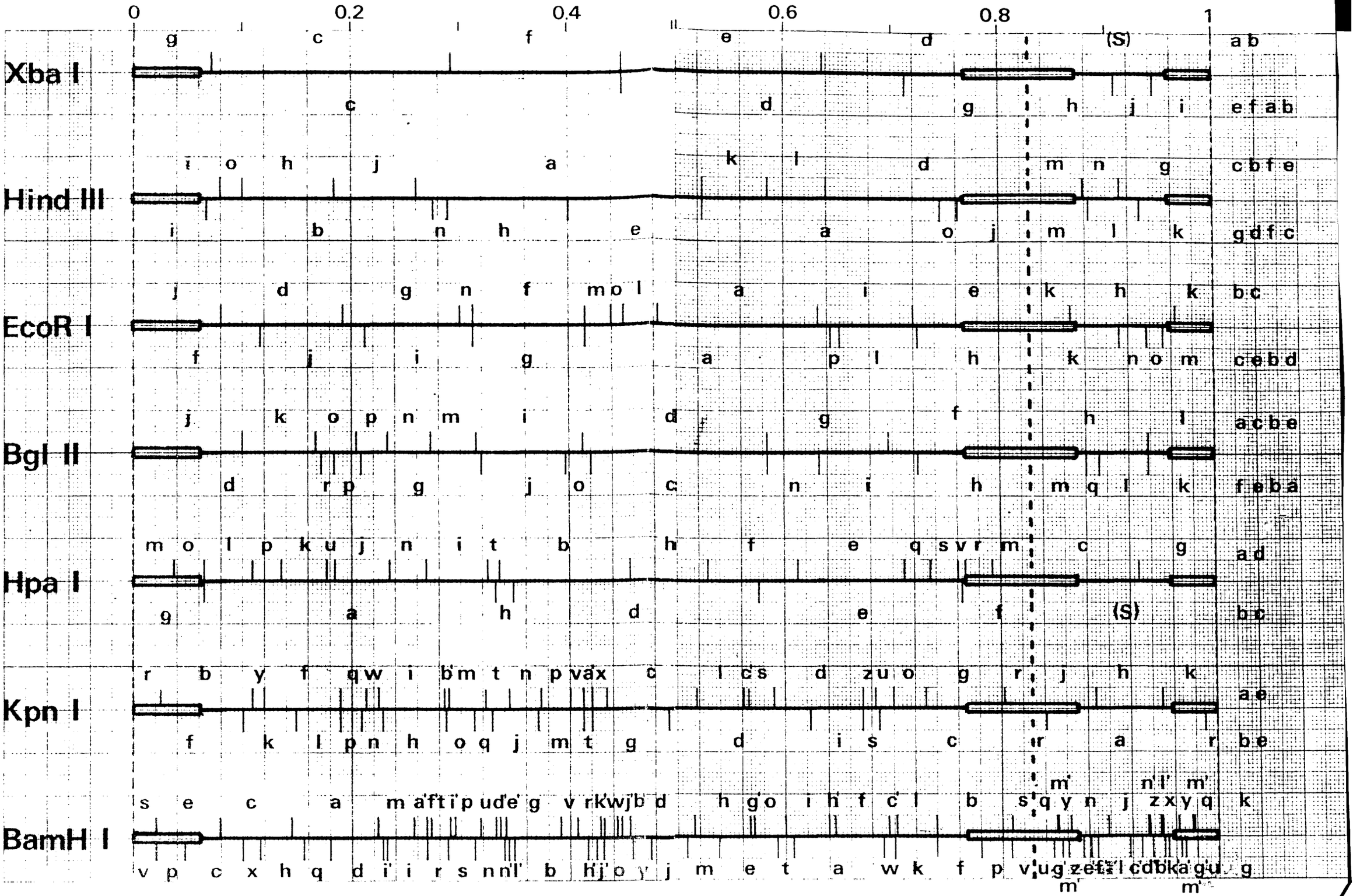
HSV-I POLYPEPTIDE NOMENCLATURE

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