ISOLATION AND STRUCTURAL ANALYSIS OF GENOMIC VARIANTS OF HERPES SIMPLEX VIRUS TYPE 2

by

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buy the long awaited hat.

Unless otherwise stated all the work described in this thesis was carried out by the author.

SUMMARY

The original aim of the project was to study recombination in HSV-2 strain HG52 using restriction enzyme sites as unselected markers. As no relevant DNA sequence data was available for HSV-2 it was decided to isolate site deletion mutants by enrichment selection of spontaneously occurring variants. The restriction enzyme Xba I was chosen as it makes only four staggered cuts in the HSV-2 genome and a virus, HG52X163X3X53, lacking all four Xba I sites was isolated following three rounds of enrichment selection. However, during the screening procedures involved in the isolation of HG52X163X3X53, a large number of variants with genomic alterations was identified and the work described in this thesis has concentrated on the analysis and characterization of these variants.

Of the variants isolated after the initial enrichment selection of HG52 DNA five have been studied in detail. Three (HG52X85/4, HG52X85/5 and HG52X86) have deletions from IR_L ranging in size from $2.5x10^6$ daltons to $6x10^6$ daltons. Under immediate early conditions VmwIE64 production by HG52X85/5 and HG52X86 in which only the 3' part of IE1 is removed is reduced despite their deletions ending approximately 1kb downstream from the 3' end of UL54 which encodes this polypeptide. HG52X85/4 which also has a deletion ending approximately 1kb downstream of UL54 but in which an entire copy of IE1 is removed makes VmwIE64 in normal amounts. Of the two remaining variants one, HG52X192, has a deletion of approximately $1x10^6$ daltons in each copy

of R_L. The final variant, HG52X19, has a deletion of approximately $9x10^6$ daltons which removes the entire internal copy of the long repeat and half of the short repeat. The long segment of the genome is fixed in the prototype orientation whilst the short region inverts inefficiently through the undeleted part of the repeats (the internal copy of the 'a' sequence being deleted). The genome has non-HSV DNA inserted across the deletion. The inserted DNA has been reiterated to give different copy numbers and hence a heterogeneous genome population. It has been demonstrated that the inserted DNA is of bovine origin - presumably the result of recombination with the calf thymus DNA used as a carrier during transfection.

To investigate if the enrichment selection procedure was responsible for the production of the unexpectedly high proportion of variants, isolates from untreated HSV stocks were studied. Of the fifty plaques of HG52 analysed, twelve differed significantly from the wild type structure. However, the plaques, five of HG52/5 and seven of HG52/10, represented only two variants with the same genome structures as HG52X86 and HG52X192 respectively. No significant variation was found in the stocks of other HSV strains examined (ie. HSV-2 strains 333 and 186 and HSV-1 strains 17 and KOS or plaque purified HG52 isolates (eg.ts1)). Variation was found in HSV-1 strain McKrae but its relevance was impossible to assess as the history of the stock is unknown. The conclusion has been drawn that variation has arisen in HG52 in the absence of enrichment selection although the structure of HG52X19 must have resulted from the transfection procedure.

Three variants, HG52X163X12, HG52X163X14 and

HG52X163X21, were isolated following the second round of enrichment selection using as the parent HG52X163 which lacks the 0.7m.c. Xba I site. HG52X163X12 has a deletion removing sequences from 0.94m.c. to 0.99m.c. (ie. part of U_S and almost all of TR_S). The isolation of this variant demonstrates that genes US 10, 11, 12 and one copy of IE3 and one copy of ori_S are non essential, at least <u>in vitro</u>. The two remaining variants, HG52X163X14 and HG52X163X21, both have deletions removing the same region as from HG52X163X12. However, the deleted sequences are replaced by sequences between 0.83m.c.-0.91m.c. in the inverted orientation thereby effectively deleting U_S between 0.94-0.96m.c. while extending the short repeats by 6kb. The only differences found between HG52X163X14 and HG52X163X21 were the loss of the 0.91m.c. Xba I site and a small insert containing a Hind III and EcoR I site at the 0.94m.c. Xba I site in the latter. It is possible that the enrichment selection procedure was responsible for the generation of these three variants as the rearrangements appear to be associated with the Xba I sites at 0.91 and 0.94m.c.

ABBREVIATIONS

BHK21/C13	baby hamster kidney cells batch 21 clone 13
BSA	bovine serum albumin
^{o}C	degrees centigrade
Ci	Curie
cpe	cytopathetic effect
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	ethylenediaminetetra acetic acid
h	hour
HSV	herpes simplex virus
IE	immediate early
IR_L	long internal repeat
IR_S	short internal repeat
k	kilo (ie. 10^3)
kbp	kilobase pairs
L	long segment
M	molar
MBq	mega Becquerels
m.c.	map coordinates
mg	milligram
min	minute
ml	millitre
mm	millimetre
mM	millimolar
MOI	multiplicity of infection
mol. wt.	molecular weight
mRNA	messenger ribonucleic acid
nm	nanometre (ie. 10^{-9}m)
NPT	non permissive temperature
ori_L	origin of replication in U_L
ori_S	origin of replication in IR_S/TR_S
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming unit

PI	post infection
RE	restriction endonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
S	short segment
SDS	sodium dodecyl sulphate
syn	syncytial
syn^+	non-syncytial (wild type)
TEMED	N,N,N',N'-tetramethylethylene diamine
TCA	trichloracetic acid
TP	tryptose phosphate (broth)
TR_L	long terminal repeat
TR_S	short terminal repeat
ts	temperature sensitive
ts^+	non-temperature sensitive (wild type)
U_L	long unique
U_S	short unique
V	volts
Vmw	molecular weight in kilodaltons of HSV induced polypeptide
v/v	volume/volume (ratio)
w/v	weight/volume (ratio)
w/w	weight/weight (ratio)
uCi	microcurie
ul	microlitre

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 OBJECTIVES

The original aim of the project was to isolate a herpes simplex virus type 2 (HSV-2), strain HG52, genome containing non-selectable markers which could be used to study HSV recombination. Removal of the Xba I endonuclease sites (two in the long unique (U_L) region and two in the short unique (U_S) region) provided four non-selectable markers in the HG52 genome. However, the emphasis of the work changed due to the isolation of a number of deletion/insertion variants in both the long and short regions of the HG52 genome. Restriction endonuclease characterization and Southern blot analysis of these novel genome structures together with studies on their biological properties became the main focus of research.

As the total DNA sequence analysis of HSV-2, strain HG52, is yet to be completed some of the interpretation of data has been inferred from the known nucleotide sequence of HSV-1. The literature on HSV relies heavily on studies of type 1 and this is necessarily reflected in the text. The introduction concentrates on those aspects of herpes virus research which are pertinent to the scope of the work presented in this thesis.

1.2 CLASSIFICATION OF HERPESVIRIDAE

More than eighty members of the Herpesviridae have been identified on the basis of the morphological characteristics which define the family (Fenner, 1976). Of these

herpesviruses, five (herpes simplex virus types 1 and 2 (HSV-1 and 2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV)) have been shown to infect Man as their primary host. Recently a sixth human herpesvirus (HHV-6), originally called human B-lymphotropic virus (HBLV), has also been described (Josephs et al., 1986; Salahuddin et al., 1986). Although originally isolated from patients with lymphoproliferative disorders it is now apparent that this is not a prerequisite for infection, with evidence of exposure to HHV-6 amongst a substantial proportion of adults (Briggs et al., 1988).

The four morphological elements which define the Herpesviridae are: (1) A central core consisting of a proteinaceous matrix (Furlong et al., 1972; Nazerian, 1974). The core, around which the double-stranded linear DNA is wound (Ben-Porat and Kaplan, 1962), probably contains the polypeptide VP21 of approximate molecular weight 43,000 daltons (Gibson and Roizman, 1972). (2) Surrounding the core is an icosahedral capsid approximately 100nm in diameter constructed from 150 hexameric and 12 pentameric capsomeres. It is thought that the hexameric capsomeres consist 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 pentameric capsomeres may contain a protein of 50,000 dalton molecular weight (Vernon et al., 1981). (3) Surrounding the capsid is the tegument (Roizman and Furlong, 1974), an amorphous layer thought to contain virion proteins VP1-3 of approximate molecular weights 273,000-239,000 daltons (Gibson and Roizman, 1972) and the 65,000 molecular weight trans-inducing factor (Batterson and Roizman, 1983; Campbell et al., 1984) and (4) An envelope

consisting of a trilaminar membrane surrounding the tegument layer to give a virion 150-200nm in diameter with glycoprotein spikes projecting from the surface (see Fig. 1).

By definition all Herpesviridae share morphological characteristics. However, they can be broken down on the basis of their biological properties into three sub-families : Alpha-, Beta- and Gamma-herpesvirinae (Roizman et al., 1978; Mathews, 1982; Roizman, 1982). Alternatively the family may be subdivided on the basis of genome structure (Honess and Watson, 1977; Roizman, 1982; Honess,1984)

1.2a CLASSIFICATION ON THE BASIS OF BIOLOGICAL PROPERTIES

Alphaherpesvirinae: Viruses in this group (eg. herpes simplex virus types 1 and 2) have a relatively rapid replicative cycle in vitro and in vivo and usually cause an acute primary infection followed by the establishment of latency in the sensory ganglia (Stevens and Cook, 1971; Baringer and Swoveland, 1973 ; Baringer, 1974).

Betaherpesvirinae: The viruses in this group are typified by human cytomegalovirus (HCMV) which has a slow replicative cycle in vitro and in vivo and usually causes asymptomatic infection in tissues including the kidneys or secretory glands. Human CMV is of importance as it may cause foetal abnormalities or severe disease in immunosuppressed individuals. It has been implicated with the European form of Kaposi's sarcoma (Giraldo et al., 1975) and cervical carcinoma (Alford and Britt, 1984; Fletcher et al., 1986).

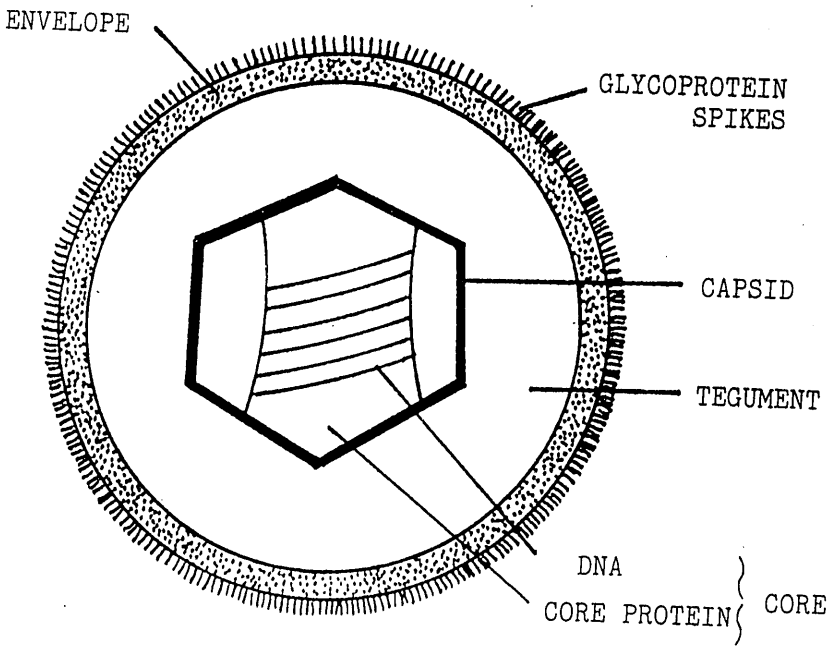
Although it is a lymphotropic virus there is evidence from sequence analysis and cross-hybridization studies that human herpes virus 6 (HHV-6) is more closely related to HCMV than to EBV suggesting its inclusion in the

FIGURE 1.

Herpes simplex virion structure

Schematic diagram of a HSV virion showing the core, capsid, tegument and envelope. The core comprises a proteinaceous mass around which the viral DNA is toroidally wound. The virion envelope contains a number of glycoproteins, visible on electron microscopy as spikes protruding from the envelope surface.

1.


ENVELOPE
GLYCOPROTEIN SPIKES
CAPSID
TEGUMENT
DNA
CORE PROTEIN
CORE

Betaherpesvirinae rather than the Gammaherpesvirinae as indicated by the cells permissive for HHV-6 infection (McGeoch, 1989; Efstathiou et al., 1988).

Gammaherpesvirinae; The viruses in this group replicate in either B or T lymphocytes and establish latency in lymphoid tissue. An example of B lymphotropic viruses is Epstein-Barr virus (EBV) the cause of infectious mononucleosis which in adults can lead to prolonged illness although the primary infection in childhood is normally asymptomatic. An association has been established between EBV and Burkitt's lymphoma (Epstein et al., 1964; de The et al., 1978) and nasopharyngeal carcinoma (zur Hausen et al., 1970; Nonoyama and Pagano, 1973; Miller, 1985).

1.2b CLASSIFICATION ON THE BASIS OF GENOME STRUCTURE

At present at least five separate groups have been identified with distinct genome structures. However, as the DNA structures of the majority of Herpesviridae are not yet known such classification is incomplete. This type of classification is somewhat arbitrary as small structural changes in the genome can easily alter the classification. For example, some pseudorabies virus (PRV) variants, because they have evolved short inverted repeats at the termini of U_L, are included in EI whereas the prototype PRV falls within group D. Likewise, variants of HSV in which sequences introduced at the thymidine kinase locus allow additional inversion (Mocarski et al., 1980; Mocarski and Roizman, 1981; Smiley et al., 1981) may be classified differently to their parental genomes. The variants like HG52X19 described in this thesis, which have a deletion removing 'a' sequences, thereby causing fixed or semi-fixed orientation

of the unique segments, may also fall into different groups from their parental genomes and even create novel groups. Figure 2 shows a diagrammatic representation of the genome structures of the herpesviruses.

Group A This group is characterized by a genome with a single isomeric form consisting of a unique portion of DNA bounded by a single direct repeat at both termini (eg Channel catfish virus (CCV)) (Chousterman et al., 1979).

Group B This group is exemplified by herpes virus saimiri (HVS) with a genome present in a single isomeric form (Bornkamm et al., 1976). The unique portion is flanked by multiple copies of a direct repeat at both termini. The number of repeat copies at each end varies widely while the overall number of repeats remains more or less constant (Stamminger et al., 1987).

Group C This group, typified by Epstein-Barr virus (EBV), has its DNA present as a single isomer bounded by multiple copies of a direct repeat at both termini (Raab-Traub et al., 1980). Additionally there are internal tandem reiterations of a different sequence from that present at the termini.

Group D The genomes of this group have two covalently bound segments; a long unique (U_L) portion of fixed orientation and a short unique (U_S) region flanked by inverted repeats which allow inversion of U_S to give two possible isomeric forms. An example of a group D virus is pseudorabies virus (PRV) (Ben-Porat et al., 1979)

Group E This group has been divided into two subgroups EI and EII. Viruses within group EI are characterized as having one long and one short unique region bounded by

FIGURE 2

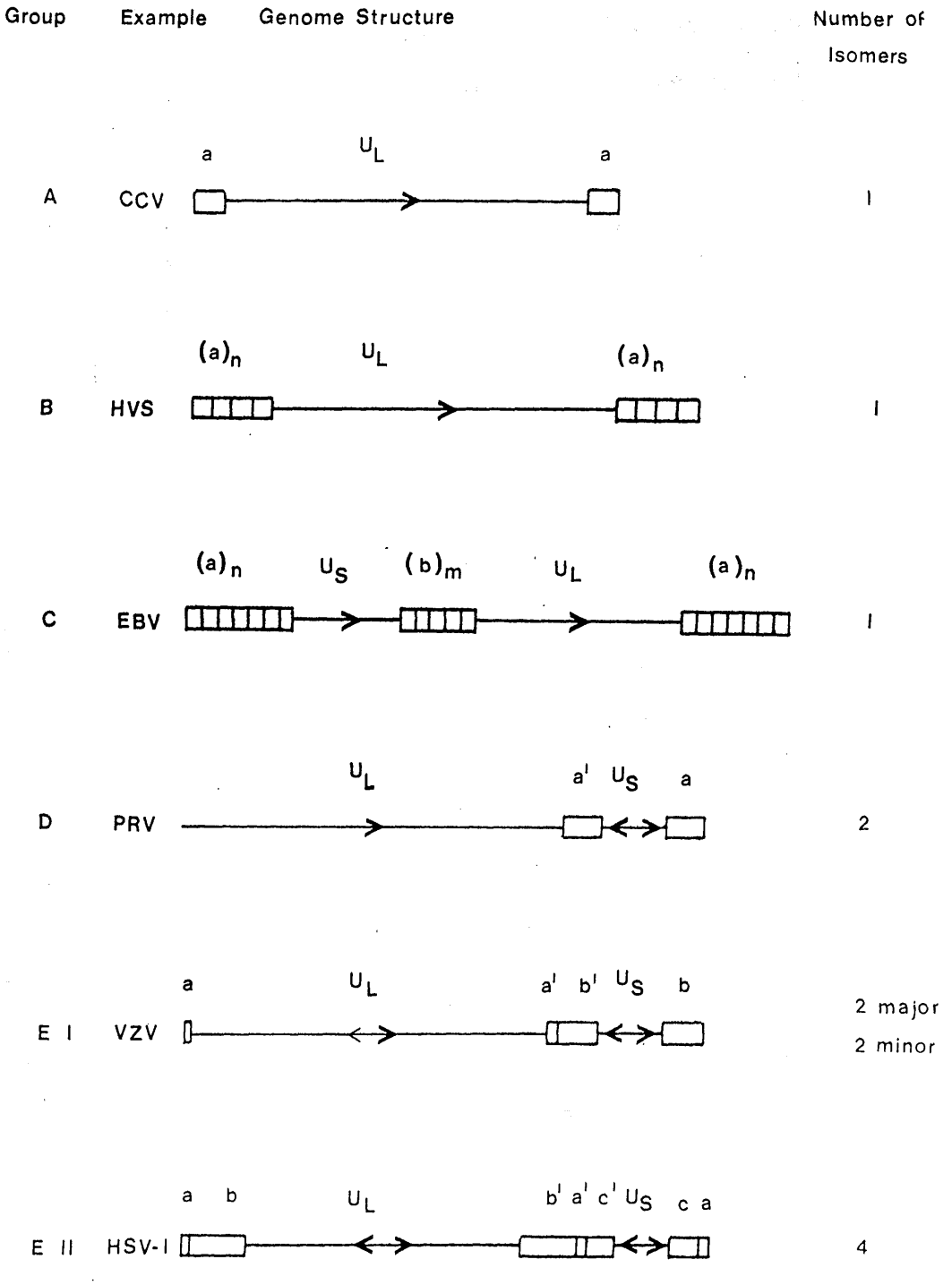
Genome structures of the herpesviruses

The structures of the herpesvirus genomes are illustrated. Repeat sequences are represented as open boxes; U_S and U_L indicate the short and long 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-E II, 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.

Adapted from MacLean (1988).

2.


Group
Example
Genome Structure
Number of Isomers
A
CCV
a
U_L
a
1
B
HVS
$(a)_n$
U_L
$(a)_n$
1
C
EBV
$(a)_n$
U_S
$(b)_m$
U_L
$(a)_n$
1
D
PRV
U_L
a' U_S
a
2
E I
VZV
a
U_L
a' b' U_S
b
2 major
2 minor
E II
HSV-I
a b
U_L
b' a' c' U_S
c a
4

unrelated inverted repeats. Within this subgroup, however, there exists variation eg. between varicella-zoster virus (VZV) and some pseudorabies virus (PRV) variants. In the case of VZV the short inverted repeats at the long termini allow only inefficient inversion with 95% of genomes being in the prototype orientation. This means that a VZV population consists predominantly of two isomers with the other two isomers being in the minority (Davison, 1984). Some variants of PRV generate four equimolar isomers due to the evolution of small inverted repeats at the flanking U_L allowing efficient inversion of the long segment (Lomnicizi et al., 1984, 1987; Lu et al., 1989).

Group EII is exemplified by HSV types 1 and 2 which, like the viruses in group EI, have genomes consisting of two unique segments, U_L and U_S, each bounded by an inverted repeat. The difference lies in the presence of a short DNA sequence directly repeated at the termini of the genome and in inverted orientation at the internal junction of the long and short repeats. HSV populations generally consist of four equimolar isomers generated by efficient inversion of the unique regions about the inverted repeats (Delius and Clements, 1976; Wilkie, 1976).

1.3 STRUCTURE AND ORGANIZATION OF THE HERPES SIMPLEX VIRUS GENOME

HSV types 1 and 2 are the most extensively characterized of the Herpesviridae both in terms of genetics and molecular biology. Both consist of linear double-stranded DNA molecules of approximately 100×10^6 dalton molecular weight (Becker et al., 1968; Frenkel and Roizman, 1971; Kieff et al., 1971; Grafstrom et al., 1974; Clements et al., 1976;

Davison, 1981).

HSV DNA consists of a linear molecule (see Fig. 3) comprising two covalently linked segments designated as long (L) and short (S). Each segment consists of a unique portion (U_L and U_S) bounded by distinct repeat sequences termed the long repeat (R_L) and short repeat (R_S). The long repeat designated ab (and b'a' in inverted orientation) and the short repeat ac (c'a' in inverted orientation) are indicated. The genome contains a terminal redundancy of approximately 250-500 base pairs (bp) designated the 'a' sequence, one or more copies of which are present in inverted orientation at the junction between the L and S segments (Sheldrick and Berthelot, 1974; Wadsworth et al., 1975; Wagner and Summers, 1978).

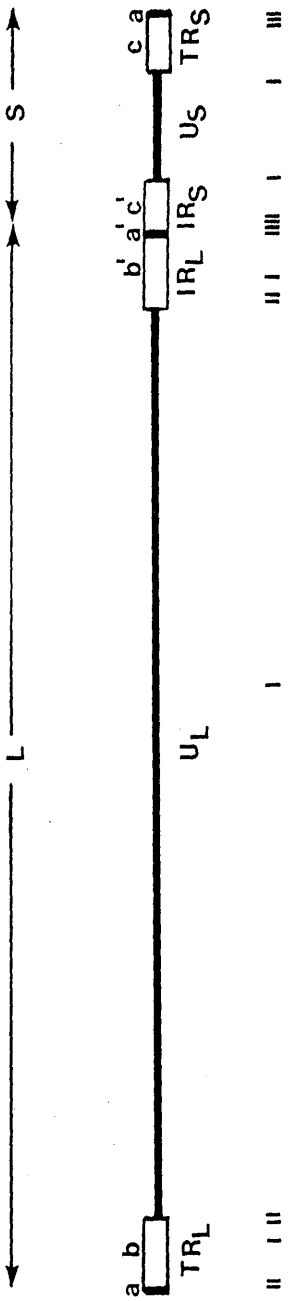
Preparations of HSV DNA consist of four equimolar isomers differing in the relative orientations of the L and S segments (Delius and Clements, 1976; Wilkie, 1976). Inversion is due to site-specific recombination mediated by the 'a' sequence (Mocarski et al., 1980; Mocarski and Roizman, 1981; Smiley et al., 1981). One genome isomer has been arbitrarily designated as having the prototype orientation (reviewed by Roizman, 1979) although the four isomers are thought to be functionally equivalent (Davison and Wilkie 1983a, b; Preston et al., 1978; Poffenberger et al., 1983; Poffenberger and Roizman, 1985).

A number of specific HSV restriction endonuclease fragments exhibit variable electrophoretic mobilities in agarose gels reflecting size variation (Locker and Frenkel, 1979; Davison and Wilkie, 1981; Lonsdale et al., 1979). The variability in terminal and L/S junction fragments is often explained by variations in the copy number of the 'a'

FIGURE 3

Gross organization of the HSV-1 genome.

A conventional representation of the HSV-1 genome is shown, with unique sequences (U_L and U_S) as solid lines and the major repeat elements (TR_L and IR_L, IR_S and TR_S) as open boxes. The locations of the long (L) and short (S) segments are marked. The a, b and c sequences and the same sequences in the opposite orientations a', b' and c' are indicated. Below the genome representation, the locations of families of high copy number, short, tandemly repeated sequences are shown with a vertical bar for each family.
Adapted from McGeoch et al. (1988b).


S
L
c a
TR_S
U_S
b' a' c'
IR_S
IR_L
U_L
a b
TR_L
III
I
IIII
II I
II I II

sequence. Analysis of other variable fragments has demonstrated the presence of short tandemly reiterated sequences present in different copy numbers in different isolates (Davison and Wilkie, 1981; Watson et al., 1981 ; Murchie and McGeoch, 1982; Rixon et al., 1984; Perry, 1986). Figure 3 indicates the location of a number of families of tandem reiterations in the HSV-1 strain 17 genome (McGeoch et al., 1988b). There is only one such repeat family in the whole of U_L (within the coding region of UL36) whereas they occur frequently within the repeat regions. The significance of such reiterationsis unclear and sequence comparison between HSV types 1 and 2 has shown that these repeat elements are poorly conserved (Davison and Wilkie, 1981; Whitton, 1984; Whitton and Clements, 1984b). Highly repetitive tandem sequences may promote unequal exchange which can reciprocally amplify and delete tandem reiterations.

The complete DNA sequences of EBV, VZV and HSV-1 have now been published (Baer et al., 1984; Davison and Scott, 1986; McGeoch et al., 1988b). The sequence of HCMV has been determined and a publication is in preparation (Chee and Barrell, 1990). HSV-1, strain 17, was shown to consist of 152,260 residues with a 68.3% overall G+C base composition rising as high as 80% in the short repeats (McGeoch et al., 1988b). In all, 72 potential genes (two of which are in the repeats) have been identified encoding 70 distinct proteins (see Fig. 4).

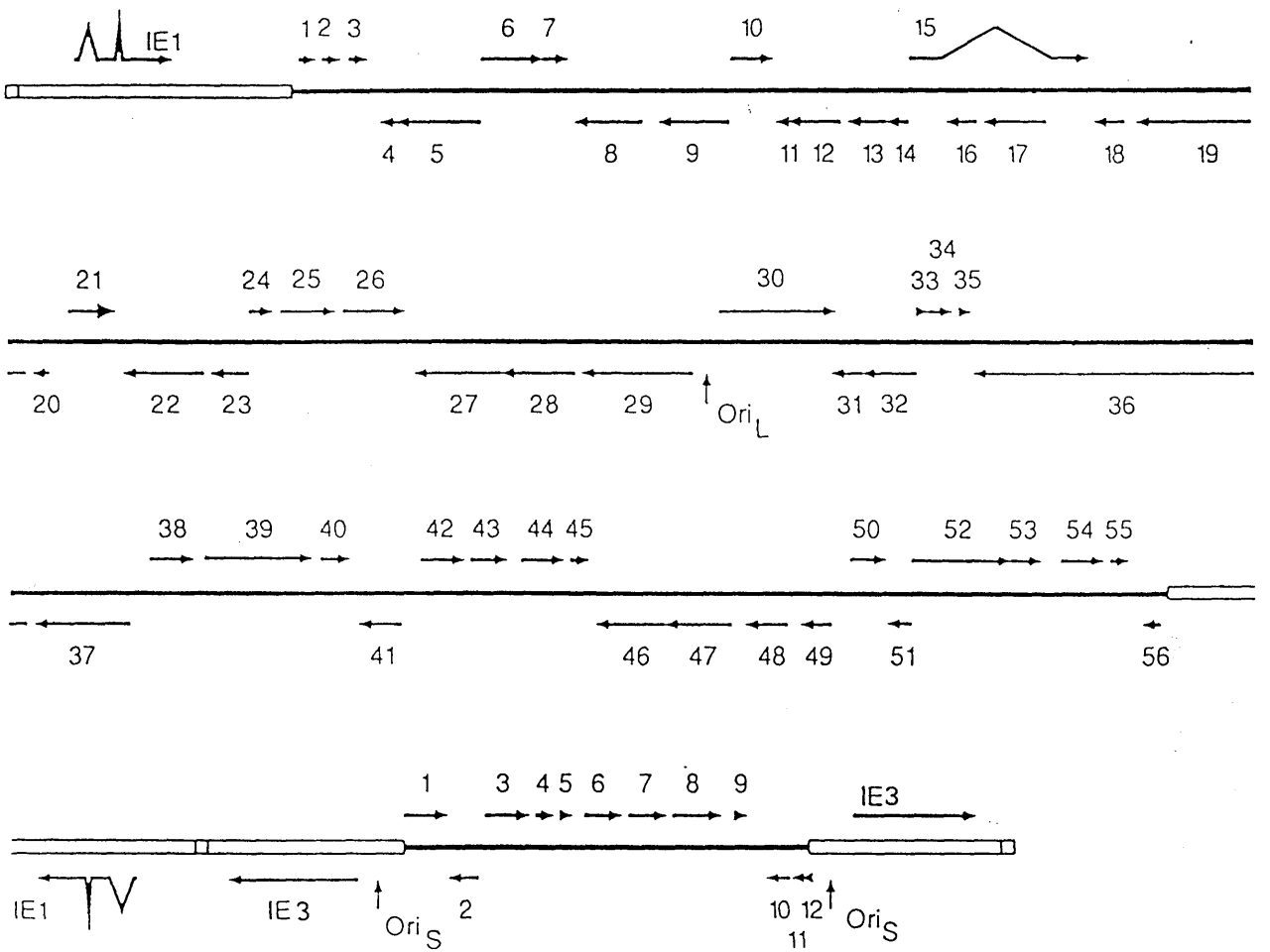
HSV-2 has not yet been completely sequenced, however, the indications from both sequence data and DNA hybridization studies (McGeoch, 1989; Davison and Wilkie, 1983c) are that HSV-1 and HSV-2 are closely related with very similar gene

FIGURE 4

Organization of the genome of HSV-1

The genome of HSV-1 is represented on four successive lines, with 40kbp per line. Locations of reading frames are shown by arrows, with splicing within coding regions indicated. In the top three lines genes UL1 to UL56 are shown as 1 to 56, and in the bottom line genes US1 to US12 as 1 to 12. Locations of the origins of DNA replication are indicated.

Adapted from McGeoch (1989).


IE1
1 2 3
6 7
10
15
4 5
8
9
11 12
13 14
16
17
18
19
21
24 25 26
30
34
33 35
20
22
23
27
28
29
Ori L
31 32
36
38
39
40
42 43
44 45
50
52
53
54 55
37
41
46
47
48
49
51
56
1
3
4 5
6
7
8
9
IE3
IE1
IE3
Ori S
2
10 12
11
Ori S

organization. The coding sequences of corresponding genes are highly conserved with 70-80% identities (McGeoch et al., 1987). The greatest degree of divergence is observed in parts of the repeat regions (Davison and Wilkie, 1981; Whitton and Clements, 1984a). The most pronounced difference observed between coding regions of the genomes so far is within US4 encoding the glycoprotein G polypeptide. Evolutionary evidence points to the difference being the result of a 1460bp deletion from the US4 gene of HSV-1 (McGeoch et al., 1987; McGeoch, 1989).

1.4 HERPES SIMPLEX VIRUS 'a' SEQUENCE

The 'a' sequence of HSV is present as direct repeats at the termini of the genome and in inverted orientation at the L/S junction (Wadsworth et al., 1975, 1976). There is only a single copy present at the short terminus and, although a single copy is most common at the long terminus and the L/S junction, up to ten copies may occur at these two locations. Variation in copy number of 'a' sequence repeats accounts for much of the observed size variability of certain L-terminus and L/S junction containing endonuclease fragments (Wagner and Summers, 1978; Locker and Frenkel, 1979; Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982b).

1.4a STRUCTURE OF THE 'a' SEQUENCE

The nucleotide sequences of the 'a' sequences of several strains of HSV types 1 and 2 have been determined (Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982b; Mocarski et al., 1985; Varmuza and Smiley, 1985). The size of the 'a' sequence (250-500bp) was found to vary both

within and between HSV strains. The 'a' sequence in each case was shown to be composed of both unique (U) and directly repeated (DR) elements with variation in copy number of the DR elements being primarily responsible for the observed size variation. Mocarski and Roizman (1981) proposed a generalized model based on HSV-1 strain F to describe the structure of HSV 'a' sequences (see Fig. 5).

The model, based on an 'a' sequence at the L/S junction is consequently in inverted orientation and lies between sequences b' and c'. The structure of such an 'a' sequence is : DR_1-U_b-$(DR_2)_n$-$(DR_4)_n$-U_c-DR_1 with the subscripts b and c reflecting proximity to the b' and c' repeat regions. The model is not without exceptions, for example, HSV-1, strain Justin, contains an additional direct repeat element $DR_{3.5}$ (Mocarski and Roizman, 1981, 1982b; Mocarski et al., 1985). However, there is a reasonable degree of conservation of 'a' sequence information between different HSV strains with the different repeat elements exhibiting similarities and certain portions of the unique elements appearing to be highly conserved (Davison and Wilkie, 1981; Deiss et al., 1986). DR_1 which varies between 17 and 21bp in length is present as a single copy directly repeated at each end of the 'a' sequence. DR_2 is a 12bp element which is present in up to 22 copies in HSV-1, strain F, but as only a single copy in HSV-2, strain HG52. As both DR_2 and DR_4 are present in only a single copy in HG52 the 'a' sequence in this case can be regarded as a single unique region flanked by a single direct repeat DR_1 (Davison and Wilkie, 1981).

Sequence data obtained for HG52X192 (Taha et al., 1989b) have demonstrated that the deletion from this variant removes the complete copy of DR1 from the internal side of

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.

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

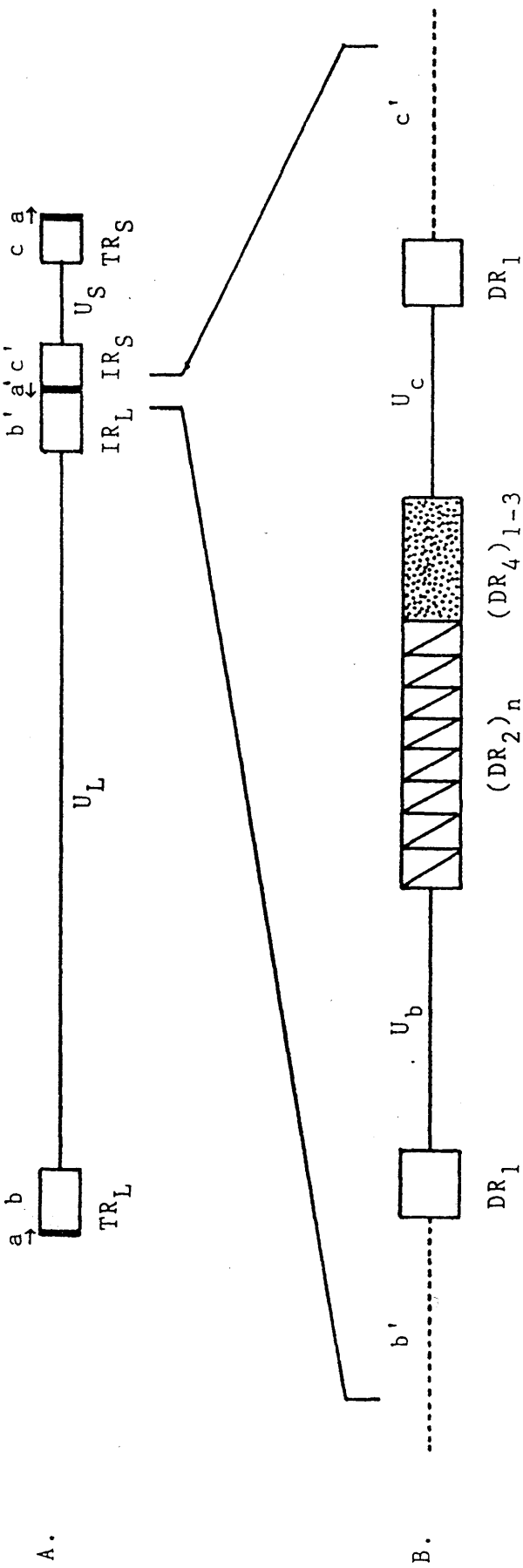
U_c: a unique sequence located towards the c' sequence.

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

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

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

Adapted from MacLean (1987).


A.
B.
a
b
TR_L
U_L
b'
a'
c'
IR_L
IR_S
U_S
TR_S
c
a
b'
U_b
DR_1
(DR_2)_n
(DR_4)_1-3
U_c
DR_1
c'

the 'a' sequences in both copies of R_L resulting in an 'a' sequence composed entirely of unique information. Preliminary experiments indicate that in other copies of the 'a' sequence within HG52X192 both copies of DR_1 are intact (A. MacLean, personal communication).

Tandem reiterations of the 'a' sequence, instead of containing two adjacent copies of DR_1, share the intervening DR_1 element. Cleavage of a double 'a' sequence would, therefore, not generate two complete copies of DR_1. Sequence analysis has shown that L and S terminal copies of the 'a' sequence, compared to those at the L/S junction, contain only one complete and one partial copy of DR_1 which together would form a complete copy. In HSV-1, strain F, the long terminal 'a' sequence DR_{1c} was shown to consist of 18bp with a single nucleotide extended 3' (18.5bp) while the short terminal DR_{1b} consisted of a single base pair with a single nucleotide extended 3' (1.5bp) (Mocarski and Roizman, 1982b). A similar situation was described in HSV-1, strain 17, where the long terminal 'a' sequence contains 20.5bp of the DR_{1c} element and the short only 0.5bp of DR_{1b} (Davison and Rixon, 1985). Circularization of the genome would, therefore, generate a double 'a' sequence with a shared DR_1 element (see Fig.6).

1.4b CIRCULARIZATION

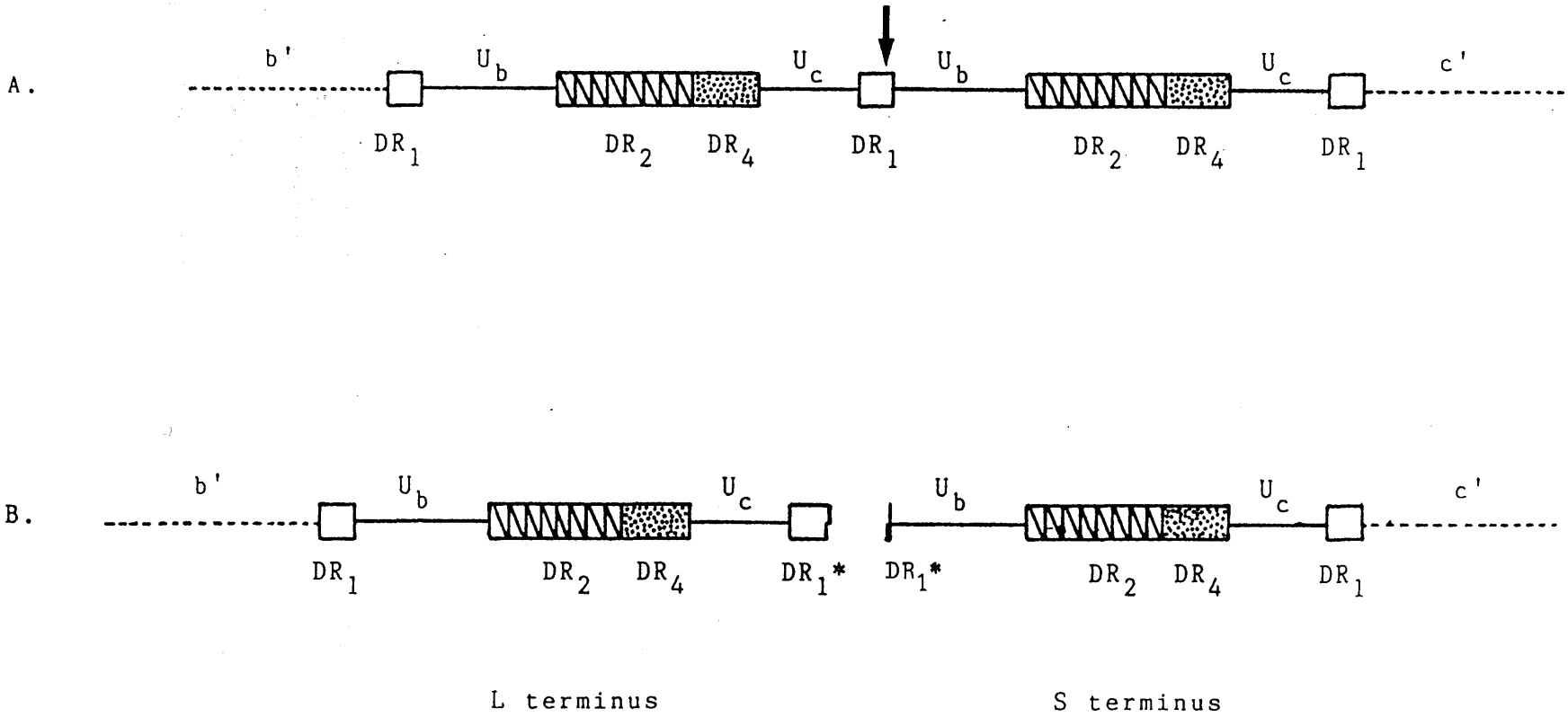
Genome circularization occurs rapidly following HSV infection in the absence of de novo protein synthesis (Poffenberger and Roizman, 1985). It is believed that circularization due to ligation of the termini is mediated by the 'a' sequence complementary single base 3' overhang ends (Mocarski and Roizman, 1982b; Davison and Rixon, 1985).

FIGURE 6

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

A. Tandem copies of the 'a' sequence share the intervening DR_1 element. The 'a' sequences are shown in the orientation seen in Fig. 5. Cleavage through the intervening DR_1 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 posess only partial DR_1 elements (DR_1*) with single nucleotide 3' extensions, such that annealing of an L and S terminus would form a complete DR_1 element as in A. Adapted from MacLean (1987).


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_1*
DR_2
DR_4
DR_1
L terminus
S terminus

It is possible that virion proteins which bind preferentially to the 'a' sequence may play a role in this process.

1.4c SITE-SPECIFIC RECOMBINATION

In HSV, inversion occurs about the L/S junction to generate four equimolar isomers with different orientations of U_L and U_S. The contributions of the four isomers are thought to be equivalent in HSV replication as it has been shown that variants fixed in three of the possible orientations grow well both *in vitro* and *in vivo* (Davison and Wilkie, 1983a; Poffenberger *et al.*, 1983; Poffenberger and Roizman, 1985; Jenkins and Roizman, 1986; Longnecker and Roizman, 1986). Isomerization has been shown to be due to site-specific recombination about the 'a' sequence which, as protein synthesis is required, is possibly mediated by trans-activating viral products (Mocarski and Roizman, 1982a). The role of the 'a' sequence in site-specific recombination is supported by the observation that certain HSV-1/HSV-2 intertypic recombinants, in which the L/S junction 'a' sequence is from one parent and the terminal 'a' sequence from the other, are fixed in a single orientation (Davison and Wilkie, 1983a); the interpretation being that lack of 'a' sequence homology inhibits normal site-specific recombination.

The ability of the 'a' sequence to promote inversion was proved by the introduction of an additional 'a' sequence into the thymidine kinase locus. This led to novel inversion of the DNA between the additional inserted 'a' sequence and relatively inverted 'a' sequences (Mocarski *et al.*,1980; Mocarski and Roizman, 1981; Smiley *et al.*, 1981).

The use of constructs with systematic 'a' sequence deletions has shown that the direct repeat elements (especially DR_2) of the 'a' sequence are particularly important in inversion. Low levels of inversion occur at DR_2 in the absence of the other repeat elements. However, there is evidence that both DR_4 and DR_2 repeat elements are necessary to allow normal high frequency inversion (Chou and Roizman, 1985; Varmuza and Smiley, 1985).

It has been proposed that the variability in copy number of DR_2 elements within single strains might be due to unequal recombination between 'a' sequences at DR_2 (Mocarski and Roizman, 1981; 1982b).

1.4d OTHER HERPES SIMPLEX VIRUS SEQUENCES CAPABLE OF MEDIATING INVERSION EVENTS

The ability of the 'a' sequence to allow site-specific recombination was originally thought to be unique. However, subsequent experiments have demonstrated that certain other sequences also allow inversion to take place. A tandemly reiterated sequence within the short repeat region of HSV-1 close to the 'a' sequence has been shown to cause high frequency inversion (Davison and Wilkie, 1981; Mocarski and Roizman, 1981; Varmuza and Smiley, 1984). Low frequency inversion has also been shown to occur about a region within the long repeat approximately 5kbp from the 'a' sequence (Longnecker and Roizman, 1986). One of the variants described in this thesis, HG52X19, has a deletion removing the entire internal copy of the long repeat and half of the internal copy of the short repeat. It was found to be fixed in the prototype orientation of the long region whilst the short region inverted at reduced frequency. As this variant

lacks the internal copies of the 'a' sequence the inversion of the short region must be mediated by the portion of the short repeat which remains undeleted (Harland and Brown, 1989).

Sequences present within the unique portions of HSV may also be capable of mediating inversion events. Experiments with HSV-1, strain F, in which various sequences were inserted in inverted orientation into the thymidine kinase locus demonstrated that DNA from the right hand end of U_L (0.706-0.744m.c.) was capable of mediating inversion (Pogue-Geile et al., 1985; Pogue-Geile and Spear, 1986). Interestingly, the recombinational events leading to such inversion also converted markers within the inverted repeat sequences to homology. Similar pressure towards homology within the normal HSV repeat elements has also been described (Stow and Stow, 1986; Sacks and Schaffer, 1987; Umene, 1987) and is probably relevant to the structure of the variant HG52X192 described in this thesis (Harland and Brown, 1985). In contrast, a number of other variants is described in this thesis in which deletions within normally diploid regions (ie. R_L and R_S) result in genomes which stably maintain only a single copy of normally repeated information (Harland and Brown, 1985; Brown and Harland, 1987). In the case of the variants HG52X85/4, HG52X85/5, HG52X86 and HG52X163X12 failure to detect recombinants deleted in both repeats or the reciprocal wild-type recombinant progeny is possibly due to the size and location of the deletions. Recombination frequency may depend on the size and nature of the repeat sequence remaining following deletion and, therefore, large deletions such as described in these variants or the proximity of the deleted portion to

the unique region might reduce the chance of recombination leading to homology.

1.4e CLEAVAGE/PACKAGING

Replication of HSV involves the formation of long head-to-tail concatamers from a circular monomeric template by a rolling circle mechanism (Jacob et al., 1979). DNA is cleaved into unit length genomes and packaged into nucleocapsids - the two processes being apparently inseparable (Ladin et al., 1982; Preston et al., 1983). Studies using defective amplicons have demonstrated that the 'a' sequence is essential for DNA cleavage and packaging to proceed (Spaete and Frenkel, 1982; Stow et al., 1983).

The processes of cleavage and packaging in HSV have been shown to be dependent on cis-acting signals within the 'a' sequence (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982; Vlazny et al., 1982; Stow et al., 1983; Deiss and Frenkel, 1986). Two signals, pac1 and pac2, which cause sequence-independent cleavage at a set distance from the signal have been identified within U_b and U_c of the 'a' sequence (Varmuza and Smiley,1985; Deiss et al., 1986). The involvement of the DR_2 and the DR_4 elements in the cleavage and packaging processes are at present unknown. It has been proposed that packaging is initiated by cleavage within the DR_1 element of the 'a' sequence adjacent to U_b at the short terminus (Deiss et al., 1986)

1.4f AMPLIFICATION OF THE 'a' SEQUENCE

It has been demonstrated that replication of a monomeric amplicon containing a single 'a' sequence yields packaged DNA molecules of approximately genome length consisting of

tandem reiterations of the amplicon with an 'a' sequence at each terminus (Stow et al., 1983; Deiss and Frenkel, 1986).

Various mechanisms to explain the amplification of 'a' sequences and their involvement in cleavage and packaging of HSV have been proposed. These models have been designed to explain (1) The presence of a single copy of the 'a' sequence at the short terminus whilst the long terminus and L/S junction in approximately 20% of genomes contain more than one copy (Wagner and Summers, 1978; Locker and Frenkel, 1979). (2) The presence of 3' single base extensions at the terminal 'a' sequences, and (3) The amplification of 'a' sequences causing generation of two terminal 'a' sequences from a junction containing only a single copy. In fact, circularization of intact HSV genomes prior to replication would generate double 'a' sequences at every second junction (Poffenberger and Roizman, 1985) which means that no amplification of 'a' sequences would be required in the majority of cases where the L terminus generated contains only a single copy of the 'a' sequence.

Varmuza and Smiley (1985) proposed two cleavage/packaging models. The first, a single-strand repair synthesis model (see Fig. 7a) proposes the generation of two terminal 'a' sequences from a joint containing a single copy. The model predicts that cleavage is initiated by single-strand nicks at either end of an 'a' sequence. The strands separate and are repaired to generate two 'a' sequence-containing termini which could then be packaged. The model offers a simple mechanism to account for 'a' sequence amplification, however, it predicts that only a small proportion of termini, generated by cleavage of double 'a' sequences, would end in a 3' single base extension.

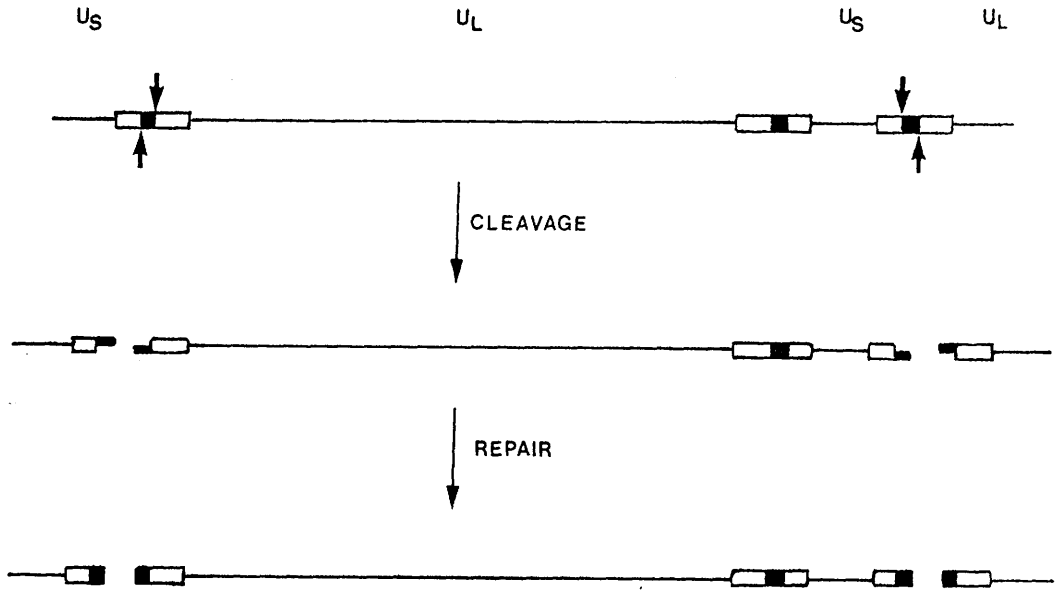
FIGURE 7

Models for amplification of 'a' sequences and roles in cleavage and packaging.

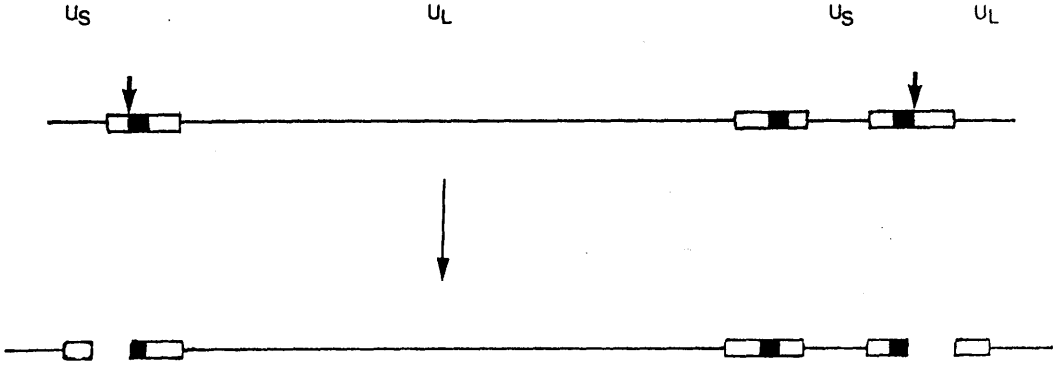
a. The single-strand nick/repair model (Varmuza and Smiley, 1985). In this model two copies of the 'a' sequence (one at the long terminus and one at the short terminus) are generated from a single shared copy. The mechanism involves staggered single-stranded nicks (↓) occuring at either end of the 'a' sequence, the strands separating and undergoing repair synthesis. This model predicts that where junctions contain two or more copies of the 'a' sequence double-stranded cleavage would occur to generate 'a' sequences with a single base extension.

b. "Theft" mechanism (Varmuza and Smiley, 1985). In this model double-stranded cleavage (↓) occurs to generate a L terminus. Packaging then proceeds until the next appropriately orientated L/S junction is encountered, at which point a second double-stranded cut generates the S terminus. The mechanism would propose that portions of DNA lacking 'a' sequences would be created which would be rapidly degraded.

7a.



7b.



DEGRADED PACKAGED DEGRADED

The second model proposed by Varmuza and Smiley (1985) involves a "theft" mechanism (see Fig. 7b). In this model L/S junctions containing a single 'a' sequence are cleaved so that only one terminus contains an 'a' sequence. This mechanism would result in the generation of L and S termini lacking 'a' sequences which would fail to package and would be rapidly degraded.

Deiss et al. (1986) also proposed two models for 'a' sequence amplification. Their "directional cleavage model" (see Fig. 7c) postulates that a packaging complex binds at random to concatameric DNA and travels in either direction until it reaches the U_c element of an 'a' sequence where it cleaves both strands with a 3' single base extension. The DNA is then packaged in the L to S direction until a directly repeated junction is encountered. A second cleavage will then occur in DR_1 proximal to the first U_b signal encountered within that junction (again producing a 3' single base extension). If it is assumed that the initial binding is entirely random then, due to the proportional sizes of the long and short regions, 80% of the time the binding will be within the long segment and 20% within the short. This in turn would generate 80% of genomes with a single copy of the 'a' sequence at each terminus and 20% with reiteration of the 'a' sequence at the long terminus. As this model is a modification of the "theft" model proposed by Varmuza and Smiley (1985) there would again be the generation of lengths of DNA lacking terminal 'a' sequences. As such structures are not observed it is necessary to postulate the repair or degradation of such termini.

The second model proposed by Deiss et al. (1986) is a

FIGURE 7

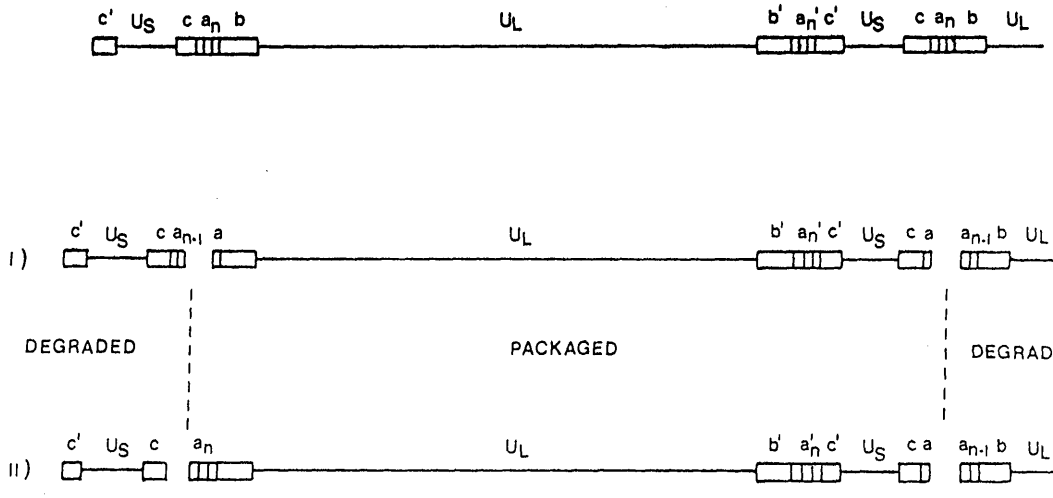
c. Directional cleavage model (Deiss et al., 1986). A packaging complex binds to the concatameric DNA and scans in either direction until it encounters an 'a' sequence U_c signal causing double-stranded cleavage at the proximal DR_1 element creating a 3' single base overhang. The genome is packaged from L to S and a second double-stranded cut occurs within DR_1 after the U_b signal in the first directly repeated 'a' sequence encountered. (n) represents the number of 'a' sequences in the junctions which are to be cleaved. I) 80% of the time the packaging complex will bind to the long segment to give genomes with a single copy of the 'a' sequence at each terminus. II) 20% of the time the packaging complex will bind to the short segment to generate genomes with the possibility of more than one copy of the 'a' sequence at the L terminus.

d. The double-strand gap/repair model (Deiss et al., 1986). This is a modification of the directional cleavage model shown in 7(c). As before the packaging complex binds and traverses the DNA at random until the U_c signal of an 'a' sequence is encountered. Then, instead of cleaving the DNA, packaging is initiated from L to S and continues until a second 'a' sequence in the same orientation is encountered. The 'a' sequences are aligned and amplified by a double-strand break/repair mechanism. Cleavage between the reiterated 'a' sequences generates four 'a' sequence-containing termini.

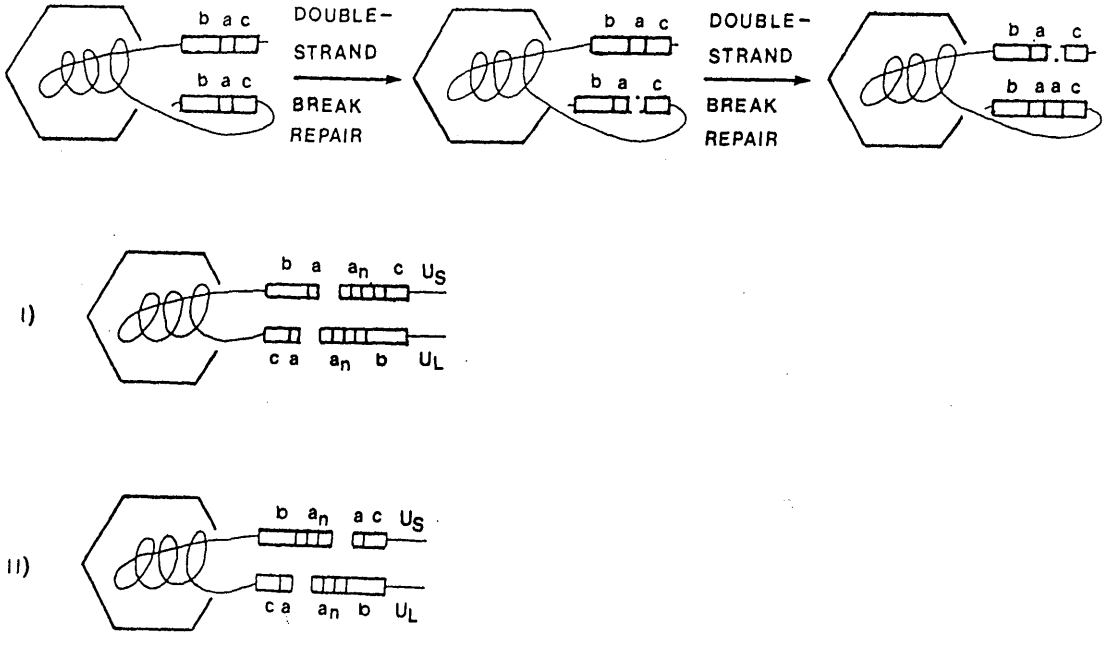
I) Represents the termini produced when the complex originally binds to L.

II) Represents the termini produced when the complex originally binds to S.

7c.


c' Us c an b
UL
b' an' c' Us c an b UL
I)
c' Us c an-1 a
UL
b' an' c' Us c a
an-1 b UL
DEGRADED
PACKAGED
DEGRADED
II)
c' Us c an
UL
b' an' c' Us c a
an-1 b UL

7d.


b a c
b a c
DOUBLE-
STRAND
BREAK
REPAIR
b a c
b a c
DOUBLE-
STRAND
BREAK
REPAIR
b a c
b aa c
I)
b a an c Us
c a an b UL
II)
b an a c Us
c a an b UL

"double-strand break and gap repair model" (see Fig. 7d). The first stage in the process would be similar to that in the previous model with a packaging complex binding and travelling at random until it recognises a U_c signal. Unlike the previous model, however, the DNA would not be cleaved but packaged in the L to S direction until a directly repeated junction is encountered. With the direct repeats juxtaposed cleavage would occur within DR_1 of one of the 'a' sequences. The cleaved ends would then align with the DR_1 elements at each end of the uncleaved 'a' sequence, generating a gap which is repaired using as a template the uncleaved 'a' sequence. A similar process would then take place to generate a second 'a' sequence for the other junction resulting in amplification of the 'a' sequence in both junctions. Cleavage generating a 3' single base extension would occur between the newly generated 'a' sequences and the previously existing ones.Like the previous model this one predicts the generation of 80% of genomes with a single long terminal 'a' sequence and has the advantage of not proposing the generation of termini lacking 'a' sequences. However, it has the drawback that some recombinants containing heterotypic 'a' sequences (Davison and Wilkie, 1983a) could not participate in this type of mechanism.

1.4g PROTEIN INTERACTIONS AT THE HERPES SIMPLEX VIRUS 'a' SEQUENCE

Little is known at present of the possible protein interactions occurring with the 'a' sequence. It has been shown by electron microscopy that a small unknown polypeptide binds to both termini and the L/S junction

region of HSV virion DNA (Wu *et al.*, 1979). *In vitro* experiments demonstrating the preferential affinity of the US11 encoded 21K and 22K molecular weight polypeptides for 'a' sequence DNA (Dalziel and Marsden, 1984) are probably not significant as *in vivo* these proteins are strongly localized in different parts of infected cells (MacLean *et al.*, 1987). Also US11 has been shown to be non-essential (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987; this thesis) with variants deleted in this gene inverting and packaging normally.

DNA-protein interaction within the DR_2 and U_b elements of the 'a' sequence has been demonstrated by DNase I footprint assay but the protein(s) has not been identified although 21/22K is definitely not involved (MacLean *et al.*, 1987).

Chou and Roizman (1989) have identified two viral factors, designated V2 and V4, which bind to the 'a' sequence of HSV-1. V2 was identified as an exonuclease with an estimated molecular weight of 80K. When purified, V4 was found to contain a host protein of approximately 300K and a 140K protein of viral origin. The binding of V2 to 'a' sequence DNA appears to be non sequence-specific whereas V4 apparently binds preferentially to the *cis*-signals of pac1 and pac2. The biological activity of V4 is being investigated by these workers.

1.5 HERPES SIMPLEX VIRUS REPLICATION

Shortly after infection HSV DNA accumulates in the cell nucleus where ligation of the terminal 'a' sequences rapidly generates circular molecules. At 37^{o}C DNA replication is first detected at approximately 3h post infection (PI), reaches its height between 9-11h PI and is more or less

complete by 16h PI (Wilkie, 1973).

Initially the monomeric circular template is probably amplified by theta replication (Ben-Porat and Tokazewski, 1977; Jean et al., 1977; McGeoch, 1987). Restriction enzyme analysis shows that newly replicated HSV DNA junction fragments are over-represented whilst terminal fragments are not detected, indicating the generation of circular or concatameric DNA. Buoyant density studies show that the latter is liable to be the predominant form (Jacob et al., 1979; Jongeneel and Bachenheimer, 1981). Long head-to-tail concatamers are generated by a rolling circle mechanism (Jacob et al., 1979). The DNA is then cleaved and packaged into nascent nucleocapsids in the cell nucleus (Vlazny et al., 1982). The processes of cleavage and packaging appear to be inseparable and may be inexorably linked.

Three forms of capsid A, B and C have been isolated from HSV infected cells. A and B are found within the cell nucleus whilst C capsids are present within virions (Gibson and Roizman, 1972, 1974). The A capsids are thought to be precursors of the other forms. They are empty; containing no core protein and no DNA. It has been proposed that the surface protein VP22a (p40, ICP35), found mainly in association with capsids containing DNA, may play a role in the encapsidation process. This protein constitutes approximately 1% of the A capsids, whereas, it accounts for approximately 15% of the B capsid protein (Heilman et al., 1979). The B capsids are non-enveloped, fast-sedimenting particles containing a protein core and DNA. As VP22a is not detected in enveloped particles it is believed to be modified in virions to the lower molecular weight protein, VP22, which in conjunction with other proteins is loosely

bound to capsids. Preston et al. (1983) described a HSV-1 temperature-sensitive (ts) packaging mutant, VP1201, which at the non-permissive temperature has a defect preventing the normal conversion of VP22a (40K mol. wt.) to the lower molecular weight protein VP22 (39K mol. wt.). The processing of VP22a to VP22 is probably essential for envelopment and may constitute a signal that the capsid is ready for envelopment.

1.5a HERPES SIMPLEX VIRUS ORIGINS OF DNA REPLICATION

It has been demonstrated that, in order to replicate and be packaged, defective molecules must contain an origin of replication and a packaging signal. Two classes of defective molecules were identified both of which contained an 'a' sequence packaging signal. In addition class I defective molecules contained sequences from the internal end of the short repeat whilst class II defectives contained additional sequences from the centre of U_L (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982). It was concluded that a herpes simplex virus genome contains three origins of replication, one copy of ori_L in the middle of U_L and two copies of ori_S, one within each of the inverted short repeats.

1.5b HERPES SIMPLEX VIRUS SHORT ORIGIN OF REPLICATION (ori_S)

Intact HSV-1 genomes contain two identical copies of ori_S located close to the internal end of each short repeat between two divergently transcribed immediate early genes at map co-ordinates 0.86 and 0.96. In HSV-1, strain 17, the minimal region containing the cis-acting sequences required for ori_S function has been determined from plasmid-based assays (Stow and McMonagle, 1983). The identified 90bp

minimal ori_S sequence has been shown to contain a 45bp imperfect palindrome with a central $(AT)_6$ region. Deletion of the $(AT)_6$ sequence from the palindrome renders the origin inactive with the plasmids containing the deleted origin failing to replicate (Stow, 1985).

Elias et al. (1986) have identified an 18bp stretch of DNA adjacent to the $(AT)_6$ sequence which forms the binding site of an HSV-1 induced nuclear protein.

The type 2 short origin of replication is present as an imperfect direct repeat of 137bp in both copies of the short repeat (Whitton and Clements, 1984a).

1.5c HERPES SIMPLEX VIRUS LONG ORIGIN OF REPLICATION (ori_L)

Analysis of class II defective genomes indicated that DNA in the middle of U_L (0.407-0.429m.c.) contains a sequence capable of acting as an origin of replication (ori_L) (Spaete and Frenkel, 1982). Initial attempts to clone this region proved unsuccessful due to the generation of spontaneous deletions of between 100 to 650bp which rendered the origin inactive. Serial passage of the deleted cloned fragments in the presence of helper virus restored the original activity as a result of recombination replacing the deleted information (Spaete and Frenkel, 1982; Weller et al., 1985). Subsequently ori_L was successfully cloned using a yeast plasmid vector in which the inserted DNA remained undeleted (Weller et al., 1985).

The nucleotide sequence of the long origins of replication of three HSV-1 strains; Angelotti (Gray and Kaerner, 1984), strain 17 (Quinn and McGeoch, 1985) and KOS (Weller et al., 1985) have been determined. They show that ori_L consists of a perfect palindrome of 144bp with a

central A+T rich region like that in ori_S with which it shares a high degree of homology. The protein binding sequence described by Elias et al.(1986) in ori_S is present within ori_L as two copies, one on each side of the A+T rich region. Recently two groups have independently shown that this origin binding protein is encoded by UL9, one of the seven genes known to be involved in viral DNA replication (Olivo et al., 1988; Weir et al., 1989). The differences in the structure and protein-binding properties of ori_L and ori_S led to the suggestion that ori_L may act as a bi-directional origin, whereas, ori_S is uni-directional (Weller et al., 1985; McGeoch, 1987).

The long origin of replication of HSV-2 located at 0.389-0.413m.c. has been sequenced and found to consist of an almost perfect palindrome of 136bp sharing a high degree of homology with its type one counterpart (Lockshon and Galloway, 1986).

The significance of HSV possessing three origins of replication is not known. A variant lacking ori_L has been isolated which grows normally in vitro and which establishes latent infection in vivo (Polvino-Bodnar et al., 1987). Viable variants of HSV types 1 and 2 lacking a single copy of ori_S have also been isolated (Longnecker and Roizman, 1986; Brown and Harland, 1987; this thesis). Failure to isolate viable HSV deletion variants lacking both copies of ori_S may not indicate that both are essential as evidence has been found of a transcript spanning ori_S (Hubenthal-Voss et al., 1987). If both copies of ori_S were deleted then both copies of the proposed gene would also be disrupted making it difficult to dissociate the two effects.

It may be significant that both ori_S and ori_L are located

between divergent transcripts encoding polypeptides with major roles in replication and transcription of the virus: ori_S is located between the 5' ends of the divergent transcripts for IE3 (encoding Vmw175) and IE4/5 (encoding Vmw68 and Vmw12) (Stow, 1982; Stow and McMonagle, 1983) whilst ori_L is situated between the divergent transcripts for the DNA polymerase (UL30) and the major DNA-binding protein (UL29) (Quinn and McGeoch, 1985).

1.5d EFFECTS OF HERPES SIMPLEX VIRUS INFECTION ON CELL METABOLISM

HSV infection of permissive cells leads to rapid inhibition of cellular DNA synthesis (Roizman and Roane, 1964) and shut-off of synthesis of the majority of cellular polypeptides (Sydiskis and Roizman, 1966, 1967). Cellular polysomes quickly disaggregate (Sydiskis and Roizman, 1967) with rapid degradation of cellular mRNA (Schek and Bachenheimer, 1985) to be replaced by polysomes associated with virally encoded mRNA (Stringer et al., 1977).

At least two viral factors have been shown to play a part in host cell shut-off. The first which induces cellular mRNA degradation is a virion component which is non-essential in tissue culture (Sydiskis and Roizman, 1967; Fenwick and Walker, 1978; Fenwick et al., 1979; Scheck and Bachenheimer, 1985; Read and Frenkel, 1983). The virion host shutoff gene has been mapped by the study of intertypic recombinant viruses and shutoff-defective mutants to the region of the HSV-1 genome predicted to encode UL41 (Kwong et al., 1988). The virion host shutoff gene of HSV-1 strain 17 causes only slight inhibition whereas HSV-2 strain G shuts off host synthesis strongly. The predicted type 2 equivalent of UL41

when inserted into the thymidine kinase gene of HSV-1 strain 17 caused strong shutoff (Fenwick and Everett, 1990). The second, required for inhibition of cellular DNA synthesis (Fenwick et al., 1979), is synthesised later in viral infection and causes complete host cell shut-off independent of the virion component (Honess and Roizman, 1974; Marsden et al., 1976).

Not all cellular genes are shut-off following infection. There is evidence that cellular promoters (eg. the human epsilon-globin gene and the rabbit beta-globin gene) suitably integrated into the host cell genome can be activated by HSV IE gene products during viral infection (Everett, 1985). Also it has been shown that the host cell RNA polymerase II is employed by the virus throughout infection (Alwine et al., 1974; Ben-Zeev et al., 1976; Costanzo et al., 1977) and that other cellular genes such as those encoding heat shock proteins appear to be up regulated following infection (Notarianni and Preston, 1982; La Thangue et al., 1984).

1.5e PROTEINS INVOLVED IN HERPES SIMPLEX VIRUS DNA REPLICATION

Genetic studies of DNA-negative temperature-sensitive mutants have shown that they fall within several complementation groups. Use of the mutants has identified DNA polymerase (Hay and Subak-Sharpe, 1976; Chartrand et al., 1979, 1980; Coen et al.,1982; Honess et al., 1984), the major DNA-binding protein (Bayliss et al., 1975; Powell and Purifoy, 1976; Powell et al., 1981), ribonucleotide reductase (Dutia, 1983; Preston, V. et al., 1984) and alkaline exonuclease (Moss, 1986) as essential proteins for

DNA replication. A number of proteins affected by such mutations in other complementation groups has not yet been identified (McGeoch, 1987; Schaffer et al., 1987).

An assay system involving the co-transfection of origin containing plasmids with restriction endonuclease digested HSV DNA or cloned fragments has helped to identify genes essential for DNA replication (Challberg, 1986). Originally it was found that a combination of five cloned Xba I fragments could supply all the trans-acting functions required for origin-dependent replication. It was also found that one of the cloned fragments could be replaced by two smaller fragments, one containing HSV encoded DNA polymerase and the other containing the gene encoding the major DNA-binding protein.

Seven genes UL5, UL8, UL9, UL29, UL30, UL42 and UL52 have been identified by this method (McGeoch et al., 1987; Wu et al., 1988) including the viral DNA polymerase (UL30) and the major DNA-binding protein (UL29). Another gene product has been identified as the 65K DNA-binding protein (UL42) of unknown function. It has recently been demonstrated that the product of UL9 is the 83K origin binding protein described by Elias et al. (1986) (Olivo et al., 1988; Weir et al., 1989). The products of the remaining genes UL5, UL8 and UL52 have been identified in HSV infected cells by immunoprecipitation with antisera raised against either synthetic peptides or fusion proteins predicted from the DNA sequence (Olivo et al., 1989). UL5 encodes a 95K, UL8 a 75K and UL52 a 115K protein. These authors demonstrated that all seven of the proteins involved in viral DNA replication are located in the nucleus, appear early in infection and are expressed despite inhibition of viral DNA synthesis.

Surprisingly, neither alkaline exonuclease nor ribonucleotide reductase was shown to be essential for *trans*-activation of DNA replication despite the fact that both were previously identified as being essential (Moss, 1986; Preston, V. *et al.*, 1984). The construction of a viable variant deleted within the ribonucleotide reductase coding region supports the view that this protein may be non-essential for DNA replication under *in vitro* conditions (Goldstein and Weller, 1988). Preston *et al.*(1988) have also described a mutant, *ts*1222, which has a single base pair deletion at the 3' end of the small subunit of ribonucleotide reductase. This mutant did not induce detectable amounts of ribonucleotide reductase activity at either 31°C or 39°C. At 31°C, however, it grew as wild type in dividing cells whilst 39.5°C was non-permissive for growth of the mutant. Their findings suggest that at 31°C growth of the mutant is supported by cellular ribonucleotide reductase whereas virally encoded ribonucleotide reductase is necessary for viral replication at the higher temperature or in resting tissue culture cells.

1.6 HERPES SIMPLEX VIRUS GENE EXPRESSION AND mRNA SYNTHESIS

1.6a HERPES SIMPLEX VIRUS TRANSCRIPTION

No HSV-induced RNA polymerase has been detected (Lowe, 1978). It is thought that viral transcription mediated by host RNA polymerase II occurs throughout infection (Alwine *et al.*, 1974; Ben-Zeev *et al.*,1976; Costanzo *et al.*,1977).

HSV transcripts, like most eukaryotic mRNAs are post-transcriptionally modified by the addition of GTP in a 5'-5' condensation reaction. HSV mRNAs are polyadenylated

(Bachenheimer and Roizman, 1972; Stringer et al., 1977) and contain a consensus 'GT box' sequence YGTGTTYY (Y=pyrimidine) situated approximately 30bp downstream of the poly A signal which has an apparently important role in mRNA termination (Gil and Proudfoot, 1984; McLauchlan et al., 1985).

The short region of HSV-1 has been completely transcript mapped (Rixon and McGeoch, 1984, 1985; McGeoch et al., 1985). Data for the long region, despite extensive mapping, remain incomplete (Wagner, 1985). Throughout the HSV genome there are 15 known 3' co-terminal families of mRNA (McGeoch et al., 1988b). This apparent clustering is particularly noticable within the short region which contains four 3' co-terminal mRNA families. In the case of US10 and US11 there is partial overlap of the coding regions of the genes with both being translated from the same strand in different reading frames. Additionally certain polyadenylation signals (eg. for the genes UL29 and UL38) only operate partially to produce 5' co-terminal mRNAs encoding a single polypeptide.

1.6b HERPES SIMPLEX VIRUS POLYPEPTIDES

Analysis of the DNA sequence of HSV-1, strain 17, has revealed the presence of 72 genes encoding 70 distinct proteins (McGeoch et al.,1988b). This figure is liable to be an under estimate of the true number as a conservative approach was adopted in interpreting the sequence data. In particular the gene content of the major repeat elements remains unresolved and any highly spliced, extensively overlapping or very small genes would not have been recognized.

The products of many genes remain to be identified and

their functions described. Approximately fifty polypeptides induced by HSV-1 have been recognized by one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Honess and Roizman, 1973, 1974; Powell and Courtney, 1975; Marsden et al., 1976). On two-dimensional SDS-PAGE at least 230 distinct polypeptide species have been identified (Haarr and Marsden, 1981). Many of these are derived from single primary translation products modified by several possible reactions including cleavage, glycosylation, phosphorylation and sulphation to give families of related polypeptides.

1.6c HERPES SIMPLEX VIRUS TEMPORAL REGULATION

HSV gene expression when analysed in the presence of metabolic inhibitors of protein and DNA synthesis has been shown to be temporally regulated, primarily at the level of transcription. Three broad temporal classes designated immediate-early (IE or alpha), early (E or beta) and late (L or gamma) have been described (Honess and Roizman, 1974; Swanstrom and Wagner, 1974; Clements et al., 1977).

With the possible exception of IE genes which demonstrate some clustering, there is no apparent relationship between the physical location of HSV genes and their temporal regulation (Clements et al., 1977; Wagner, 1985). Two of the IE genes, IE1 and IE3, encoding VmwIE110 and VmwIE175 are present within the repeat regions and are consequently diploid.

Immediate-early polypeptides have been shown, both by the analysis of temperature sensitive mutants and plasmid-based transient expression systems, to play a major role in the control of gene expression.

1.6d HERPES SIMPLEX VIRUS IMMEDIATE-EARLY GENE EXPRESSION

Five major IE genes (IE 1-5) encoding VmwIE110, VmwIE63, VmwIE175, VmwIE68 and VmwIE12 have been identified for HSV-1 with equivalent polypeptides having been found in HSV-2 infected cells (Clements et al., 1979; Preston, 1979a; Easton and Clements, 1980).

Immediate-early RNA is transcribed in the absence of de novo protein synthesis (Kozak and Roizman, 1974; Clements et al., 1977) although Vmw65, the major structural component of the virion has been shown to stimulate IE RNA transcription by 5-10 fold (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).

At 37^{o}C detectable levels of IE RNA are present in HSV infected cells at 1h post infection. The rate of synthesis increases until 2h PI with levels of mRNA increasing until approximately 3h PI. There is evidence of down-regulation of IE expression by both VmwIE175 and by early gene products although IE RNA is still present at late times in infection (Preston, 1979a; Gelman and Silverstein, 1986; Honess and Roizman, 1974, 1975; Godowski and Knipe, 1986; Harris-Hamilton and Bachenheimer, 1985).

In plasmid-based expression systems it has been shown that VmwIE110 has a stimulatory effect on all IE promoters (O'Hare and Hayward, 1985b; Gelman and Silverstein, 1986, 1987). At low molar ratios VmwIE175 also has a slight stimulatory effect. At high molar ratios, however, VmwIE175 inhibits expression from its own promoter and those of other IE genes except for IE2 where there appears to be a general stimulatory effect independent of molar ratios (DeLuca and

Schaffer, 1985; O'Hare and Hayward, 1985b; Gelman and Silverstein, 1986, 1987).

VmwIE110

VmwIE110 appears to be non-essential *in vitro* although it is thought to stimulate gene expression and play a role in initiating infection. Variants of both HSV-1 and HSV-2 deleted in one copy of IE1 have been shown to grow normally in tissue culture (MacLean and Brown, 1987b; Harland and Brown, 1985; this thesis). Also, viable HSV-1 constructs deleted in both copies of VmwIE110 have been described (Stow and Stow, 1986; Sacks and Schaffer, 1987). The growth of these variants was significantly impaired compared to the parental wild-type strains with yields following low-multiplicity infection being reduced by up to 100 fold. It was suggested (Stow and Stow, 1986) that this effect might be due to the lack of stimulation of VmwIE175 production in the absence of VmwIE110. At low multiplicities of infection of virus deleted in IE gene 1 this might mean that the levels of VmwIE175 fail to reach some critical level required to allow progression to the early and late phases of infection.

In vivo studies using the mouse footpad model suggest that a variant of HSV-1, strain 17, *dl* 1403, deleted in both copies of VmwIE110 is capable of establishing a latent infection and of reactivating spontaneously, albeit possibly delayed (Clements and Stow, 1989). The delay in reactivation, however, may be due to the fact that the deletion also removes the latency associated transcript (LAT) whose deletion has been implicated with delayed reactivation (Steiner *et al.*, 1989; Leib *et al.*,1989b). The same variant, *dl* 1403 when used in the mouse eye model (Leib

et al., 1989a) caused latent infection and could reactivate spontaneously. This contrasts with two other VmwIE110 deletion variants of strain KOS which went latent but which did not reactivate spontaneously possibly due to strain differences or to the existence of additional undetected mutations elsewhere in their genomes.

Studies using the deletion variant dl 1403 demonstrated that it is able to establish and maintain a latent infection in vitro (Russell et al., 1987). Unlike the control viruses ts k which has a temperature sensitive lesion in IE3 and in 1411 which has an 8bp linker insertion inactivating VmwIE175 superinfection with dl 1403 failed to reactivate HSV-2 suggesting a role for VmwIE110 as a trans-activator of gene expression required for reactivation in the in vitro system. The failure of dl 1403 to reactivate from in vitro latency in contrast to the in vivo results supports the hypothesis that reactivation is triggered by cellular mechanisms with similar trans-activating properties to VmwIE110.

VmwIE63

The isolation of a number of temperature sensitive mutants mapping within IE2 (UL54), encoding VmwIE63, has indicated that it is an essential gene (Sacks et al., 1985). At the non-permissive temperature these mutants show gross impairment in the production of late polypeptides. This observation is supported by plasmid-based transient expression assays which demonstrate that in conjunction with VmwIE110 and VmwIE175, VmwIE63 causes increased transcription from a late promoter, VP5, of the major capsid protein Vmw155 (Everett, 1986). McCarthy et al. (1989) confirmed that VmwIE63 is essential by the construction of deletion mutants which were viable only in a complementary

cell line. They proposed an essential role for VmwIE63 in the modulation of early and late gene expression at the transcriptional level.

MacLean and Brown (1987b) have isolated a variant of HSV-1, 1703, which fails to synthesise detectable levels of IE2 mRNA or VmwIE63 under immediate-early conditions. As IE2 has been shown to be an essential gene they suggested that VmwIE63 may not be essential at immediate-early times but may be required at early times to stimulate late gene expression. The variant 1703 has a deletion of approximately $5x10^6$ molecular weight from 0.76l-0.8lm.c. which removes part of UL55, all of UL56 and the 3' part of one copy of IE gene 1. As the deletion does not extend into IE gene 2 they postulated that a secondary mutation occurring in the promoter region of the gene may have changed its category from immediate-early to early.

More recently there has been a suggestion (J. McLauchlan, personal communication) that as the deletion in 1703 removes the polyadenylation signal of the IE1 gene, the message could read through to the next suitable signal. Due to the orientation of the genes in this region of the genome (see Fig. 4) this would mean that the message would continue through to the polyadenylation signal of UL51 and contain the coding information for the start of IE gene 1, part of UL55, all of UL54, UL53 and UL52 in an antisense form and a normal sense UL51. It is known that antisense RNA can function to repress the expression of viral genes (reviewed by Green et al., 1986) by the formation of an antisense RNA:mRNA hybrid structure. It is possible, therefore, that processing and transport out of the nucleus of the RNA of UL52, UL53 and UL54 would be at best inefficient. As the

antisense RNA would be under the control of the IE1 promoter it would be transcribed less at later times in infection allowing increased synthesis of the UL52, UL53 and UL54 gene products. Two type 2 deletion variants, HG52X85/5 and HG52X86 (Harland and Brown, 1985; this thesis) also show reduced or undetectable levels of VmwIE64, the type 2 equivalent of VmwIE63. As the deletions in both variants, like that in 1703, do not extend as far as UL54 this supports the suggestion that the deletion may be the cause of the effect on VmwIE63 rather than a secondary mutation.

VmwIE175

The isolation of a number of ts mutants has shown IE3 to be an essential gene (Marsden et al., 1976; Watson and Clements, 1978, 1980; Preston, 1979a; Dixon and Schaffer, 1980; DeLuca et al., 1984). This is supported by the isolation of variants deleted in both copies of IE3 which only grow in cell lines expressing VmwIE175 (DeLuca et al., 1985). Variants of both HSV-1 and HSV-2 lacking only one copy of IE3 grow normally in tissue culture (Longnecker and Roizman, 1986; Brown and Harland, 1987; this thesis)

At the non-permissive temperature most ts mutants in IE3 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). The isolation of ts mutants in IE gene 3 which express early but not late genes and the finding that some mutants are DNA negative whilst others replicate DNA normally (DeLuca et al., 1984) led to the suggestion that VmwIE175 is also important in the regulation of DNA replication and late gene expression possibly through different domains of the polypeptide. The majority of ts

mutants, however, demonstrate the involvement of VmwIE175 in early gene expression and immediate-early repression (Dixon and Schaffer, 1980; Watson and Clements, 1980). This latter function is supported by *in vitro* translation assays demonstrating the down-regulation of the IE3 promoter by VmwIE175 whilst early and late promoters are up-regulated (Pizer *et al.*, 1986).

VmwIE68

A mutant deleted in IE4 has been isolated and found to be host-range restricted (Post and Roizman, 1981; Ackermann *et al.*, 1985; Sears *et al.*, 1985a). It has, therefore, been suggested that VmwIE68 is an essential polypeptide which can, in certain cell lines, be substituted by host cell factors. The pattern of gene expression in non-permissive cells infected with the mutant led to the suggestion that VmwIE68 plays a role in late gene expression.

VmwIE12

The isolation of viable variants of both HSV-1 and HSV-2 in which IE5 has been deleted has demonstrated the gene to be non-essential for growth in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987; this thesis). A possible role for VmwIE12 in the regulation of early genes has been proposed by O'Hare and Hayward (1985a).

1.6e SEQUENCE REQUIREMENTS FOR HERPES SIMPLEX VIRUS IMMEDIATE-EARLY PROMOTERS

Immediate-early promoters have been shown to consist of a minimal transcription unit, required for basal level expression, which is regulated by upstream sequences which give temporal control (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 sequences required to allow basal level expression have been shown to include the CAP site, TATA box and G-C rich elements. All HSV-1 and HSV-2 IE genes contain at least one copy of an upstream regulatory element with a consensus sequence TAATGARATTC (R=purine) which is responsive to the major virion component Vmw65.

1.6f HERPES SIMPLEX VIRUS EARLY GENE EXPRESSION

It transpires that the division of HSV gene expression into three distinct classes is an over simplification. Early gene transcription is maximal between 4h and 6h post infection, normally following IE gene transcription. However, early gene expression in certain cases has been shown to be independent of IE gene expression prompting the sub-division of the early class into $beta_1$ and $beta_2$ genes depending on the absence or presence of the requirement for functional IE gene products (Pereira et al., 1977; Roizman and Batterson, 1985).

The concept of a spectrum of temporal regulation rather than three distinct classes is further indicated by the blurring of the division between early and late genes. For example, the production of certain polypeptides such as glycoprotein D, whilst being detected at early times, is stimulated following DNA synthesis (Gibson and Spear, 1983; Johnson et al., 1986). Various subdivisions, including early-late, leaky-late, beta-gamma and $gamma_1$ have, therefore, been proposed to accurately describe the expression of genes which do not fall neatly within the strictures of the three tier classification (Harris-Hamilton

and Bachenheimer, 1985; Roizman and Batterson, 1985; Wagner, 1985).

Similar patterns of stimulation by IE gene products, of both early and early-late genes have been demonstrated in short-term transfection assays. Individually both VmwIE110 and VmwIE175 have been shown to stimulate expression from early promoters but together they appear to act synergistically to give increased expression (Everett, 1984a, 1986; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985; Gelman and Silverstein, 1986).

1.6g SEQUENCE REQUIREMENTS FOR HERPES SIMPLEX VIRUS EARLY PROMOTERS

Sequence requirements for the HSV thymidine kinase (tk) promoter (true early) and for the glycoprotein D (gD) promoter (early-late) have been extensively investigated (McKnight and Kingsbury, 1982; McKnight et al., 1984; Eisenberg et al., 1985; El Kareh et al., 1985; Jones et al., 1985; Coen et al., 1986; Everett, 1983, 1984b).

In each case there appear to be at least four functionally important regions required for efficient transcription: a region within the untranslated leader close to the RNA CAP site, a TATA box, and two separate G-C rich regions. Additionally the tk promoter requires a CAAT box function which is apparently missing from the gD promoter.

1.6h HERPES SIMPLEX VIRUS LATE GENE EXPRESSION

Late gene products are detected initially at approximately 2h post infection and continue to accumulate up to 16h post infection (Wilkie, 1973; Roizman, 1979).

As previously stated, the division between early and late

gene expression is not clear-cut, with some essentially late genes being transcribed to some extent in the absence of DNA synthesis. These genes are variously described as early-late, leaky-late or gamma$_1$ genes. The second group which requires DNA replication prior to transcription consists of true-late or gamma$_2$ genes (Roizman and Batterson, 1985; Wagner, 1985; Powell et al., 1975; Godowski and Knipe, 1985; Johnson et al., 1986).

Findings from plasmid-based assays show that late promoters are essentially similar to early promoters in their regulation. As in the case of early promoters, VmwIE110 and VmwIE175 individually have been shown to stimulate expression from late genes whilst together they act synergistically to give increased expression (Johnson, 1987; Shapira et al., 1987; DeLuca and Schaffer, 1985).

The requirement for DNA synthesis to allow expression from a true-late gene has also been demonstrated in a plasmid-based assay by Johnson and Everett (1986a). Their data support the view that the increase in expression from late genes following novel DNA synthesis is not due to a simple increase in template numbers. They, therefore, propose that the promoters may be more susceptible to transcription factors following DNA replication.

1.6i SEQUENCE REQUIREMENTS FOR HERPES SIMPLEX VIRUS LATE PROMOTERS

Work by different groups supports the view that the only sequence requirement for true-late gene promoters is the presence of a TATA box (Johnson and Everett, 1986b; Homa et al., 1986; Shapira et al., 1987).

1.7 GENETICS OF HERPES SIMPLEX VIRUS

The isolation of HSV mutants has proved extremely useful in identifying the positions and functions of a number of genes. The most useful group of mutants has been the temperature-sensitive (ts) mutants which have helped in the mapping and identification of many polypeptides. Temperature-sensitive mutants which grow in permissive conditions (eg. 31°C) but not in non-permissive conditions (eg. 38.5°C) are the most common example of conditional lethal mutants. Conditional lethal mutations by their nature must lie within essential genes. In contrast the isolation of viable deletion variants has proved useful in identifying genes non-essential in tissue culture, or in conjunction with complementary cell lines to unambiguously identify essential genes.

1.7a TEMPERATURE-SENSITIVE MUTANTS OF HERPES SIMPLEX VIRUS

The most common type of temperature sensitivity allows the virus to grow at a lower temperature (31-34°C), whereas, it fails to replicate at an elevated temperature (38-39.5°C).

Temperature-sensitive lesions are normally the result of a point mutation, leading to a single amino acid substitution within a polypeptide, rendering it non-functional at the non-permissive temperature. The nature of the alteration makes such mutants prone to spontaneous reversion and in some cases leakiness.

The ability of two ts mutants to complement one another allowing replication at the non-permissive temperature indicates that the lesions must be in different genes (Brown et al., 1973). In certain cases, however, low level

complementation may occur if the lesions lie within different regions of the same gene allowing intragenic complementation (Jamieson and Subak-Sharpe, 1974). At least thirty five complementation groups have been recognized in HSV indicating that the virus contains at least this many genes essential for growth in tissue culture (Schaffer et al., 1978, 1987).

The accurate map locations of temperature-sensitive mutations has been achieved by marker rescue of lesions with specific cloned HSV DNA fragments (Wilkie et al., 1974, 1978; Stow et al., 1978; Preston, 1981). Most temperature-sensitive mutants have been isolated following the treatment of replicating virus with mutagenic drugs such as 5 bromo-2'-deoxyuridine (BUdR) or with ultra violet radiation (Schaffer et al., 1970, 1973; Timbury, 1971; Esparza et al., 1974). These treatments can create multiple mutations which may require to be resolved by recombination with wild-type virus (Preston, V. et al., 1984). A number of temperature-sensitive mutants has also been engineered by in vitro mutagenesis of specific cloned fragments of HSV DNA which are then recombined with wild-type virus (Chu et al., 1979; Sandri-Goldin et al., 1981).

1.7b DRUG-RESISTANT MUTANTS OF HERPES SIMPLEX VIRUS

The range of drug-resistant mutants is limited by the range of effective anti-viral preparations. All have lesions within the genes encoding either thymidine kinase or DNA polymerase (Honess et al., 1984; Chiou et al., 1985; Larder et al., 1987).

The nucleoside analogue 5 bromo-2'-deoxyuridine (BUdR) has an antiviral effect being phosphorylated by thymidine kinase (tk) with the phosphorylated form, in turn, inhibiting DNA polymerase (Dubbs and Kit, 1964). The presence of BUdR,

therefore, causes suppression of wild type HSV growth. The growth of the mutant 2011 is somewhat reduced in $LMTK^+$ cells in the presence of BUdR due to the presence of cellular tk. However, in $LMTK^-$ cells, 2011 grows equally well with or without BUdR.

Another antiviral drug, acycloguanosine (ACG), also inhibits viral DNA synthesis following phosphorylation by the viral tk. The majority of ACG-resistant mutants proved to be tk negative. However, in some cases it was found that the alteration leading to drug-resistance lay, not in the inability of the viral tk to phosphorylate the drug, but was due to changes in the DNA polymerase which no longer recognized the phosphorylated ACG (Crumpacker et al., 1980; Field et al., 1980; Darby et al., 1981; Larder and Darby, 1985; Larder et al., 1987).

Phosphonoacetic acid (PAA) is an antiviral drug which acts directly on the DNA polymerase by binding to the pyrophosphate binding site (Leinbach et al., 1976). A number of PAA-resistant mutants have been described which have lesions within the DNA polymerase gene (Hay and Subak-Sharpe, 1976; Purifoy and Powell, 1977).

1.7c PLAQUE MORPHOLOGY MUTANTS OF HERPES SIMPLEX VIRUS

Most HSV wild-type strains create plaques in cellular monolayers as the result of the rounding up of infected cells. Attempts to identify variants on the basis of differences in the size or shape of plaques are normally too variable or subjective to be useful. However, morphologically distinct variants producing syncytial (syn) plaques, consisting of multinucleate cells formed by the fusion of infected cells, have been isolated (Hoggan and

Roizman, 1959; Brown et al., 1973). The lesions causing syncytia formation have been mapped to at least seven separate loci (reviewed by Marsden, 1987).

1.7d DELETION VARIANTS OF HERPES SIMPLEX VIRUS

The isolation of viable variants has identified a number of genes non-essential for growth in tissue culture. Polypeptides identified as being non-essential by this means include thymidine kinase (Sanders et al., 1982), glycoprotein C (Little et al., 1981), VmwIE110 (Stow and Stow, 1986; Sacks and Schaffer, 1987), the products of genes UL55 and UL56 coding for the predicted polypeptides 20K and 22K respectively (MacLean and Brown, 1987b, c) and all the genes in U_S with the exception of glycoprotein D (Longnecker and Roizman, 1986, 1987; Umene, 1986; Brown and Harland, 1987; Longnecker et al., 1987; Harland and Brown, 1988; this thesis).

The use of variants deleted in specific genes in conjunction with biochemically transformed cell lines containing the same stably integrated HSV genes has allowed the unambiguous identification of essential genes. Originally it was shown that cells transformed with IE3, encoding VmwIE175, were capable of sustaining the growth at the non-permissive temperature of a temperature-sensitive mutant of HSV-1 with a lesion in that gene (Davidson and Stow, 1985). Subsequently variants with deletions in IE3 were constructed which grew only in the complementary cells proving the essential nature of the gene (DeLuca et al., 1985; Smith and Schaffer, 1987). Similar experiments have proved glycoprotein B and VmwIE63 to be essential polypeptides (Cai et al., 1987; McCarthy et al., 1989).

Herpes simplex virus genomes with restriction enzyme site-deletions have also been isolated. Originally restriction enzyme site-deletion mutants were isolated in adenovirus type 5 (Jones and Shenk, 1978, 1979). The method employed to obtain such mutants was enrichment selection of genomes pre-existing within a population. The procedure involved cleavage of viral DNA with restriction enzyme, ligation of the fragments and transfection of the resultant DNA to select viable genomes. As genomes with fewer restriction enzyme sites are more likely to survive the procedure to produce viable virus the method selects for naturally occuring mutants lacking restriction enzyme sites. Herpes simplex virus restriction enzyme site-deletion mutants have been isolated either by enrichment selection (Brown et al., 1984; Harland and Brown, 1985, 1988; Brown and Harland, 1987; MacLean and Brown, 1987a; this thesis) or following site directed mutagenesis by recombination of wild type virus with oligonucleotide sequences containing a conservative single base alteration which disrupts the site (MacLean and Brown, 1987a). Mutants with deleted restriction enzyme sites have proved useful in recombination studies (MacLean, 1988), as tools in superinfection experiments to study latency (Cook and Brown, 1987) and as eukaryotic vectors as possible recipients of cloned viral fragments at desired positions (F. Rixon personal communication).

1.8 THE EVOLUTION OF HERPESVIRUSES

Similarities between different members of the herpesvirus families, for example the alphaherpesviruses HSV-1 and VZV, have led to the conclusion that they are closely related, probably evolving from a common ancestral virus (Davison and

Wilkie, 1983c; Honess, 1984; McGeoch, 1987). This close relationship, based on antigenic cross-reaction and DNA cross-hybridization, is strongly supported by the fact that VZV complements certain HSV-1 ts mutants with lesions in, for example, VmwIE63 and VmwIE175 (Felser et al., 1987). Subsequent analysis of the DNA sequences of HSV-1 and VZV has confirmed a high degree of homology with many of the genes, especially within U_L, being co-linear (Davison and McGeoch, 1986; Davison and Scott, 1986; McGeoch, 1987).

On the basis of antigenic cross-reaction and DNA cross hybridization there is no indication of any close relationship between members of different herpesvirus sub-families. However, similarities at the level of gene homology were detected by comparison of the DNA sequences of VZV (an alphaherpesvirus) with EBV (a gammaherpesvirus) (Davison and Taylor, 1987). The related genes are located within U_L in three blocks which remain fairly consistent although the order of the blocks themselves differs in the two genomes. The long segment of the herpesviruses contains those genes which are more highly conserved and is less variable than the short segment which differs both between and within herpes family sub-groups (McGeoch, 1987).

The theory that herpes viruses may have evolved from eukaryotic cellular DNA is supported by comparison of homologies between viral and cellular genes (McGeoch, 1987). Homologies have been detected between herpes genes and cellular genes encoding both subunits of ribonucleotide reductase (Nikas et al., 1986), thymidylate synthetase (Davison and Scott, 1986; Honess et al., 1986; Thompson et al., 1987), a protein kinase (McGeoch and Davison, 1986) and DNA polymerase (Earl et al., 1986; McGeoch, 1987). This type

of approach may elucidate the genetic content of ancestral herpes viruses (Honess, 1984).

1.8a G+C CONTENT OF HERPESVIRUSES

It has been suggested that, with notable exceptions such as VZV, herpes viruses with similar biological properties show similar G+C content: alphaherpesviruses having high G+C content, betaherpesviruses intermediate G+C content and gammaherpesviruses low G+C content (Honess and Watson, 1977; Honess, 1984). This generalization is not borne out by the accurate G+C determination of those genomes which have been completely sequenced. Herpes simplex virus type 1 has an overall G+C content of 68.3% (McGeoch et al., 1988b) while the level for the closely related alphaherpesvirus, VZV, is 46% (Davison and Scott, 1986) and the more distantly related gammaherpesvirus, EBV, has a G+C content of 60% (Baer et al., 1984).

It is open to speculation whether the observed variations in G+C content arise as a consequence of cellular factors directing the evolution of viral changes or conversely if some genetically predetermined factors within the viruses determine the differences in G+C content which might in turn have an effect on cell tropism.

1.8b REPEAT REGIONS OF HERPESVIRUSES

Herpes simplex virus genomes consist of two unique portions of DNA, U_L and U_S, each flanked by inverted repeat regions, R_L and R_S, with 'a' sequences present at the termini and in inverted orientation at the L/S junction. Herpes simplex virus genomes exist as four equimolar isomers due to high frequency inversion about the L/S junction

(Sheldrick and Berthelot, 1974; Wadsworth et al., 1975; Delius and Clements, 1976; Roizman, 1979; Roizman et al., 1979). The reason for the observed isomerization in HSV is not clear although it is known that at least three of the four isomers are biologically active (Davison and Wilkie, 1983b; Poffenberger and Roizman, 1985; Jenkins and Roizman, 1986; Longnecker and Roizman, 1986). The genes contained within the repeats are diploid. However, the available evidence showing that the repeat regions of related viruses contain different genes (Davison and McGeoch, 1986) together with the proven viability in tissue culture of deletion variants with single copies of normally diploid genes suggests that such duplication is not essential (Davison et al., 1981; Brown et al., 1984; Harland and Brown, 1985; Brown and Harland, 1987; MacLean and Brown, 1987b; this thesis).

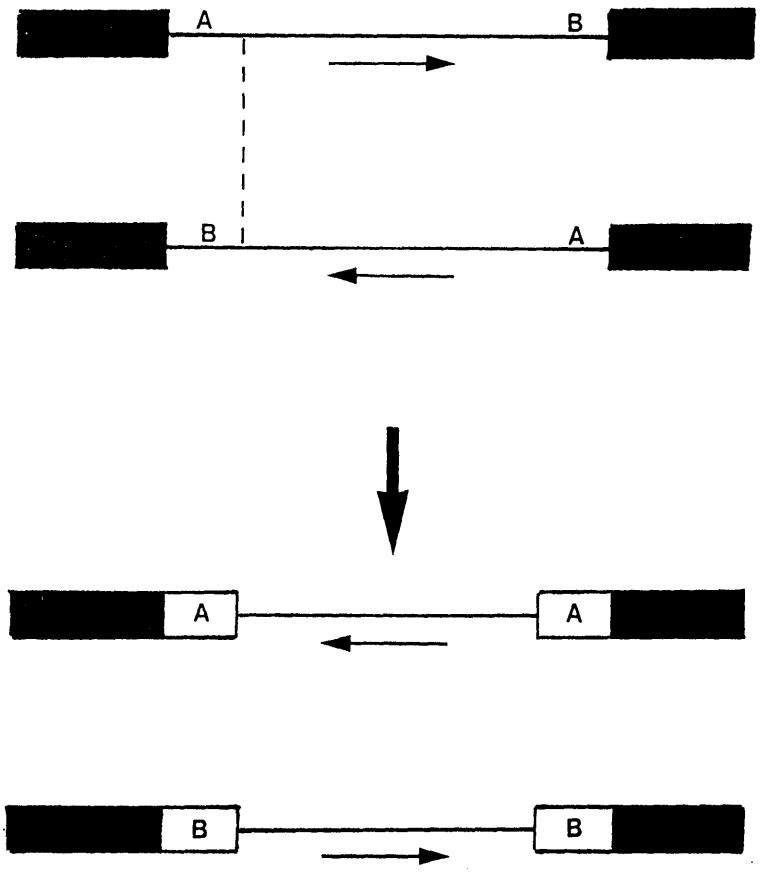
The repeat elements do not represent fixed compartments in terms of the contained genes and they are also flexible in size. It has been proposed that herpesvirus inverted repeats can expand and contract (Honess, 1984; McGeoch, 1984; Whitton and Clements, 1984b; Davison and McGeoch, 1986). A model involving illegitimate recombination within the unique segments of two identical genomes inverted relative to each other (see Fig. 8) has been proposed to explain expansion of the repeat elements (Whitton and Clements, 1984b). This model could account for both unaltered genome size if the parental genomes are perfectly aligned in inverted orientation, or for changes in overall size due to unequal exchange. The model proposes that limitations on the contraction or expansion of repeat elements would be imposed by the deletion or alteration of

FIGURE 8

A model for the expansion of repeat regions through non-homologous recombination.

The upper two lines represent one of the unique segments (long or short) of the parental genome. Repeat region is shown as a solid box; unique region depicted as single line; the orientation of the unique portion is indicated by an arrow; the dotted line represents the crossover point. Each recombination yields two distinct progeny molecules with expanded repeats (lower two lines).

The figure depicts two elements perfectly aligned in opposite orientations yielding progeny with expanded repeats but unaltered in overall size. Unequal exchange would produce genomes of different sizes.


A
B
B
A
A
A
B
B

essential genes or regulatory sequences. The isolation of variants of both HSV-1 and HSV-2 with expanded short and long repeat regions (Umene, 1986; Brown and Harland, 1987; MacLean and Brown, 1987c; this thesis) substantiates this model.

The simplest mechanism to explain contraction of the repeat elements would be the deletion of information from one copy of the normally diploid inverted repeat regions. It is proposed that limitations would be imposed by essential functional elements within the repeat (Whitton and Clements, 1984b). If the deleted portion were adjacent to unique sequences this would cause elongation of the unique portion and contraction of the repeat. This explanation has been proposed by MacLean and Brown (1987c) to explain the structure of a variant of HSV-1 with a contracted long repeat region.

A complicated evolutionary model involving this type of repeat expansion and contraction as a possible mechanism to explain the relationship between the short regions of VZV and HSV-1 has been proposed by Davison and McGeoch (1986).

1.9 RECOMBINATION

1.9a RECOMBINATION IN HIGHER EUKARYOTES

Due to the complexity of recombinational events between chromosomes, investigation of eukaryotic genetic exchange has mainly centered on plasmids or viruses, with the hope that an understanding of the mechanisms employed in these simpler systems may shed light on the more complicated processes of cellular recombination.

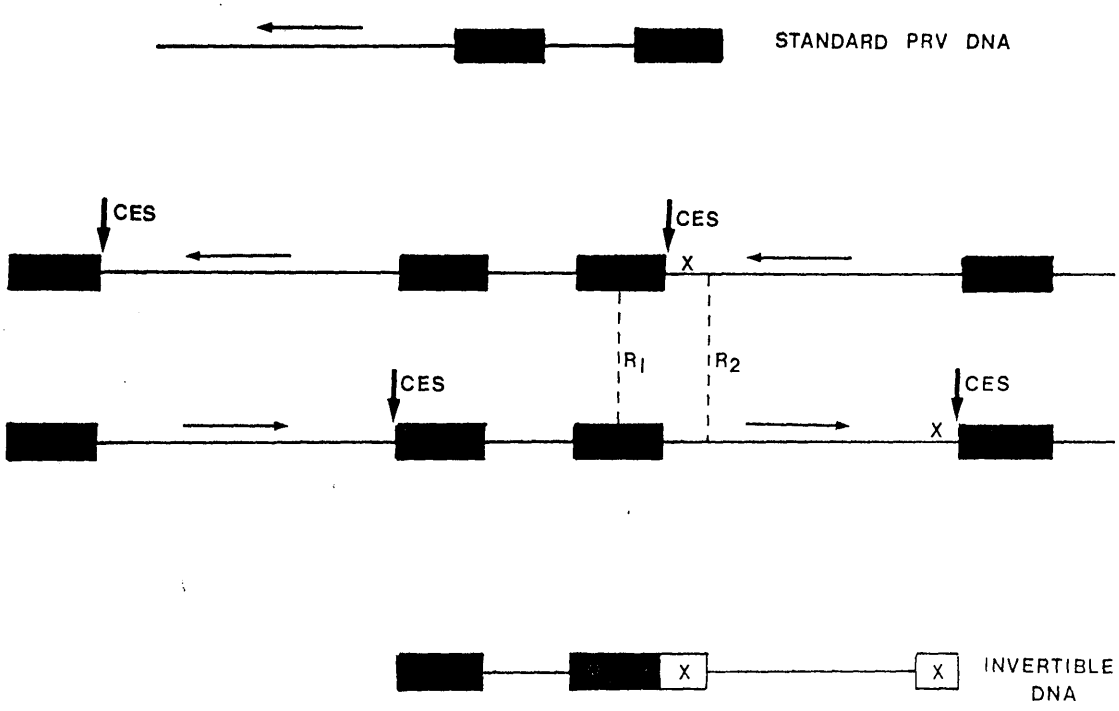
It has been demonstrated that recombination occurs

between plasmid molecules following transfection (Folger et al., 1984; Song et al., 1985; Anderson and Eliason, 1986; Chakrabarti and Seidman, 1986). In this system recombination is mediated by cellular functions.

Recombination has been shown to occur in a number of viruses including HSV (Wildy, 1955; Subak-Sharpe, 1969), pseudorabies virus (PRV) and adenovirus (Frost and Williams, 1978). The genome of PRV consists of a non-inverting long component (L) and a short component (S), bounded by inverted repeats, which does invert. Lu et al. (1989) have reported the isolation of a number of variants in which the L component is bracketed by inverted repeats allowing inversion. They conclude that the variants must have arisen by recombinational events involving concatameric or circular DNA (see Fig. 9). Intertypic recombination has been demonstrated within homologous regions of different adenovirus strains (Boursnell and Mautner, 1981; Mautner and Boursnell, 1983; Young and Silverstein, 1980). Intertypic recombinants were detected by selection of ts^+ progeny following infection with two ts adenovirus strains, thereby, allowing detection of crossovers between the ts markers (Sambrook et al., 1975). Limitations of this system, for example, failure to detect those genomes with even numbers of crossovers between the markers or in which the crossovers render the virus inviable, may be overcome by the use of Southern blotting techniques with specific probes to detect novel viral DNA restriction profiles. Restriction enzyme analysis of intertypic recombinants showed that, not only did the number of such recombinants increase with time, but that the number of crossovers within the ts^+ progeny also increased markedly indicating the participation of

FIGURE 9

Model from Lu et al., (1989) showing how pseudorabies virus (PRV) genomes with invertible L components may be generated. Line one depicts a standard PRV genome (not drawn to scale) with a long unique component and a short unique portion bracketed by inverted repeats which allow inversion of the S component. Lines two and three show how two recombinational events (one homologous (R_1) in the inverted repeat and one illegitimate (R_2) in the long unique segment) between standard concatameric DNA molecules in opposite orientations may give rise to a genome in which sequence (X), normally present at the left end of the genome is translocated to the right end of L. Deletion of sequences normally present at the right end of L would occur. Only if these sequences are non-essential will the generated genomes be viable. The necessity to postulate two recombinational events arises due to the size of the progeny of a single crossover. Due to the positions of the cleavage/encapsidation signals (CES) single crossovers would result in progeny almost twice the size of standard genomes which would probably not be encapsidated.


STANDARD PRV DNA
CES
CES
X
R1
R2
CES
CES
X
X
INVERTIBLE DNA

recombinant progeny molecules in further recombinational events (Young and Silverstein, 1980).

There is a link between adenovirus recombination and replication with recombination initially being detected immediately after the start of DNA replication and increasing in frequency into the late phase of DNA replication (Young and Silverstein, 1980). This link is supported by experiments showing that blocking adenovirus DNA replication by the addition of cytosine arabinoside slows down and reduces the extent of recombination (Young et al., 1984) and by evidence from electron microscopic studies demonstrating interactions between full length duplexes and replicative intermediates during the DNA-replication phase of adenovirus infection (Munz et al., 1983).

Replication and recombination in adenovirus might be linked as a consequence of structural or functional aspects of replication. A possible structural link being the production during the replicative cycle of single-stranded DNA sequences which might represent candidates for initiating genetic exchange (Flint et al., 1976). Shared functional aspects of replication and recombination might involve proteins such as the DNA-binding protein, DNA polymerase or cellular polypeptides. The observation that wild-type adenovirus DNA recombination is delayed at 40.9^{o}C despite active replication implies that there is some thermolabile factor which directly or indirectly affects viral recombination without affecting DNA replication (Young et al., 1984).

It has been shown that in cells deficient for DNA repair, ts mutants recombine to yield ts^{+} progeny at the same frequency as in normal cells indicating that the host cell

repair system need not be involved in adenovirus recombination (Young and Fisher, 1980). Interestingly, somewhat contradictory results have been obtained when studying a different adenovirus recombination system in which overlapping sub-genomic fragments of adenovirus were co-transfected (Chinnadurai et al., 1979). Recombination here occurs in the absence of viral replication indicating a possible role for host cell factors in recombination.

1.9b HERPES SIMPLEX VIRUS RECOMBINATION

Recombination between ts mutants of HSV to yield ts^+ progeny (Subak-Sharpe, 1969) has allowed the construction of linkage maps for different HSV strains (Brown et al., 1973; Schaffer et al., 1974; Brown and Ritchie, 1975; Timbury and Calder, 1976; Parris et al., 1980). Apart from HSV-2, strain HG52, where there is a close correlation, it transpired that, although the order was generally correct, the calculated genetic distance between the markers did not agree with their subsequently obtained physical map locations. This was thought to be due to multiple crossovers between distant markers (Stow et al., 1978; Wilkie et al., 1978).

The restrictions imposed by the limited homology between HSV-1 and HSV-2 have meant that intertypic recombination has not provided much information regarding the mechanisms of recombination although valuable information has been gained regarding the mapping of 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).

An interesting observation has been made by Batra and Brown (1989) who isolated unselected HSV-1 McKrae/ HSV-2 HG52 recombinants. They demonstrated preferential recombination between intact genomes and restriction endonuclease fragments containing an origin of replication. They speculated from their results that either (1) the presence of an origin of replication within a fragment allows amplification increasing the chance of recombination or that (2) recombination may be potentiated by the replicative process including, for example, the generation of single-stranded DNA. The functional relationship between recombination and replication was supported by the finding that even in large fragments such as HSV-2 Xba I c (0-0.45m.c.) recombination always occured to include the part of the fragment (0.35-0.45m.c.) containing an origin of replication indicating that amplification of the fragment was not the primary reason for the preferential recombination with origin-containing fragments. However, as the numbers of recombinants isolated by them was small due to the lack of any selection the significance of their observations is hard to evaluate.

To investigate the possibility of recombination being biased by the increased recombinational potential of particular sequences it would be desirable to study homotypic recombination. To this end it has been suggested that recombination between homotypic genomes containing different restriction enzyme sites as unselected markers could be studied (Brown et al., 1984). Using such a protocol to investigate recombination between two HSV-1 strains, differing in eight restriction enzyme sites no recombinational hot spot was apparent (Umene, 1985) although

it is probable that the markers would have to be closer together to yield significant data.

The four genomic isomers of HSV make analysis of recombination complicated and it is not clear whether recombination involves all four isomers, the circular molecules or concatameric DNA (Honess, 1984; Davison and Wilkie, 1983a; Poffenberger et al., 1983; Poffenberger and Roizman, 1985; Jacob et al., 1979).

There is some evidence on the basis of time course experiments and tri-parental crosses that progeny as well as parental genomes take part in recombination (Ritchie et al., 1977; MacLean, 1988). It is unclear at present what roles are played by viral and host cell factors in recombinational events (Dasgupta and Summers, 1980).

1.10 AIMS OF PROJECT

The original aim of the project was to isolate a HSV-2 , strain HG52, mutant lacking all four wild-type Xba I sites and to use the sites as unselected markers to study intratypic recombination in HSV-2. The isolation of Xba I site-deletion mutants by enrichment selection involved the restriction endonuclease screening of large numbers of individual plaque isolates. This analysis of HSV-2, strain HG52, showed that the percentage of viable spontaneous variants within a population was unexpectedly high and, therefore, most of the work presented in this thesis involved characterization and investigation of the biological properties of a number of these variants. Consequently, although a genome devoid of all four Xba I sites was isolated, no recombination studies using this genome have been carried out at present.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

VIRUS

Herpes simplex virus type 2, strain HG52 (Timbury, 1971), was the main parental strain used throughout this study. Stocks of HSV-2 , strain 186 (Esparza *et al.*, 1974), HSV-2, strain 333 (Seth *et al.*, 1974), *ts*1 from HG52 (Timbury, 1971), HSV-1, strain 17 (Brown *et al.*, 1973), HSV-1, strain KOS (Aron *et al.*, 1975) and HSV-1, strain McKrae (Williams *et al.*, 1965), were also used. All stocks, except HSV-1, strain McKrae, were grown from elite stocks held within the Institute of Virology. Apart from HG52 and *ts* 1 the precise passage numbers of the elite stocks are not known. HG52 and *ts* 1 were isolated by M. Timbury and had been grown sequentially from a single purified plaque into a 4x1/2 inch tube of BHK21/C13 cells, a 20 ounce bottle and a ten burrler stock. An inoculum of this was used to produce a further ten burrler stock, a titrated aliquot of which is distributed as the elite stock. Strains 186 and KOS were both received from P. Schaffer and subsequently grown once to produce the elite stock. Strain 17 came from J. Hay and was passaged once to give the elite stock. The strain 333 was received from F. Rapp and subsequently grown up twice to yield the elite stock. HSV-1 strain McKrae (passage number unknown) was supplied by J.M. Hill, Lousiana State University, New Orleans La., USA to S.D. Cook who grew up a stock, some of which was kindly supplied for this study.

CELLS

For the routine growth of virus and for most of the experimental work BHK21/C13 cells, a polyoma transformed line derived from baby hamster kidney (Macpherson and Stoker, 1962), were used. In the production of immediate early polypeptide extracts, F2002, human foetal lung cells were used (Flow Laboratories).

TISSUE CULTURE MEDIA AND SOLUTIONS

The Eagle's medium used for cell propagation was Glasgow minimum essential medium (Gibco Laboratories) supplemented with 100 units of penicillin-streptomycin per ml. Phosphate-free, sulphate-free and methionine-free Eagle's media, also tryptose phosphate broth (TP), phosphate buffered saline (PBS), trypsin and versene were prepared in the Institute of Virology as were the calf and pooled human sera.

The following abbreviations are used throughout the text for various media and solutions:

ECX%	Eagle's medium containing X% calf serum
ETCX%	Eagle's medium containing 10% TP and X% calf serum
EHuX%	Eagle's medium containing X% human serum
Trypsin/versene	Trypsin/versene (1/5)
PBS Ca	PBS containing 5% calf serum
PIC	Phosphate-free Eagle's medium containing 1% calf serum
$ESO_4^-C2\%$	Sulphate-free Eagle's medium containing 2% calf serum
Emet/5C2%	Eagle's medium containing 1/5 of the normal concentration of methionine and 2% calf serum

RADIOCHEMICALS

(^{32}P) orthophosphoric acid in 1ml of a 0.02M HCl solution (NEX-054) was obtained from New England Nuclear, DuPont (U.K.) Ltd., Stevenage, Hertfordshire. L-(^{35}S) methionine SJ204 30TBq/m mol; deoxycytidine 5'-(a-^{32}P) triphosphate, triethylammonium salt 110TBq/m mol; deoxyguanosine 5'-(a-^{32}P) triphosphate, triethylammonium salt and (^{35}S) sulphate SJ51 were all obtained from Amersham International plc., Buckinghamshire.

CHEMICALS

Agarose type 1; 5% bovine albumin; deoxyribonucleic acid from calf thymus type 1; deoxyribonucleic acid sodium salt type III from salmon testes; cycloheximide; 2' deoxyguanosine 5'-triphosphate sodium salt grade 1; thymidine 5'-triphosphate sodium salt and 2'-deoxyadenosine 5'-triphosphate disodium salt were all supplied by Sigma Chemical Co. Ltd., Fancy Rd., Poole, Dorset.

Temed (N, N, N', N'-tetramethylethylenediamine) was supplied by Biorad Laboratories, Richmond, California.

Ammonium persulphate was from Serva Feinbiochemica, Heidelberg.

Koch-Light Laboratories Ltd., Haverhill, Suffolk supplied trichloroacetic acid (TCA), acrylamide and caesium chloride.

Nitrocellulose sheets were obtained from Schleicher and Schuell, Dassel, W. Germany.

Tris (Tris (hydroxymethyl)aminomethan) was from Boehringer, Mannheim House, Bell Lane, Lewes, East Sussex.

Ecoscint was supplied by Nuclear Medical Electronic Systems and Services Ltd., 2 Hutton Square, Brucefield

Industrial Park, Livingston, Scotland.

Sodium heparin, 1,000 units/ml was from Leo Laboratories Ltd., Princes Risborough, Buckinghamshire.

EN^{3}HANCE Autoradiography enhancer was from DuPont (U.K.) Ltd., Stevenage, Hertfordshire

The remaining chemicals were mainly supplied by BDH Chemicals Ltd., Poole, England and were of analytical or reagent grade.

MISCELLANEOUS MATERIAL

Tissue culture Petri dishes were supplied by Sterilin Ltd., Feltham, England.

Sterile 15ml and 50ml tubes were obtained from Becton, Dickinson and Co., 1950 Williams Dr., Oxnard, CA, USA.

0.5ml, 1.5ml and 15ml tubes were from Sarstedt, W. Germany

Kapton tape was from Scotch Brand tape, St. Paul, MN, USA

XS1 X-Omat S (1596) X-ray film was from Kodak and Agfa Scopix CR 3B was from Agfa Gevaert.

Automatic pipette tips were from Gordon Keeble Ltd..

ENZYMES

Ribonuclease T1 grade V, ribonuclease type II A and protease type 14 were obtained from Sigma Chemical Co. Ltd., Fancy Rd., Poole, Dorset.

The restriction endonucleases BamH I, Bgl II, EcoR I, Hind III, Hpa I, Kpn I, and Xbal I were obtained from Bethesda Research Laboratories, Gibco Ltd., P.O. Box 35, 3 Washington Rd., Paisley

BUFFER SOLUTIONS

The following buffer solutions were routinely used:

HEBS: 137mM NaCl, 5mM KCl, 0.2mM Na_2HPO_4, 9mM D-glucose and 21mM Hepes-NaOH pH 7.05

NTE: 100mM NaCl, 1mM EDTA and 10mM Tris-HCl pH 7.4

TE: 1mM EDTA and 10mM Tris-HCl pH 7.4

RSB: 10mM KCl, 1.5 mM $MgCl_2$ and 10mM Tris-HCl pH 7.4

SSC: 150mM NaCl and 15mM sodium citrate pH 7.5

BUFFER FOR PHENOL SATURATION: 50mM NaCl, 10mM EDTA and 10mM Tris-HCl pH 7.5

ELECTROLYSIS BUFFER FOR AGAROSE GELS: 30mM NaH_2PO_4, 1mM EDTA and 36mM Tris-HCl pH7.8. If DNA was to be visualized under U.V., ethidium bromide at 0.5 ug/ml was added.

RE STOP: 10% Ficoll 400, 105mM EDTA, 150mM NaH_2PO_4 and 180mM Tris-HCl pH 7.8. Bromophenol blue was added to give colour to samples.

SAMPLE BUFFER FOR POLYPEPTIDES: 10% glycerol, 70.8mM SDS, 717mM 2-mercaptoethanol and 48.7mM Tris-HCl pH 6.7

RESOLVING GEL BUFFER: 14mM SDS and 1.5M Tris-HCl pH 8.9

SPACER GEL BUFFER: 14mM SDS and 487mM Tris-HCl pH 6.7

ACRYLAMIDE GEL TANK BUFFER: 3.5mM SDS, 53mM glycine and 52mM Tris.

CLONED HSV-2 FRAGMENTS

The following fragments were used during the course of this study:

BamH I a'	pGZ66
BamH I b'	pGZ67
BamH I g	pGZ1
Hind III a	pGZ26
Hind III b	pGZ11
Hind III e	pGZ14

Hind III h pGZ15

Hind III k pGZ12

Hind III l pGZ28

Hind III o pGZ13

METHODS

2.1 GROWTH OF CELLS

BHK21/C13 cells were grown at 37°C in 2.5 litre roller bottles containing 100ml of ETC10% in an atmosphere of 95% air and 5% CO_2. To harvest, the cells were washed twice with trypsin/versene, allowed to detatch from the surface, and resuspended in 20ml of ETC10% to give approximately $1.5x10^7$ cells per ml. Roller bottles were seeded at a density of $3x10^7$ cells/bottle (ie. split 1 in 10). Fifty millimetre diameter plastic Petri dishes were seeded at $3x10^6$ cells per dish in 4ml of ETC10% and were used after 16-24h at 37°C. Twenty four well tissue culture dishes were seeded at $5x10^5$ cells per well in 0.5ml of medium.

2.2 GROWTH OF VIRUS STOCKS

Virus stocks were prepared by infecting almost confluent monolayers of BHK21/C13 cells in roller bottles with 20ml of ETC10% containing $1x10^6$ plaque forming units (pfu) (ie. a multiplicity of infection (MOI) of approximately 1pfu/300 cells). The bottles were incubated at 31°C for 3-7 days until complete cytopathetic effect (cpe) was observed. The infected cells were shaken into the medium (if necessary with the aid of sterile glass beads) and pelleted by centrifugation at 2,000rpm for 10 min at 4°C. The supernatant was spun at 12,000 rpm for 2h to retrieve the cell released virus and the virus pellet resuspended in a small volume of the supernatant by sonication. The cell pellet was resuspended in a quantity of supernatant (approximately 0.5ml/roller bottle) and the cells disrupted

in a sonic bath. Cellular debris was removed by centrifugation at 2,000 rpm for 10min and re-extracted as before. The cell released and cell associated virus was aliquoted separately or combined and stored at -70°C. Sterility checks were performed using blood agar and, if sterile, the stock was titrated.

2.3 TITRATION OF VIRUS STOCKS

Serial ten fold dilutions of the virus were made in PBS Ca and 0.1ml inocula added to BHK21/C13 cell monolayers in 50mm Petri dishes from which the growth medium had been removed. The virus was allowed to absorb for 45min at 37°C, 4ml of EHu5% added and the cells incubated at 31°C or 38.5°C for 3 or 2 days respectively.

Pooled human serum contains neutralizing antibodies which prevent the spread of virus through the medium. To prevent serum clotting, which can have a deleterious effect on the monolayer, two drops of heparin (1,000 units/ml) were added to 20ml of serum prior to its addition to the growth medium.

Monolayers were exposed to Geimsa stain for 20min. at room temperature, washed with water and the plaques counted using a dissecting microscope. Counts from plates with fewer than twelve plaques were not considered to be statistically reliable whereas greater than four hundred plaques were often too close together to count.

2.4 PLAQUE PURIFICATION OF VIRUS

Virus was titrated under EHu5% at a dilution calculated to give plates with a small number of well separated plaques. The medium was removed and the plate washed four times with PBS Ca. The infected cells forming a single

plaque were taken up into a GK11 tip, using an automatic pipette, and transferred to 0.5ml of PBS Ca; the operation being carried out using the dark field illumination of a Wild M7A stereoscopic dissecting microscope. The infected cells were disrupted by sonication to release virus.

After three rounds of plaque purification, the virus from individual plaques was grown up to give a 50mm plate stock which was subsequently used to inoculate roller bottles for working stocks of virus.

2.5 SINGLE CYCLE GROWTH EXPERIMENTS

The medium was removed from 35mm Petri dishes containing almost confluent monolayers of BHK21/C13 cells ($1x10^6$ cells). The cells were infected at a multiplicity of infection (MOI) of 5 plaque forming units (pfu) per cell in 0.1ml of inoculum and incubated at 37^oC for 45min to absorb the virus. The plates were then washed twice with PBS Ca, overlaid with 2ml of ETC10%, and returned to 37^oC for selected periods (0-24h). Virus was harvested by scraping the cells into the medium and sonicating prior to titration. All experiments using mutant virus were carried out with the appropriate wild type virus controls.

2.6 RESTRICTION ENZYME ANALYSIS OF ^{32}P LABELLED VIRAL DNA

The method used was a modification of that described by Lonsdale (1979). Twenty four well tissue culture plates were seeded with $5x10^5$ BHK21/C13 cells in 0.5ml of PIC and incubated at 37^oC. On the following day when the monolayers were confluent, virus was added at a MOI of 10pfu/cell and the plates incubated at 37^oC for 1h. The medium was then removed, the cells washed with 0.8ml and overlaid with

0.45ml of PIC. After 2h at 31°C, 50uCi of ^{32}P orthophosphate was added in 50ul of PIC and the plates incubated at 31°C for approximately 48h until there was extensive cytopathic effect (cpe).

To harvest the DNA, the cells were lysed by adding 0.5ml of 5% SDS to each well. The DNA was extracted once with saturated phenol and pelleted by a low speed spin at room temperature after precipitation with twice the volume of analar ethanol. The dried sample was resuspended in 0.2ml of water and digested for a minimum of 2h at 37°C with a mixture of RNase A and T_1 (5 units T_1 and 2.5 units of A per sample). A 10ul sample of the DNA was precipitated with trichloroacetic acid and the incorporated ^{32}P was measured using a scintillation counter. After equalizing the counts by the addition of water, the samples were digested with restriction endonucleases in the appropriate buffer and run on agarose gels of concentrations depending on the expected sizes of the DNA fragments. The agarose concentrations for the various enzymes used were as follows: 0.5% for Xba I; 0.6% for Hind III, EcoR I, Bgl II, Kpn I and Hpa I; 1.2% for BamH I.

The gels were dried onto glass plates at 80-90°C for approximately 3h and autoradiographs were obtained by exposing XS1 film directly to the gels for a period dependent on the ^{32}P incorporation into the DNA, usually 24h.

2.7 PREPARATION OF VIRAL DNA

Large scale preparations of viral DNA for use in transfection and Southern blotting were made as follows: ten roller bottles containing almost confluent monolayers of

BHK21/C13 cells were infected with virus at a MOI of 0.003pfu/cell. The infection was continued at 31°C until there was extensive cpe (usually for 3-7 days). The cells were shaken into the medium and spun down at 2,000rpm for 10 minutes. The supernatant was retained and the cytoplasmic fraction of the cell pellet was added to it prior to spinning at high speed to pellet the virus. (The nuclei were extracted from the cell pellet by low speed centrifugation following treatment with 0.5% NP40 in RSB). The viral pellet containing cell released and cytoplasmic virus was resuspended in NTE buffer and EDTA and SDS added to a final concentration of 10mM and 2% respectively to cause lysis of the virus. Viral DNA was extracted twice with saturated phenol and once with chloroform: isoamyl alcohol (3 methyl-1- butanol) (24:1) prior to dialysing against 0.1xSSc for approximately 16h.

2.8 CAESIUM CHLORIDE GRADIENT BANDING OF VIRAL DNA

DNA samples were adjusted to give a refractive index of 1.401 by the addition of CsCl and run at 40,000rpm for approximately 16h at room temperature in a TV865B rotor. A hole was pierced in the top of the sealed tube to break the vacuum and fractions collected by dripping through a 21G syringe needle pushed into the base of the tube. To ascertain which fractions contained DNA, 5ul samples were run on a 0.9% agarose gel containing ethidium bromide. The DNA was visualized under short wave (260nm) U.V. light and photographed using polaroid film. Fractions containing DNA were pooled and dialysed overnight against TE buffer. To quantitate the DNA a range of volumes (4-20ul) was run adjacent to DNA of known concentration, on an ethidium

bromide containing, 0.9% agarose gel. The gel was photographed under U.V. light, the intensity of the bands compared to the intensity of the standard and hence the concentration of the DNA estimated.

2.9 ISOKINETIC SUCROSE GRADIENT BANDING OF VIRAL DNA

Preformed sucrose gradients were prepared from 5% sucrose in NTE and 29.8% sucrose in NTE. A 10ml gradient was formed by introducing the solutions via a tube to the base of a TST41 centrifuge tube. Initially 5.9ml of the 5% sucrose solution was placed in a sealed mixing chamber. As the gradient was poured the 5% sucrose was replaced by 29.8% sucrose. When the gradient had been formed, 0.5ml of 60% sucrose in NTE was introduced at the base of the centrifuge tube to act as a cushion to stop the DNA from pelleting. Approximately 1ml of the viral DNA preparation was added to the top of the tube and the gradient spun at 35,000rpm for 5h at 18^{o}C. Fractions were collected by dripping through a 21G syringe needle pushed into the base of the tube. The smallest fragments run at the top of the gradient and come off last. To ascertain which fractions contained DNA, 5ul samples were run on a 0.9% agarose gel containing ethidium bromide. The DNA was visualized under short wave (260nm) U.V. light and photographed. Fractions containing apparently unfragmented DNA were pooled and precipitated in twice the volume of ethanol at -20^{o}C overnight.

2.10 DNA-DNA HYBRIDIZATION

The method used was a modification of that described by Southern (1975). One microgram samples of HSV DNA were digested with restriction endonucleases and run on the

appropriate concentration of agarose gel containing 0.5ug/ml of ethidium bromide. To denature the DNA, the gel was soaked for 45min in approximately 2litres of GS I (3M NaCl and 0.6M NaOH) followed by the same length of time in GS II (0.6M NaCl and 1M Tris-HCl pH8). Transfer of the DNA to nitrocellulose was achieved by lying the gel on a wick of blotting paper and drawing 10xSSC through the gel and nitrocellulose sheet. This process relies on the capillary action of absorbant paper above the nitrocellulose. To increase the efficiency of the system, it was primed by wetting the first two sheets of absorbant paper above the nitrocellulose. When the single stranded DNA had transferred to the nitrocellulose, the sheet was cut into appropriate strips and baked for 2h in a vacuum oven at 80^{o}C. The strips were pre-hybridized for approximately 2h at 75^{o}C in hybridization buffer (3xSSC, 0.1% Ficoll 400, 0.1% bovine serum albumin (BSA), 0.1% polyvinylpyrrolidone, 0.1mg/ml single-stranded salmon sperm DNA and 10mM Tris HCl-pH7.5). Hybridization was carried out with a ^{32}P labelled probe in fresh hybridization buffer in a shaking water bath for 16-48h at 75^{o}C. After hybridization, the strips were washed in several changes of wash buffer (2xSSC, 0.1% SDS and 5mM sodium phosphate buffer pH7) at 60^{o}C over a number of hours.

2.11 NICK TRANSLATION OF DNA

The method used was a modification of that described by Rigby et al. (1977). The probe was prepared by nick translation of 0.5ug of HSV DNA. The DNA, in the presence of 0.1% BSA and 1x nick translation buffer (5mM $MgCl_2$, 1mM dithiothreitol and 50mM Tris-HCl pH7.8), was treated with DNase at 10^{-4}mg/ml to nick the DNA, and 5 units of

polymerase I to translate the nick.

Escherichia coli DNA polymerase I adds nucleotide residues to the 3'-hydroxyl terminus created when one strand of a double-stranded DNA molecule is nicked. Due to its exonuclease activity the enzyme also removes nucleotides from the 5' end of the nick. As nucleotides are removed from the 5' end and fresh ones are added to the 3' end of the nick, the nick itself is translated along the DNA.

To produce a probe by nick translation, template DNA nucleotides are replaced with radioactive nucleotides to give ^{32}P labelled DNA. Probes were prepared from reaction mixtures containing an excess of three unlabelled deoxynucleotide triphosphates (dNTPs) and a lesser quantity of the fourth ^{32}P labelled dNTP. As HSV DNA consists of a majority of G and C residues, the label used was normally ^{32}P dCTP. Typically the reaction mixture contained 0.15mM dATP, 0.15mM dTTP, 0.15mM dGTP and 6.6pM ^{32}P dCTP. After mixing well the reaction was maintained at 15^{o}C for 90min. To stop the reaction, the DNA was extracted by adding sodium acetate (pH7) to a final concentration of 1M and precipitating in 40% isopropanol on dry ice. The probe was re-extracted with 0.3M sodium acetate and 30% isopropanol and, after precipitation on dry ice, the pellet was resuspended in an 80% formamide aqueous solution and boiled for 5min to denature the DNA.

2.12 DIGESTION OF HSV DNA WITH RESTRICTION ENDONUCLEASES

Because of the range of buffers required if the manufacturer's recommended conditions were used for each restriction enzyme, the following three reliable standard buffers were used routinely.

(1) For Xba I, EcoR I, Hind III, Hpa I and Bgl II digestion:
100mM NaCl, 6mM $MgCl_2$, 6mM Tris-HCl pH7.4 and 0.01% BSA.
(2) For BamH I digestion:
100mM NaCl, 6mM $MgCl_2$, 5.7mM 2 mercaptoethanol, 6mM Tris-HCl pH7.5 and 0.01% BSA.
(3) For Kpn I digestion:
10mM $MgCl_2$, 1mM DTT, 10mM Tris-HCl pH7.5 and 0.01% BSA.

To achieve complete digestion, lug samples of HSV DNA were digested with 1-2 units of enzyme for 4h at 37°C. If the digested DNA was to be run on an agarose gel RE stop (1/4) was added. When Xba I digested DNA was used for transfection, the endonuclease reaction was stopped by phenol extraction, the DNA precipitated in ethanol and resuspended in HEBS buffer. In order to recover viable virus from transfection of Xba I digested DNA partial digestion was employed. Digestion was allowed to proceed for times between 1 and 4h, the DNA transfected and any resultant plaques isolated.

2.13 TRANSFECTION OF VIRAL DNA

The method employed was a modification of the calcium phosphate DNA infectivity technique of Graham and Van der Eb (1973) (Stow and Wilkie, 1976). Four micrograms of intact or partially digested HSV DNA and 4ug of calf thymus carrier DNA were made up in Hebs buffer (pH7.05). Calcium chloride was added to a final concentration of 0.13M and the resulting fine suspension (0.4ml) was added to a 50mm dish of almost confluent BHK21/C13 cells from which the growth medium had been removed. After incubation at 37°C for 40min, the plates were overlaid with 4ml of EC5% and incubation continued at 37°C. At 4h post infection the cells were

washed with EC5% and exposed to 1ml of 25% v/v DMSO in Hebs buffer for 4min. After washing with EC5%, incubation was continued at 31°C in 4ml of EC5%+ 5%Hu until plaques were observed (4-12 days). Depending on the density of the plaques, either well separated plaques were picked directly or the plates were washed to remove the human serum, the cells harvested and the virus titrated to facilitate the picking of individual plaques.

Calf thymus DNA was purified prior to use as carrier DNA by sequential phenol/chloroform extractions of a 10mg/ml solution. Sodium chloride was added to a final concentration of 1.5M and the DNA precipitated by adding twice the volume of ethanol and leaving for approximately 16h at -20°C. The DNA obtained was dried and made up in distilled water to give a stock solution of 10mg/ml.

2.14 COMPARISON OF ENCAPSIDATED DNA WITH TOTAL VIRAL DNA

Fifty millimetre dishes of BHK21/C13 cells which had been grown overnight in phosphate-free Eagle's medium with 1% calf serum (PIC) were infected with virus at a MOI of 10pfu/cell. Three hours post infection 200uCi of ^{32}P orthophosphate was added and the plates incubated at 31°C for two days. Total cellular and encapsidated DNA (DNase resistant) was prepared using the method described by Stow et al. (1983). To prepare total cellular DNA, the growth medium was removed and the monolayers incubated for 4h at 37°C with 2ml of lysis buffer (0.6% SDS, 10mM EDTA, 10mM Tris-HCl pH7.5) containing 0.5mg/ml of protease type 14 which had been autodigested for more than 2h at 37°C prior to use. Sodium chloride was added to a final concentration of 0.2M and sequential extractions with phenol and

chloroform performed before precipitating the DNA with ethanol. The resulting pellet was dissolved in water and digested with a mixture of RNase A and T_1 for 2h at 37°C.

To prepare encapsidated DNA the monolayer was scraped into 1ml of RSB and Nonidet P40 added to a final concentration of 0.5%. The samples were then digested with 200ug of DNase I for 2h at 37°C prior to the addition of 1ml of 2x lysis buffer and protease type 14 to a final concentration of 0.5mg/ml. After a further incubation of 2h at 37°C the DNA was extracted and RNase treated as described for the total cellular extract.

The TCA precipitable counts were measured and the samples were equalized prior to restriction endonuclease digestion and running on gels.

2.15 PREPARATION OF PLASMID DNA BY THE ALKALINE LYSIS METHOD

An overnight culture was grown in 5ml of L-broth containing the appropriate antibiotic (usually 100ug/ml of ampicillin or 12.5ug/ml of tetracycline). This culture was transferred to a 2 litre flask with 350ml of L-broth containing the same concentration of antibiotic and incubated overnight at 37°C in an orbital shaker. When the culture had reached the required density, the suspension was spun at 8,000rpm in a GSA rotor to pellet the bacteria. The supernatant was discarded and the bacterial pellet resuspended in 7ml of solution I (25mM Tris-HCl pH8.0, 50mM D-glucose and 10mM EDTA with 5mg/ml of lysozyme added just prior to use). The mixture was left at room temperature for 5min to allow the lysozyme to lyse the bacterial cell walls; 14ml of freshly prepared solution II (0.2M NaOH and 1% SDS) was added and mixed by gentle inversion of the tube which

was then placed on ice for 10min, following which 10.5ml of an ice cold solution of 5M potassium acetate pH4.8 was added. (The solution which was 5M with respect to acetate was prepared from 60% 5M potassium acetate and 11.5% glacial acetic acid.) The tube was inverted sharply several times and put on ice for 10min prior to centrifugation at 20,000rpm for 20min at 4^{o}C to pellet the cellular DNA and bacterial debris. The supernatant was treated with serial phenol: chloroform extractions before precipitating the DNA by adding two volumes of ethanol, mixing and leaving at room temperature for 15min. The DNA was recovered by spinning at 12,000rpm for 30min at room temperature (to avoid precipitation of salt); the pellet was washed with 70% ethanol at room temperature and dried in a vacuum desiccator before being resuspended in 1ml of water.

2.16 ^{35}S METHIONINE LABELLING OF HSV INFECTED CELL POLYPEPTIDES

Twenty four well tissue culture dishes were seeded with 2x10^{5} BHK21/C13 cells per well in 1ml of EC10% and incubated at 37^{o}C overnight. When the cells were approximately 80% confluent, they were infected at a MOI of 20pfu/cell. The virus was allowed to absorb for 1h at 37^{o}C when monolayers were washed with methionine-reduced medium (E met/5 C2%) prior to overlaying with 450ul of the same medium. After a further 3h incubation at 37^{o}C, 10uCi of ^{35}S methionine was added to each well in 50ul of E met/5 C2% and the samples returned to 37^{o}C overnight. On the following day the growth medium was removed, the cells washed once with pre-warmed PBS and harvested into 150ul of 1:3 sample buffer. The samples were transferred to vials and heated to 95^{o}C for

2min to reduce the viscosity. The samples were run on denaturing polyacrylamide gels of the appropriate concentrations.

2.17 SULPHATE LABELLING OF GLYCOPROTEIN E

Twenty four well tissue culture dishes were seeded with $5x10^5$ cells and incubated overnight at 37°C. The medium was removed and the cells infected with 20pfu/cell of virus in 0.2ml of sulphate-free Eagle's medium with 2% calf serum (E SO_4^- C2%). The virus was allowed to absorb for 1h at 37°C. The cells were then washed, overlaid with 450ul of E SO_4^- C2% and incubated at 37°C for a further 3h. The medium was removed and 0.2ml of PBS containing 100uCi of $^{35}SO_4$ was added and the incubation continued for a further 3h at 37°C. The monolayers were washed twice with pre-warmed complete PBS and the samples harvested into 150ul of 1:3 sample buffer.

2.18 IMMEDIATE EARLY VIRAL POLYPEPTIDE SYNTHESIS

Thirty millimetre Petri dishes were seeded with $1x10^6$ BHK21/C13 cells in 2ml of ETC10% and incubated at 37°C overnight to give confluent monolayers. On the following day cycloheximide was added to a final concentration of 100ug/ml and the plates returned to 37°C for 15min. (Cycloheximide is a protein synthesis inhibitor, the addition of which will allow mRNA transcription but not its translation. In the presence of cycloheximide, mRNA accumulates in cells.) After the short pre-treatment with cycloheximide, the medium was removed and the cells infected at a MOI of 100pfu/cell in the presence of 100ug/ml cycloheximide. The plates were incubated for 1h at 38°C to allow absorption of the virus,

then washed twice with methionine-reduced medium (E met/5 C2%) and overlaid with 2ml of the same medium containing 100ug/ml of cycloheximide. The incubation was continued for a further 5h at 38^{o}C allowing a build up of IE mRNA. (Without IE polypeptide synthesis the cascade effect causing transcription of early and late mRNA does not proceed and only immediate early mRNA is present even at later times in infection when early polypeptides would normally be present).

Fifteen minutes before the end of the 5h incubation Actinomycin D, a RNA synthesis inhibitor, was added to a final concentration of 2.5ug/ml. At the end of the incubation period the medium was removed and the infected monolayers washed four times with pre-warmed complete PBS. Each wash was left on for approximately 1min to allow the cycloheximide to leach out completely. The Actinomycin D block is irreversible.

Two hundred microcuries of ^{35}S methionine label was added in 1ml of PBS and the plates incubated for 1h at 38^{o}C prior to washing once with PBS and harvesting in 350ul of 1:3 sample buffer. The samples were run on polyacrylamide gels of the appropriate concentrations. (The removal of the cycloheximide block allows the accumulated mRNA to be translated whilst the continued presence of the Actinomycin D means that any further transcription is halted. The label is, therefore, incorporated only into IE polypeptides.)

2.19 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Slab gels were formed between glass plates separated by 1.5mm thick perspex spacers and sealed with Kapton tape. Two

types of resolving gel were used:

(1) Single concentration gels were prepared from 7.5% acrylamide cross-linked with one part in forty (W/W) N',-methylenebis acrylamide in 375mM Tris pH8.9, 3.5mM SDS with 0.0004% ammonium persulphate and 0.0004% N, N, N', N', tetramethylethylenediamine (TEMED) added to cause polymerisation. The stacking gel used was 5% acrylamide also in the proportion of forty parts acrylamide to one of N'-methylenebis acrylamide in a buffer containing 122mM Tris-HCl pH6.7 and 3.5mM SDS and polymerised with 0.0008% ammonium persulphate and 0.0004% TEMED.

(2) Gradient gels containing a 5% to 12.5% linear gradient of acrylamide cross-linked with one part in twenty (W/W) N'-methylenebis acrylamide in 375mM Tris-HCl pH8.9 and 3.5mM SDS and polymerised with 0.0008% ammonium persulphate and 0.0004% TEMED. The stacking gel was 5% acrylamide cross-linked with one part in twenty of N'-methylenebisacrylamide in 122mM Tris-HCl pH6.7 and 3.5mM SDS polymerised with 0.0008% ammonium persulphate and 0.0004% TEMED.

CHAPTER 3

RESULTS

It has been shown by the use of conditional lethal mutants that both intertypic and intratypic recombination occurs with HSV-1 and HSV-2. However, the mechanisms involved in recombination require further study to elucidate viral and host contributions and to examine the possibility that particular base sequences may be more recombinogenic than others. It has been postulated that banks of tandemly reiterated sequences for example, might predispose the genome to recombine or possibly some functional aspect of the sequence such as a protein binding site might have an effect on the frequency of recombination in that region. Analysis of intertypic recombination between HSV-1 and HSV-2 is limited by the extent of non-homology between the genomes (estimated to be 45% from nucleic acid hybridization). These constraints are not applicable in intratypic studies. However, in order to study intratypic recombination, genomic markers have to be introduced which do not significantly alter the complete homology. The disruption of a restriction endonuclease site can be caused by as little as one base pair change making such markers ideal to study recombination. The aim of this project was to isolate mutants of HSV-2, strain HG52, lacking restriction endonuclease sites whose absence could be used as non-selected markers in recombination studies. HSV-2 genomes with multiple non-selected markers could be used in studies of recombination frequency between selected conditional lethal (ts) markers over a period of time in order to map

recombinational "hot spots" as well as determining the role of particular genes and parental and progeny molecules in recombination. However, during the course of the project, the emphasis of the work changed due to the unexpected isolation of a number of deletion and insertion variants. It was considered that these novel variants were worthy of investigation as their DNA structures might provide information on the essential nature of specific genes and the function of virus encoded products in vitro and in vivo. For this reason much of the work concentrated on the analysis of these spontaneously generated novel genomes and relating structural changes in their genomes to changes in function.

3.1 ISOLATION OF HSV-2, STRAIN HG52, GENOMES LACKING Xba I RESTRICTION ENZYME SITES

AS the HSV-2, HG52 genome has not been completely sequenced, it was not possible to remove restriction endonuclease sites by site directed mutagenesis using synthetic oligonucleotides. Therefore, to isolate genomes lacking Xba I sites, an enrichment selection technique was employed. This was a modification of the method described by Jones and Shenk (1978) for adenovirus type 5. Within a virus population genomes lacking individual restriction endonuclease (RE) sites are more likely to survive after RE digestion than those with a full complement of sites. However, when HG52 DNA was completely digested with Xba I no plaques were obtained upon transfection. When transfected onto a confluent monolayer of BHK21/C13 ($4x10^6$) cells, lug of undigested HG52 DNA yielded in excess of 500 plaques per plate after 4-5 days incubation at 37°C. Transfection of the

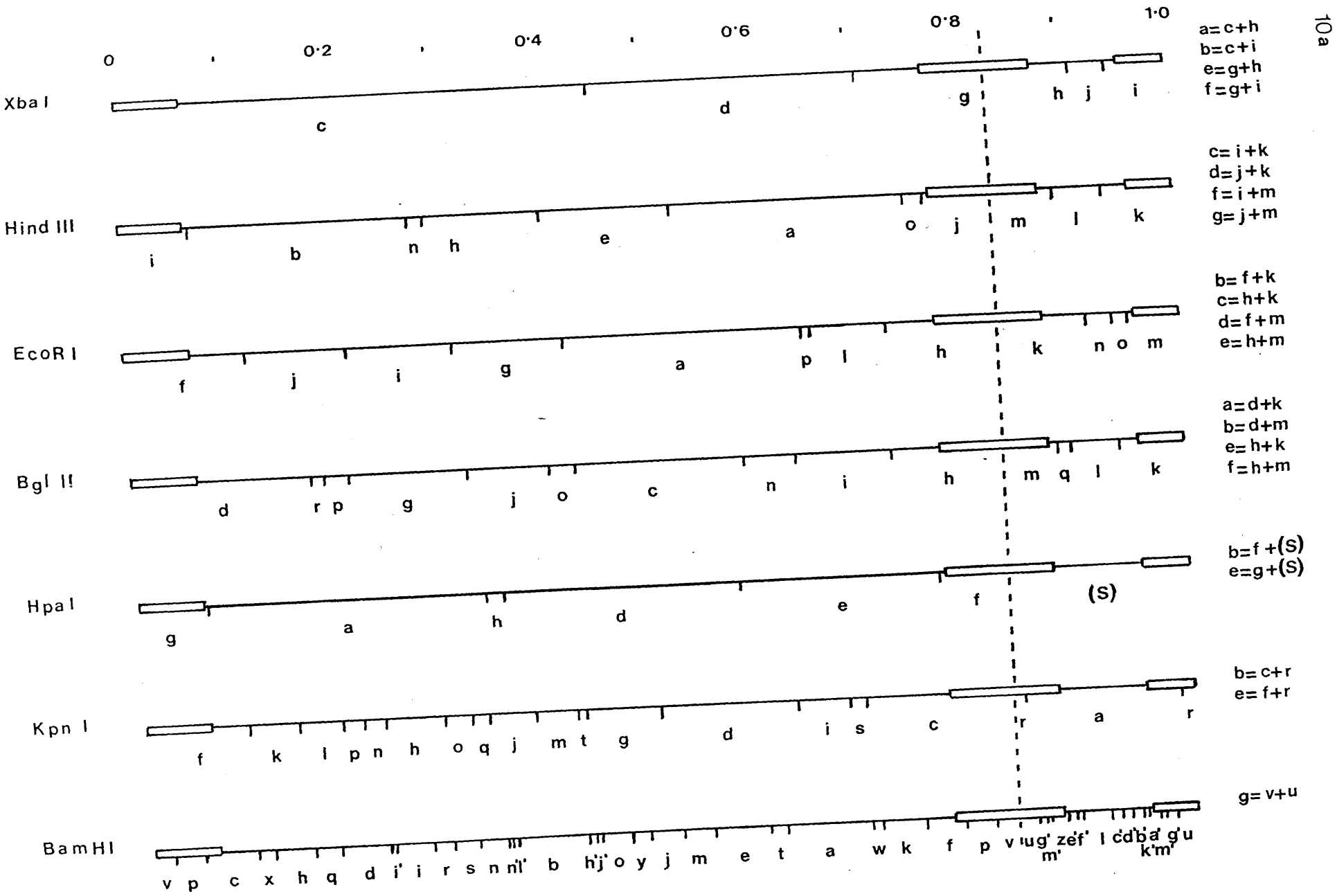
same quantity of HG52 DNA which had been completely digested with Xba I (5 units/ug DNA for 4h at 37°C) failed to produce any plaques. It was, therefore, decided to obtain partial digests of the DNA by reducing the quantity of Xba I and using shorter periods of incubation. Digestion with Xba I at 1 unit/ug of DNA for 1h or 2h caused a ten fold reduction in the infectivity of the HG52 DNA. Initially one hundred plaques were picked from transfection plates of DNA digested for each of one and two hours. The two hundred plaques were grown up to give 50mm plate harvests which were analysed by RE digestion of their ^{32}P labelled DNA.

Xba I cleaves the HG52 genome twice within the long unique and twice within the short unique region to give a total of ten fragments (Fig. 10). Fig. 10a shows the RE maps for HG52 for all the restriction enzymes used in this study. Fig. 10b shows the theoretical distribution of the fragments, their molecular weights and molarities. The Xba I d (mol. wt. $24x10^6$ daltons, 0.45-0.7 map co-ordinates (m.c.)) and j (mol. wt. 4.4 $x10^6$, 0.91-0.94m.c.), one molar fragments are from the long and short unique regions respectively. The c (mol. wt. $45x10^6$, 0-0.45m.c.) and g (mol. wt. $12x10^6$, 0.7-0.82m.c.) fragments are half molar and comprise the terminal and internal long ends of the genome respectively. Similarly h (mol. wt. $8x10^6$, 0.82-0.91m.c.) and i (mol.wt. $5.6x10^6$,0.94-1.00m.c.) are half molar and comprise the internal and terminal short ends respictively. The remaining four observed fragments are quarter molar joints a ((c+h) mol. wt. $53x10^6$), b((c+i) mol. wt. $50.6x10^6$), e((g+h) mol. wt. $20x10^6$) and f ((g+i) mol.wt. $17.6x10^6$) each from one isomeric form of the genome.

Of the two hundred plaques analysed from the initial

Figure 10a

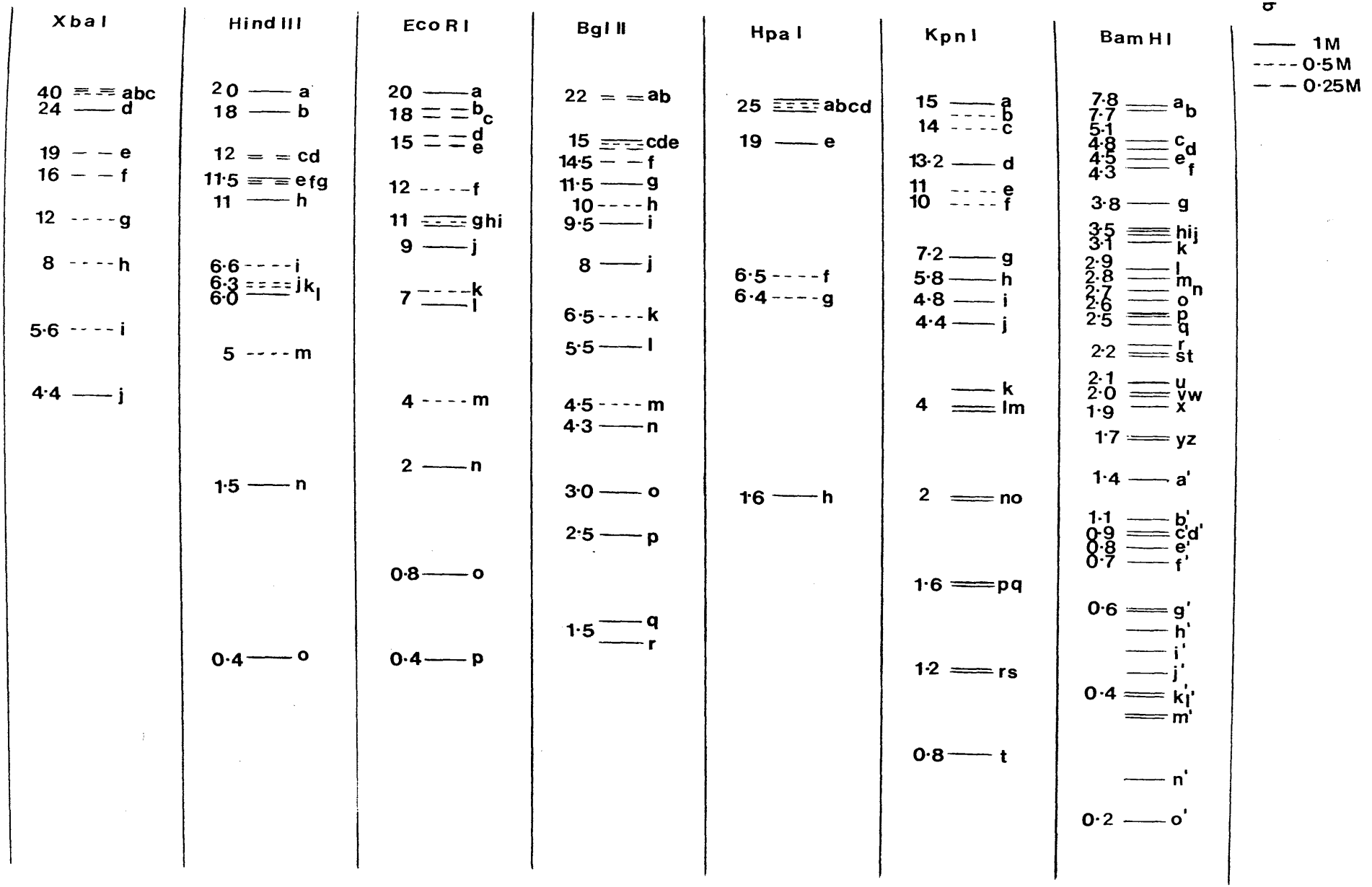
Restriction endonuclease maps for the DNA of HSV-2 strainHG52 (Chartrand et al., 1981) for all the restriction endonucleases used in this study. The origin of the L/S joint fragments is shown on the right.


0
0·2
0·4
0·6
0·8
1·0
Xba I
c
d
g
h j i
a=c+h
b=c+i
e=g+h
f=g+i
Hind III
i
b
n h
e
a
o j
m l k
c=i+k
d=j+k
f=i+m
g=j+m
EcoR I
f
j
i
g
a
p l
h
k
n o m
b=f+k
c=h+k
d=f+m
e=h+m
Bgl II
d
r p
g
j
o
c
n
i
h
m q l k
a=d+k
b=d+m
e=h+k
f=h+m
Hpa I
g
a
h
d
e
f
(S)
b=f+(S)
e=g+(S)
Kpn I
f
k
l p n
h
o q
j
m t
g
d
i
s
c
r
a
r
b=c+r
e=f+r
BamHI
v p
c
x
h q
d
i' i
r
s
n n'l'
b
h'j'
o
y
j
m
e
t
a
w k
f
p
v
u g'
m'
z e'f' l
c d'b'a'
k'm'
g'u
g=v+u

10a

Figure 10b

Theoretical distribution of the HG52 restriction endonuclease fragments and their approximate molecular weights ($x10^6$ daltons) for the restriction endonucleases used in this study (Davison, 1981). A key to the representation of different molarities is shown on the right.


Xba I
40 abc
24 d
19 e
16 f
12 g
8 h
5·6 i
4·4 j
Hind III
20 a
18 b
12 cd
11·5 efg
11 h
6·6 i
6·3 jk
6·0 l
5 m
1·5 n
0·4 o
Eco R I
20 a
18 bc
15 de
12 f
11 ghi
9 j
7 k
l
4 m
2 n
0·8 o
0·4 p
Bgl II
22 ab
15 cde
14·5 f
11·5 g
10 h
9·5 i
8 j
6·5 k
5·5 l
4·5 m
4·3 n
3·0 o
2·5 p
1·5 q
r
Hpa I
25 abcd
19 e
6·5 f
6·4 g
1·6 h
Kpn I
15 a
b
14 c
13·2 d
11 e
10 f
7·2 g
5·8 h
4·8 i
4·4 j
k
4 lm
2 no
1·6 pq
1·2 rs
0·8 t
Bam HI
7·8 ab
7·7
5·1
4·8 cd
4·5 e
4·3 f
3·8 g
3·5 hij
3·1 k
2·9 l
2·8 mn
2·7
2·6 o
2·5 pq
r
2·2 st
2·1 u
2·0 vw
1·9 x
1·7 yz
1·4 a'
1·1 b'
0·9 c'd'
0·8 e'
0·7 f'
0·6 g'
h'
i'
j'
0·4 k'l'
m'
n'
0·2 o'
10q
1M
0·5M
0·25M

transfection only two were found to exhibit genomes lacking an Xba I site. In both cases the 0.7m.c. site between the Xba I g and d fragments had been lost. These variants were designated HG52 Xba 94 (HG52X94) and HG52 Xba 163 (HG52X163).

3.1a HG52X94

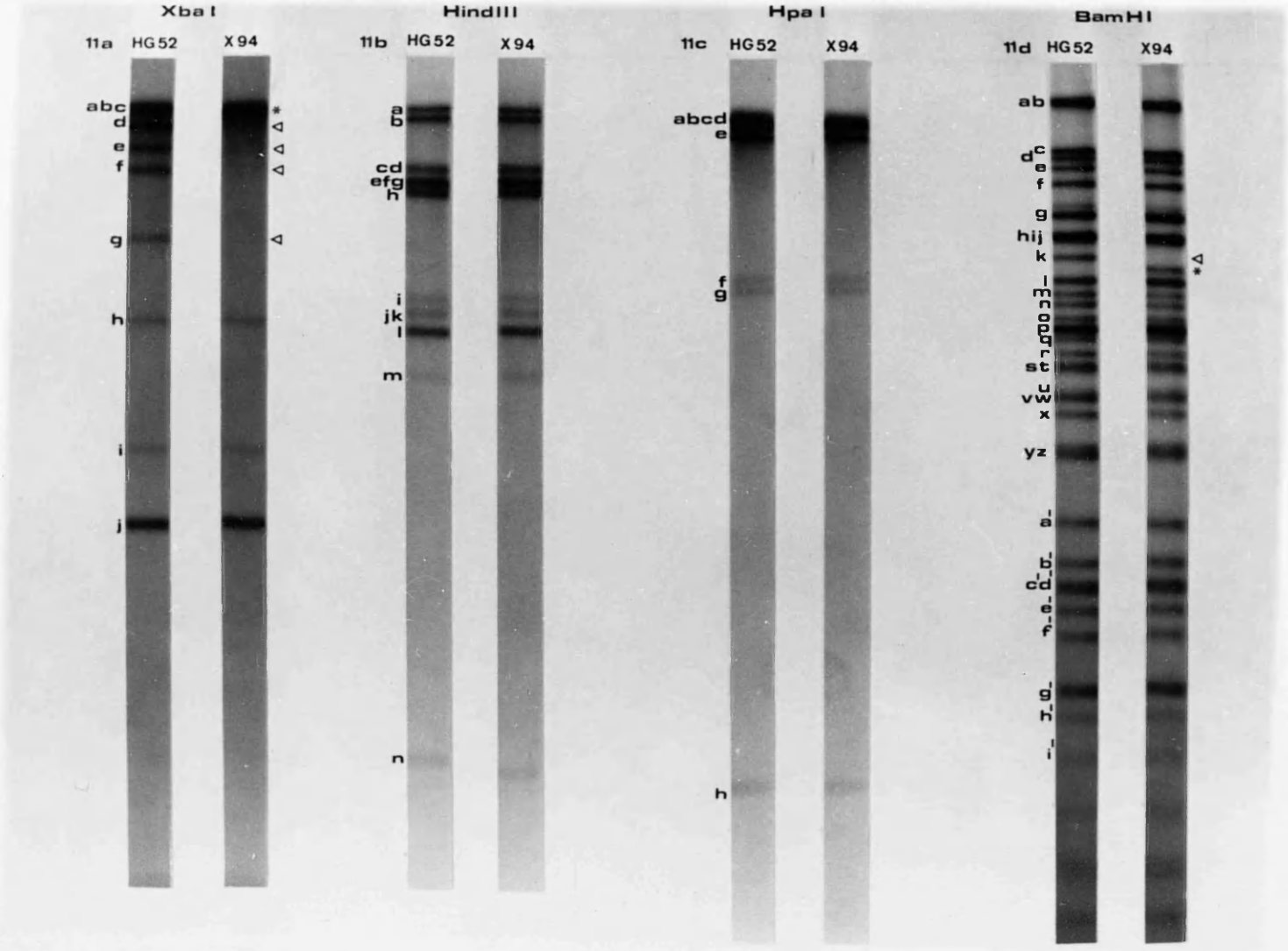
On digestion with Xba I the DNA profile of HG52X94 was markedly altered from the parental HG52 (Fig. 11a). The d, e, f and g bands were absent whilst there was an increase in the molarity of the bands running with the a, b and c fragments at the top of the gel. The three Xba I fragments which comprise the short region, h, j and i, appeared to be of normal size and intensity. The Xba I profile suggested loss of the site at 0.7m.c. giving rise to a novel fragment of $36x10^6$ daltons due to the fusion of the g and d fragments. The joint fragments e and f which contain g would consequently be lost and replaced by two novel joint fragments d+g+h ($44x10^6$ mol. wt.) and d+g+i ($41.6x10^6$ mol.wt.). The three large novel bands would co-migrate with the a, b and c fragments thereby explaining the increased intensity in that region.

The Hind III and Hpa I profiles of HG52X94 (Figs.11b and 11c) appear to be identical to the parental HG52. The Hind III a and the Hpa I e fragments which contain the 0.7m.c. Xba I site are, however, large fragments ($20x10^6$ and $19x10^6$ mol. wt.) and only large deletions or insertions would be detectable. However, digestion with a restriction endonuclease which cuts HG52 DNA more frequently, BamH I (Fig. 11d), demonstrated a deletion of approximately one hundred and fifty base pairs ($0.1x10^6$ mol. wt.) within the k

Figure 11(a-d)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* of the HSV-2 wild type strain HG52 and the variant HG52X94 obtained after one round of enrichment selection of HG52 DNA. (a) Xba I digests, (b) Hind III digests, (c) Hpa I digests and (d) BamH I digests.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


Xba I
11a HG52 X94
abc
d
e
f
g
h
i
j
Hind III
11b HG52 X94
a
b
cd
efg
h
i
jk
l
m
n
Hpa I
11c HG52 X94
abcd
e
f
g
h
BamHI
11d HG52 X94
ab
c
d
e
f
g
hij
k
l
m
n
o
p
q
r
s
t
u
v
w
x
yz
a
b
c
d
e
f
g
h
i

fragment which contains the 0.7m.c. Xba I site. Therefore, in HG52X94 the 0.7m.c. Xba I site has been lost by deletion of approximately 150bp.

3.1b HG52X163

On digestion with Xba I, Hind III and Hpa I (Figs. 12a, b and c) HG52X163 appeared identical to HG52X94 although it could not have been clonally related having been isolated from a different transfection plate. When digested with BamH I (Fig. 12d) HG52X163 also exhibited an alteration in the mobility of the k band. However, in contrast to HG52X94 the band was larger than in HG52. The size of the novel band indicated that in HG52X163 the Xba I site at 0.7m.c. has been lost by insertion of approximately one hundred base pairs of DNA causing disruption of the recognition sequence for the restriction enzyme. The BamH I profile of HG52X163 also differed from that of HG52 by an apparent increase in the mobility of the BamH I u fragment, causing it to comigrate with v and w, thus indicating a deletion of approximately 0.1×10^6 daltons from BamH I u.

To investigate the unlikely possibility that sequences deleted from BamH I u had been inserted into BamH I k, a Southern blot was performed. Figure 12e shows a blot of BamH I digests of HG52 and HG52X163 hybridized to a wild type, HG52, probe. In Fig. 12f BamH I digests of HG52 and HG52X163 blotted and probed with BamH I g(v+u) are shown. The probe, as expected, hybridized to the g, u and v wild type fragments. Due to the presence of varying copy numbers of the 'a' sequence at the long terminus, the BamH I v fragment is variable in size on a population basis. As the short terminus has only one copy of the 'a' sequence the BamH I u

Figure 12(a-d)

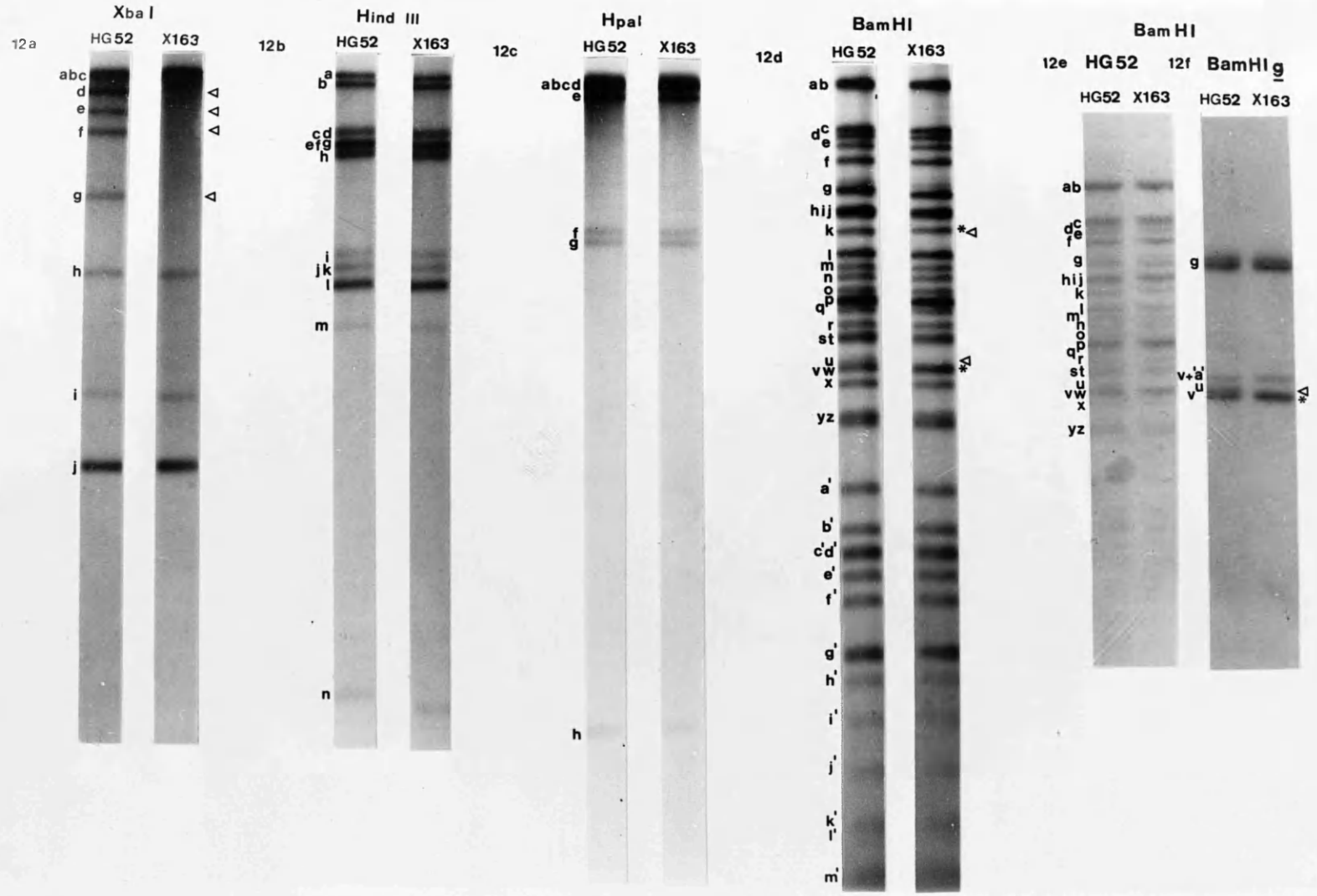
Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* of the HSV-2 wild type strain HG52 and the variant HG52X163 obtained after one round of enrichment selection of HG52 DNA. (a) Xba I digests, (b) Hind III digests, (c) Hpa I digests and (d) BamH I digests.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.

Figure 12(e+f)

Autoradiograph of nitrocellulose blot strips from a single gel containing BamH I restriction fragments of HG52 and HG53X163 to which nick-translated HSV-2 DNA probes have been hybridized. The probes were (e) HG52 and (f) pGZ1 a recombinant plasmid containing BamH I g(v+u) of HG52.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


12a
Xba I
HG52
X163
abc
d
e
f
g
h
i
j
12b
Hind III
HG52
X163
a
b
cd
efg
h
i
jk
l
m
n
12c
Hpa I
HG52
X163
abcd
e
f
g
h
12d
Bam HI
HG52
X163
ab
c
d
e
f
g
hij
k
l
m
n
o
p
q
r
st
u
vw
x
yz
a'
b'
c'
d'
e'
f'
g'
h'
i'
j'
k'
l'
m'
12e
HG52
12f
Bam HI g
HG52
X163
HG52
X163
ab
c
d
e
f
g
hij
k
l
m
n
o
p
q
r
st
v+a'
vc
vw
x
yz
g

fragment is homogeneous in size. The different numbers of copies of the 'a' sequence in BamH I v also accounts for the pronounced heterogeneity of the BamH I g fragment. The HG52Xl63 track demonstrated a reduction in the size of both copies of BamH I u causing them to run with v, confirming the deletion of 150bp from both copies of BamH I u. There was, however, no hybridization to the novel BamH I k fragment in HG52Xl63 indicating that the inserted sequences at the 0.7m.c. site had not arisen from the sequences deleted from the BamH I u fragments. The origin of the inserted DNA remains unknown.

3.1c HG52Xl63X3

HG52Xl63 which lacks the 0.7m.c. Xba I g/d site was used as the parental virus for a further enrichment selection experiment designed to isolate mutants lacking the remaining Xba I sites. HG52Xl63 DNA was digested with two units of Xba I per microgram of DNA for two hours at 37^{o}C. Of forty plaques isolated after transfection of the digested DNA one, HG52Xl63X3, in addition to the missing 0.7m.c. Xba I site, also lacked the Xba I site at 0.91m.c..

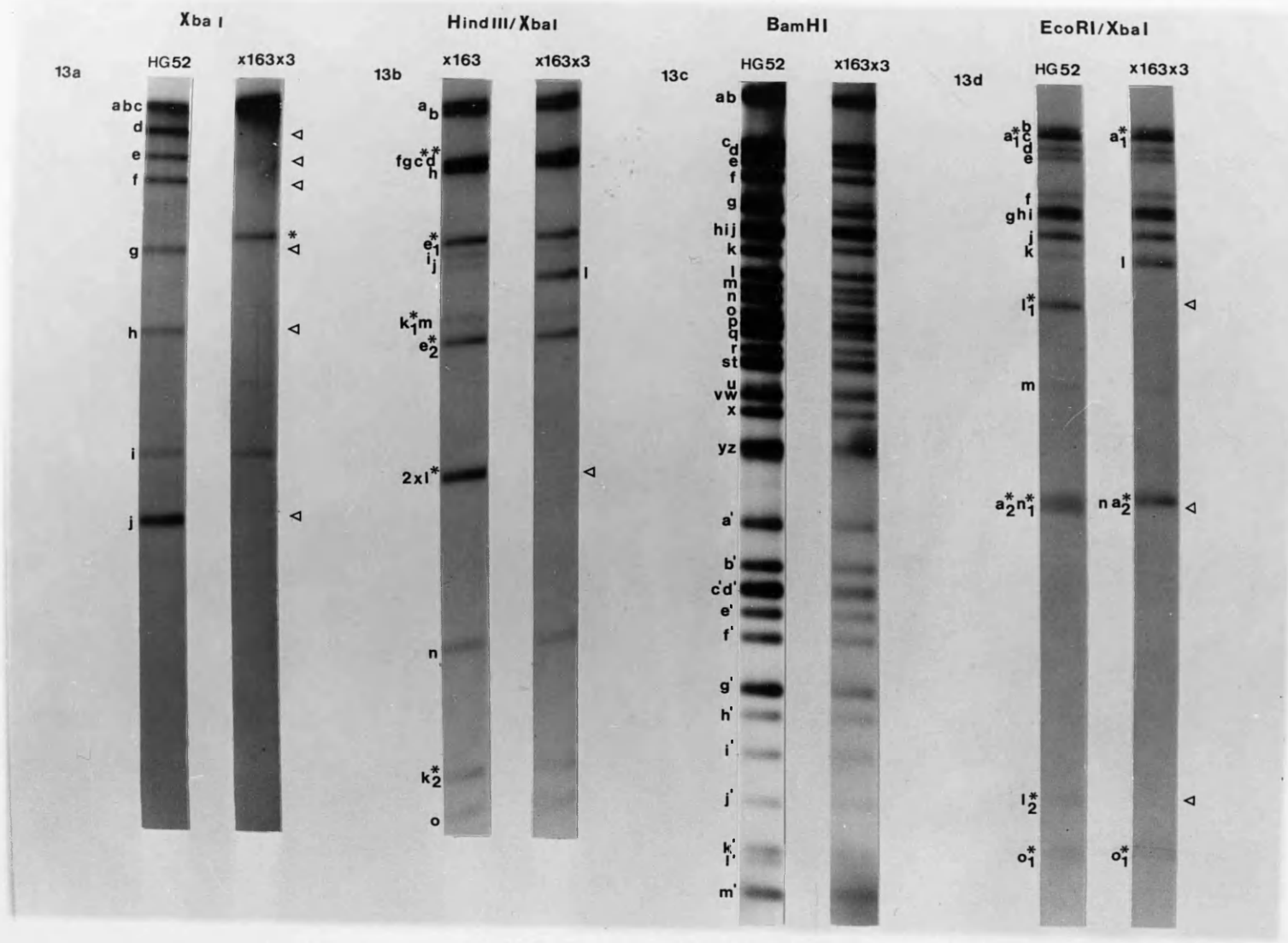
On digestion with Xba I, HG52Xl63X3 (Fig. 13a) showed missing d, e, f, g, h and j bands and a novel half molar band of approximately 12.5x10^{6} daltons. The parental genome, HG52Xl63, already showed absence of the d, e, f and g bands due to the lack of the 0.7m.c. Xba I g/d site. The absence of the h and j bands is explained by loss of the 0.91m.c. Xba I site creating a novel half molar fusion fragment of approximately 12.5x10^{6} daltons. The increase in the sizes of the h containing joints due to the addition of the j fragment would not be detectable as their molecular weights

Figure 13(a-d)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* of the variant HG52Xl63X3 obtained after enrichment selection of HG52Xl63 DNA compared with either the wild type HG52 or the parental HG52Xl63. (a) HG52 and HG52Xl63X3 digested with Xba I, (b) HG52Xl63 and HG52Xl63X3 digested with Hind III/Xba I, (c) HG52 and HG52Xl63X3 digested with BamH I and (d) HG52 and HG52Xl63X3 digested with EcoR I/Xba I.

Letters refer to specific fragments (in the case of the Hind III/Xba I and EcoR I/Xba I digests the fragments are labelled as for Hind III and EcoR I respectively). The novel fragments created by cutting of EcoR I or Hind III fragments with Xba I are given a subscript (1) denoting the higher molecular weight fragment and (2) the smaller.

◁ denotes missing fragment, * denotes novel fragment.


Xba I
13a
HG52
x163x3
abc
d
e
f
g
h
i
j
HindIII/Xbal
13b
x163
x163x3
a b
fgc d h
e1 i j
k1 m
e2
2xl
n
k2
o
l
BamHI
13c
HG52
x163x3
ab
c d e f g hij k l m n o p q r st u vw x yz
a'
b'
c'd'
e'
f'
g'
h'
i'
j'
k' l'
m'
EcoRI/Xbal
13d
HG52
x163x3
a1 b c d e
f ghi j k
l1
m
a2 n1
l2
o1
a1
l
na2
o1

are large and separation in that region of the gel, poor.

To confirm the loss of the Xba I site at 0.91m.c. a double digest of HG52X163X3 with Xba I and Hind III was performed (Fig.13b). The parental virus, HG52X163, contains three Xba I sites: the c/d site at 0.45m.c., the h/j site at 0.91m.c. and the j/i site at 0.94m.c.. On digestion with Hind III/Xba I the Xba I c/d site at 0.45m.c. cuts Hind III e (0.4m.c.-0.52m.c.) to give two molar bands of approximately $5x10^6$ and $7x10^6$ daltons which run just below m and above i respectively. The Xba I site at 0.91m.c. bisects the Hind III l (0.88m.c.-0.93m.c.) fragment to give two new molar bands which are both approximately $2.5x10^6$ daltons and which run together half way between n and m. The Xba I j/i site at 0.94m.c. lies within the Hind III k (0.93m.c.-1m.c.) fragment and cuts it to give two bands: a one molar band of $0.7x10^6$ daltons which runs between n and o and a half molar band of approximately $5.6x10^6$ daltons which probably runs with m or e^*_2. The cut within Hind III k also creates novel joint fragments c*(i+k*) and d*(j+k*) which run with f, g and h. When HG52X163X3 was digested with Xba I/Hind III the Hind III l fragment was not bisected and ran in the normal position of Hind III l confirming that the Xba I site at 0.91m.c. was absent.

When HG52X163X3 was digested with BamH I (Fig 13c) the l fragment (0.88m.c.-0.91m.c.), which contains the 0.91m.c. Xba I site, remained unaltered in its mobility suggesting that the site had been lost by a small change, possibly a single base substitution affecting the recognition sequence for Xba I, and not by a large deletion or insertion.

An EcoR I/Xba I digest of HG52X163X3 compared to HG52 is also shown (Fig. 13d), as such digests were used to screen

for Xba I site loss from subsequent enrichment selection experiments (to distinguish genomes cut at 0.45m.c. from those which remained uncut). When HG52 DNA is digested with EcoR I/Xba I the profile is predominantly like that for EcoR I alone except that the EcoR I a fragment is cut at the 0.45m.c. site to give two molar bands of which one runs with c and the other with n_1*; EcoR I l is cut by Xba I at 0.7m.c. to give two molar bands one of which runs below the normal EcoR I l and one above EcoR I o^*_1; the 0.91m.c. Xba I site cuts the EcoR I n band to give a one molar band marginally smaller than the normal n and one small molar band which has migrated off the gel; the Xba I site at 0.94m.c. cuts EcoR I o to give two molar bands of which the smaller has been lost from the gel. When HG52X163X3 is cut with EcoR I/Xba I, only the EcoR I a and o fragments are cut. As the Xba I sites at 0.7m.c. and 0.91m.c. have been lost the l and n fragments have returned to the positions they would occupy on digestion with EcoR I alone.

3.1d HG52 GENOMES LACKING ALL FOUR Xba I SITES

HG52X163X3 which lacks the 0.7m.c. and 0.91m.c. Xba I sites was used as the parental virus from which mutants lacking the two remaining Xba I sites were isolated. HG52X163X3 DNA was digested with Xba I at 1 unit per microgram of DNA at 37^{o}C for 1, 3 or 4.5h and then transfected onto monolayers of BHK21/C13 cells. The treatment with Xba I caused a drop in infectivity of approximately twenty fold in each case. Thirty two plaques each were picked from the transfections of the DNA digested for the three periods. Of the ninety six plaques, twenty had apparently lost the Xba I j/i (0.94m.c.) site, two had lost

the 0.45m.c. Xba I c/d site and five had lost both to give genomes containing no Xba I sites, There appeared to be no difference between the numbers of site deletion mutants isolated after the different periods of Xba I digestion of the DNA.

3.1e HG52X163X3X45

HG52X163X3X45 is an example of the twenty plaques which had apparently lost the Xba I j/i (0.94m.c.) site. It can be seen (Fig. 14) that the Xba I/EcoR I profile differs from the parental HG52X163X3 (Fig. 13d) in that the EcoR I o band is no longer cut by Xba I but runs in the normal position for EcoR I o. The restriction digest data, therefore, demonstrate that this isolate contains only one Xba I site, namely, the c/d site at 0.45m.c. which lies within EcoR I a.

3.1f HG52X163X3X27

HG52X163X3X27 is one of the two isolates which contain only the Xba I site at 0.94m.c.. The 0.45m.c. Xba I site is no longer present and on digestion with EcoR I/Xba I (Fig. 15) the EcoR I a band can be seen in its normal position. The only difference between the EcoR I/Xba I double digest profile of HG52X163X3X27 and that of the wild type, HG52, cut with EcoR I alone is that EcoR I o is cut by Xba I at the 0.94m.c. site.

3.1g HG52X163X3X53

HG52X163X3X53 is one of the five isolates which on digestion with EcoR I/Xba I gave a profile identical to that obtained with EcoR I alone. Figure 16a shows a comparison of HG52 digested with EcoR I alone and HG52X163X3X53 digested

Figure 14

Autoradiograph of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* comparing an EcoR I digest of HG52 with an EcoR I/Xba I digest of HG52X163X3X45.

Letters refer to specific fragments. In the case of the EcoR I/Xba I digest the fragments are as for EcoR I with the novel fragments created by cutting with Xba I being given a subscript (1) denoting the higher molecular weight and (2) the smaller.

◁ denotes missing fragment, * denotes novel fragment.

Figure 15

Autoradiograph of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* comparing an EcoR I digest of HG52 with an EcoR I/Xba I digest of HG52X163X3X27.

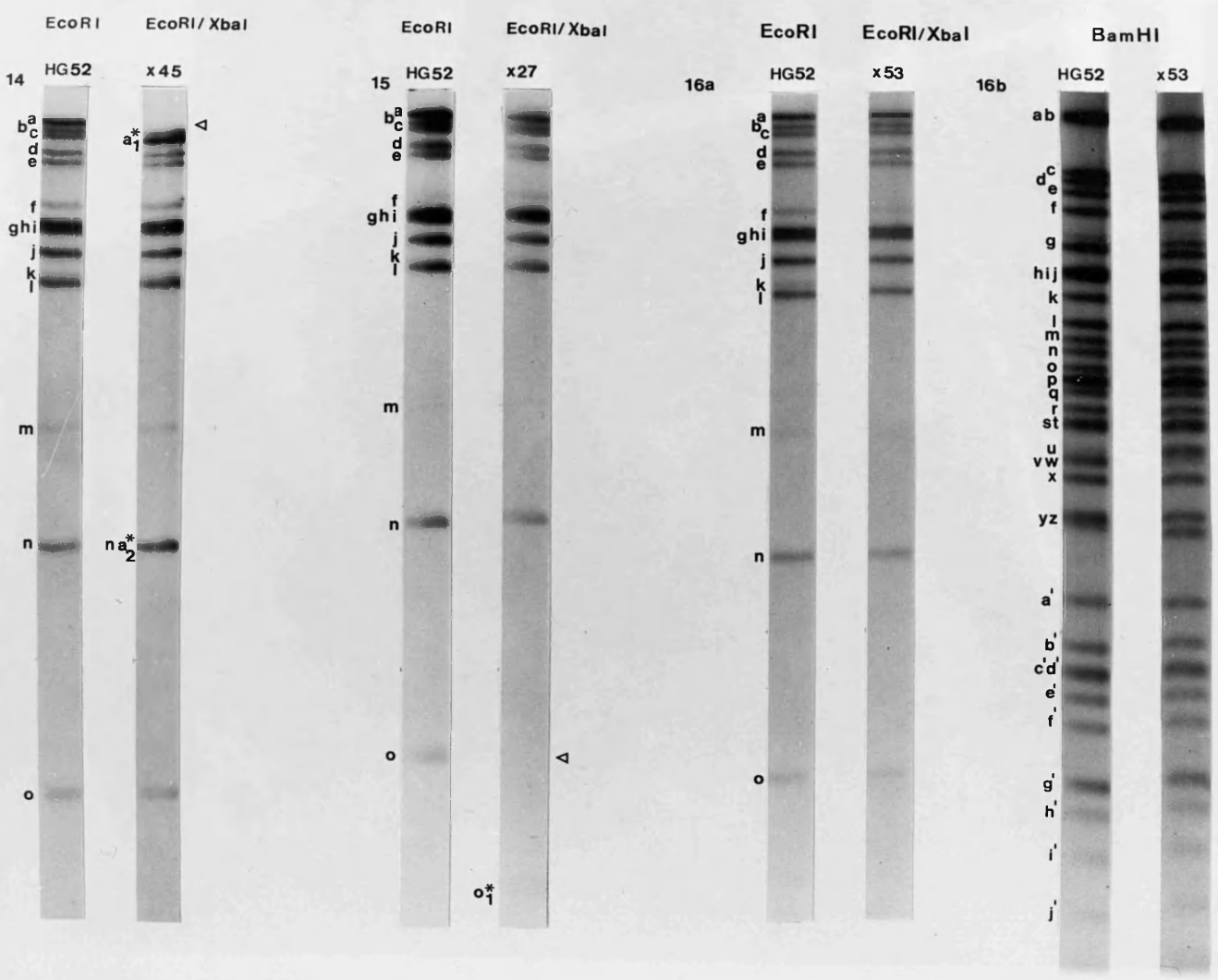
Letters refer to specific fragments. In the case of the EcoR I/Xba I digest the fragments are labelled as for EcoR I with the novel fragment created by cutting with Xba I being given a subscript (1) denoting the higher molecular weight the smaller fragment having run off the gel. ◁ denotes missing fragment, * denotes novel fragment.

Figure 16(a+b)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo*.

(a) An EcoR I digest of HG52 compared with an EcoR I/Xba I digest of HG52X163X3X53. (b) BamH I digests of HG52 compared with HG52X163X3X53.

Letters refer to specific fragments.


14
EcoRI
HG52
EcoRI/ XbaI
x45
15
EcoRI
HG52
EcoRI/ XbaI
x27
16a
EcoRI
HG52
EcoRI/XbaI
x53
16b
BamHI
HG52
x53

with EcoR I/Xba I. It can be seen that none of the EcoR I a, l, n or o fragments in HG52Xl63X3X53 is cut proving that this variant must lack all four of the Xba I sites present in HG52. It can also be seen that the EcoR I l fragment of HG52Xl63X3X53 is slightly larger than in HG52 due to the previously described 150bp insertion at the 0.7m.c. Xba I site in the parental genome, HG52Xl63. It would be expected that EcoR I m and k might also show changes in mobility due to the indicated deletion of approximately $0.1x10^6$ daltons from both copies of BamH I u. Any increase in the mobility of k would be difficult to detect due to the presence of the altered l fragment but there is some indication of a slight increase in the mobility of the m fragment seen more clearly in an EcoR I digest of another of the HG52Xl63 progeny viruses HG52Xl63X3 (Fig. 13d).

Digestion of HG52Xl63X3X53 with BamH I (Fig. 16b) demonstrated that the Xba I sites at 0.45m.c. and 0.94m.c. had been lost without significantly altering the mobility of the small fragments in which they occur (ie. BamH I o and b' respectively). It can be concluded, therefore, that these sites have been lost by minor changes in the genome possibly involving small insertions/deletions or a base change disrupting the recognition sequence for the restriction enzyme.

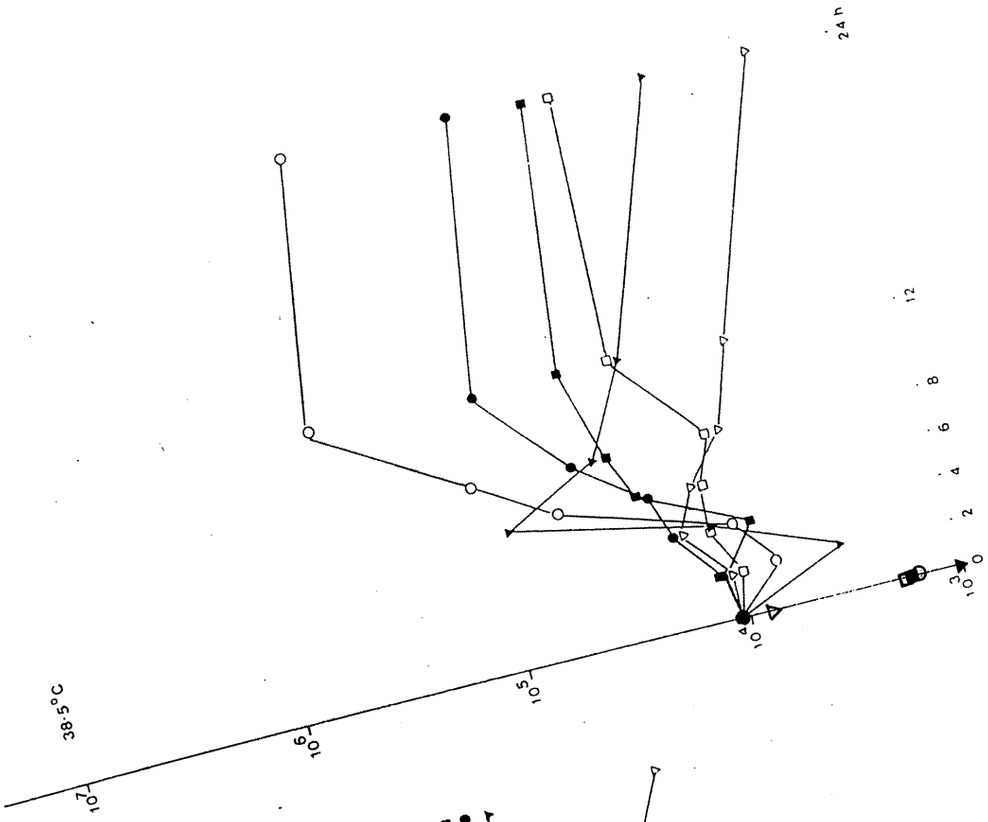
3.1h GROWTH CHARACTERISTICS OF THE Xba I SITE LOSS MUTANTS HG52X94, HG52Xl63, HG52Xl63X3, HG52Xl63X3X27, HG52Xl63X3X45 AND HG52Xl63X3X53

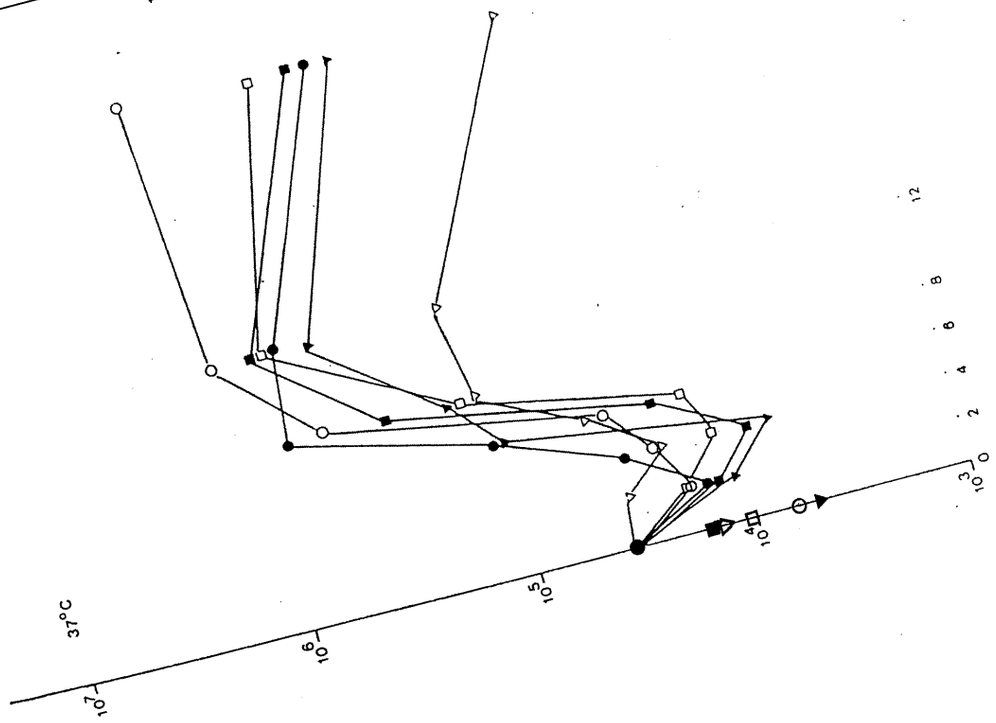
One step growth experiments were performed on all of the site deletion mutants except HG52Xl63X3X27 (Fig. 17). Twenty four hour growth experiments were carried out in BHK21/Cl3

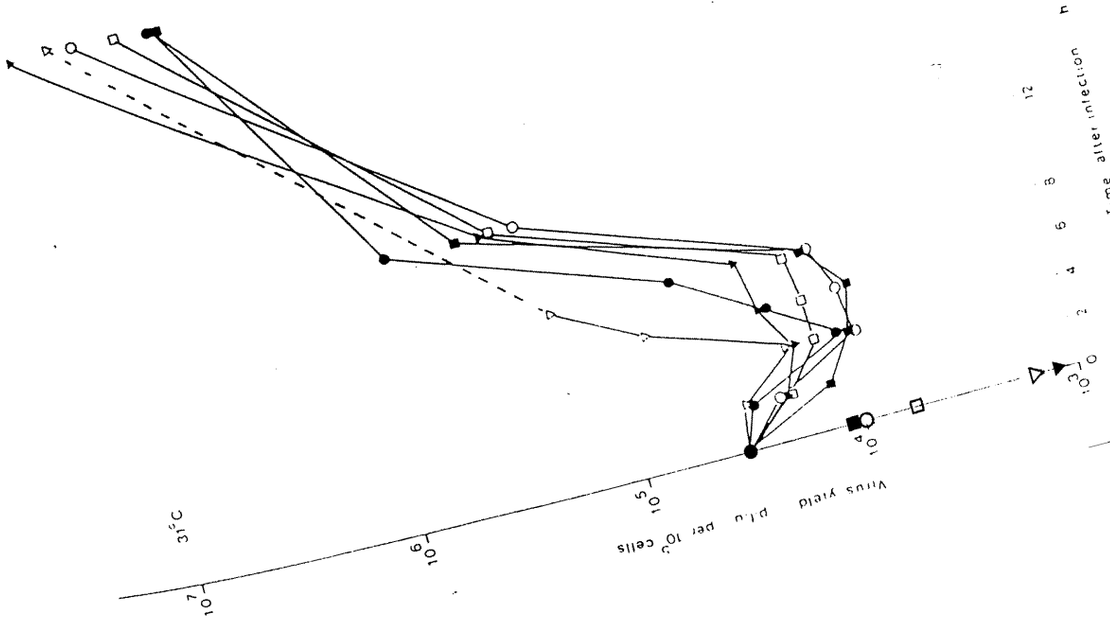
Figure 17

One step growth curves of HSV-2 strain HG52 ●, HG52X94 ○, HG52X163 ■, HG52X163X3 □, HG52X163X45 ▼ and HG52X163X3X53 ▽ in BHK21/C13 cells at 31°C, 37°C and 38.5°C. Cells were infected at a MOI of 5pfu/cell. After absorption for 45min at the appropriate temperature the monolayers were washed twice with phosphate-buffered saline, overlaid with ETC10% and incubated at the appropriate temperature. Cultures were harvested at the times indicated and virus titres measured by plaque assay on BHK21/C13 cells. The titres have all been standardized to the HG52 zero time figure.

To allow comparison zero hour samples were standardized to that of HG52. Actual zero hour values are marked on axis.


38·5°C
24 h


37°C
24 h


37°C
Virus yield p.f.u. per 10⁷ cells
Time after infection

cells at 31°C, 37°C and 38.5°C and the virus yields titrated at 31°C. At 31°C all of the site deletion mutants grew at least as well as the HG52 wild type strain from which they were derived. At 37°C, however, there was an indication that HG52X163X3X53 was slightly impaired in its growth. The others grew as well as the wild type strain with HG52X94 growing particularly well. At 38.5°C, HG52 is somewhat temperature restricted and the apparent advantage of HG52X94 at 37°C became more pronounced at the higher temperature. The other viruses appeared to be impaired to a greater or lesser degree. HG52X163 and HG52X163X3 grew slightly less well than HG52. The graph of HG52X163X3X45 indicated that this mutant grew reasonably well at the higher temperature but growth was not sustained, possibly indicating heat lability. The graph of HG52X163X3X53 at 38.5°C, however, clearly depicts the growth failure of a temperature sensitive mutant. In addition the HG52X163X3X53 37°C and 38.5°C virus yields when titrated at 31°C formed tiny plaques. The titres of a typical stock of HG52X163X3X53 are shown below:

31°C 1.8×10^6 pfu/ml 37°C 1.7×10^5 pfu/ml 38.5°C $< 10^3$ pfu/ml

Although a burrler stock of HG52X163X3X27 was not grown and consequently no one step growth experiment performed, a 50mm plate stock was grown and proved to be temperature sensitive:

31°C 8.5×10^6 pfu/ml 37°C 1.7×10^5 pfu/ml 38.5°C $< 10^2$ pfu/ml

The fact that both HG52X163X3X53 and HG52X163X3x27 are temperature sensitive led to the conclusion that the DNA alteration causing loss of the 0.45m.c. Xba I site may have resulted in the synthesis of a temperature sensitive polypeptide. All of the HG52 mutants lacking the 0.45m.c.

Xba I site were subsequently found to be temperature sensitive. From the single cycle growth experiments it is concluded that the site loss mutants grow well in tissue culture at 31°C to yield high titre stock and that HG52X163X3X27 and HG52X163X3X53 are temperature sensitive at 38.5°C.

One variable which may affect lytic growth is the particle: pfu ratio of the virus stock. The particle: pfu ratios of the stocks used in the growth experiments are shown below:

HG52	1,263:1
HG52X94	224:1
HG52X163	24:1
HG52X163X3	737:1
HG52X163X3X45	776:1
HG52X163X3X53	2,771:1

The particle: pfu ratio of the HG52X163X3X53 stock was rather high. However, as that of the wild type stock was also quite high it is difficult to draw conclusions from these data.

3.1i POLYPEPTIDES INDUCED BY Xba I SITE LOSS MUTANTS

HG52X94 and HG52X163 have each lost the 0.7m.c. Xba I site due to deletion of approximately 150bp or insertion of approximately 100bp or respectively. HG52X163 was the parental genome from which subsequently HG52X163X3 was isolated which in addition to the 0.7m.c. Xba I site also lacks the 0.91m.c. Xba I site. HG52X163X45 lacks the 0.7, 0.91 and 0.94m.c. Xba I sites and HG52X163X3X53 lacks all four Xba I sites (ie. 0.45, 0.7, 0.91 and 0.94m.c.) and is

temperature sensitive at 38.5°C.

There are a few alterations apparent in the polypeptide profiles of the Xba I deletion mutants compared to the parental HG52 (Fig. 18). HG52X94 appears to produce reduced amounts of thymidine kinase (if any at all). HG52X163 and its progeny viruses all exhibit some alteration in the mobility of a polypeptide in the 36/37kd region. In addition HG52X163X3X45 shows some alteration in the mobility of a polypeptide of approximately 132/134kd molecular weight. The significance, if any, of these alterations has not been further studied for the purpose of this thesis.

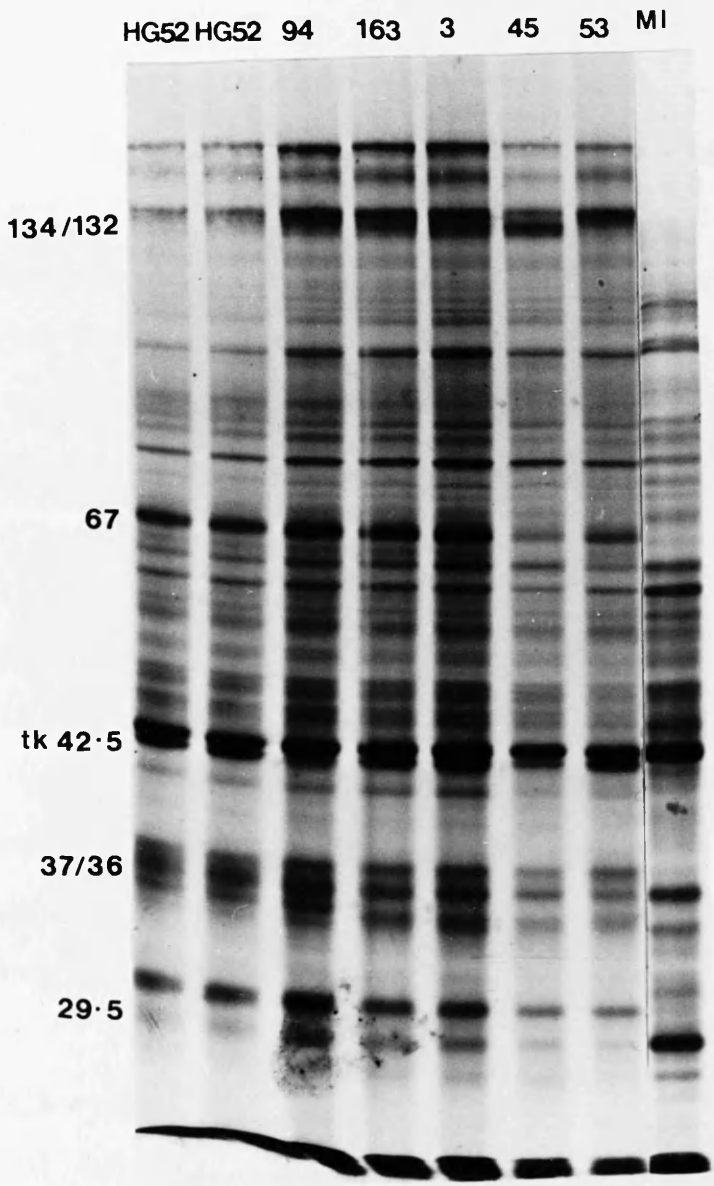
3.2 VARIANTS ISOLATED AFTER TRANSFECTION OF Xba I TREATED HG52 DNA

Restriction enzyme analysis of the two hundred plaques picked after transfection of partially Xba I digested HG52 DNA revealed a number of variants with altered genome structures. The frequency of isolation of variants was 5% of which the majority exhibited deletions from the long internal repeat region of the genome contained within Xba I g. Alterations in other portions of the long region would probably not have been detected as isolates were normally screened only with Xba I to detect site losses. The long terminal Xba I c fragment (0m.c.-0.45m.c.) and its joints are too large to detect changes in mobility due to similar deletions or insertions into the terminal fragment. The short termini, contained in the Xba I h and i fragments, exhibited some variability in size but no major alterations.

A quarter of the two hundred plaques were analysed with EcoR I which allowed detection in TR_L (EcoR I f) of equivalent sized deletions as seen in IR_L (Xba I g). No such

Figure 18

Autoradiograph of ^{35}S methionine labelled infected cell polypeptides induced in BHK21/C13 cells and separated by SDS-PAGE. Cells were infected at 38.5^{o}C and polypeptides analysed on a 7.5% gel. Lanes 1 and 2 HG52, lane 3 HG52X94, lane4 HG52X163X3, lane 5 HG52X163X3X45, lane 6 HG52X163X3X53 and lane 7 mock infected. Numbers show the apparent molecular weight (x10^{3} daltons) of HSV-2 infected cell polypeptides.


HG52
HG52
94
163
3
45
53
MI
134/132
67
tk 42·5
37/36
29·5

deletions were observed.

Initial analyses of the DNA of individual plaques often revealed a mixture of genome structures within a single isolate. Therefore, stocks which appeared to contain variants were subjected to three rounds of stringent plaque purification involving the picking of well separated plaques grown in the presence of neutralizing antibody. In a number of cases it proved impossible to obtain a pure stock of a variant which had been observed in a mixture, indicating that these variants were either unstable or required helper virus for their growth. They were not investigated further.

As variants with deletions from IR_L were isolated relatively frequently, only four were analysed in detail. These were designated HG52X85/4, HG52X85/5, HG52X86 and HG52X192.

3.2a HG52X85

HG52X85 showed a complicated profile when digested with Xba I (Fig. 19a). After plaque purification it became clear that the original isolate contained two separate variants, HG52X85/4 and HG52X85/5, each exhibiting different sized deletions from Xba I g.

3.2b HG52X85/4

Analysis of HG52X85/4 indicated a deletion of approximately $6x10^6$ daltons of DNA from the long internal repeat region of the genome. On digestion with Xba I (Fig. 19b), the novel g band was approximately $6x10^6$ instead of $12x10^6$ daltons and ran just above the $5x10^6$ dalton i band. The two joints e(g+h) and f(g+i) were also reduced in size by the same amount to run below their normal positions.

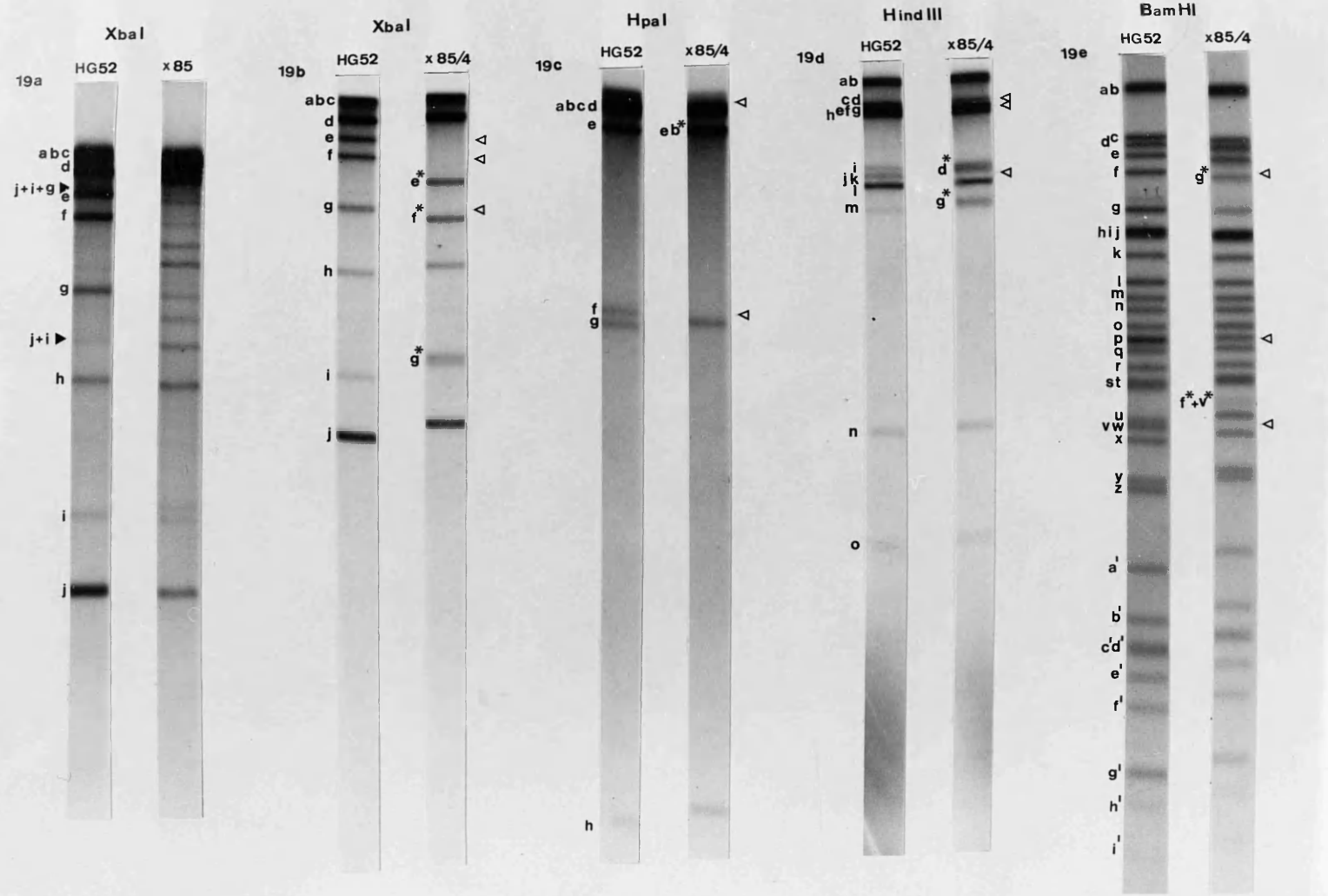
Figure 19a

Autoradiograph of Xba I restriction digests of viral DNA ^{32}P labelled in vivo of HG52 and HG52X85. In the wild type track, letters refer to specific fragments and fragments due to partial digestion are marked ▶. As the original HG52X85 isolate proved to be a mixture, novel and missing fragments are not marked.

Figure 19(b+e)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled in vivo of HG52 and one of the variants from the isolate HG52X85, ie. HG52X85/4.
(b) Xba I digests, (c) Hpa I digests, (d) Hind III digests and (e) BamH I digests.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


19a
XbaI
HG52
x85
abc
d
j+i+g
e
f
g
j+i
h
i
j
19b
XbaI
HG52
x85/4
abc
d
e
f
g
h
i
j
e*
f*
g*
19c
HpaI
HG52
x85/4
abcd
e
f
g
h
eb*
19d
HindIII
HG52
x85/4
ab
cd
hefg
i
jk
l
m
n
o
d*
g*
19e
BamHI
HG52
x85/4
ab
dc
e
f
g
hij
k
l
m
n
o
p
q
r
st
u
vw
x
y
z
a'
b'
c'd'
e'
f'
g'
h'
i'
g*
f*+v*

Digestion with Hpa I (Fig. 19c) confirmed the findings from the Xba I digest and demonstrated that the deletion does not extend far into the long unique region as the e fragment was normal but the Hpa I f band was lost. The profile was consistent with the loss of more than $5x10^6$ daltons from Hpa I f to give a band of less than $1.5x10^6$ daltons which would have run off the gel and a novel b joint (f*+ [S]) which now ran just below e.

Digestion with Hind III (Fig. 19d) revealed that, whilst Hind III o was apparently normal, almost the entire j fragment was deleted. The two joint fragments containing j, d(j+k) and g(j+m), were reduced in size to run just above k and m respectively. The novel j fragment size can be calculated from the size of the novel joints to be approximately $0.2x10^6$ daltons, indicating a deletion of approximately $6.1x10^6$ daltons and, therefore, the loss of almost the entire internal long repeat. This digest indicates that there has been retention of the 'a' sequence contained in the long internal repeat as the long portion of the genome inverts to give the novel j containing joints, d and g, and the i containing joints, c and f, in normal quarter molar proportions.

Further evidence for the deletion of information from IR_L was provided by digestion with BamH I (Fig. 19e). BamH I f and one copy each of p and v had apparently been lost and a novel g* joint comprising the remainder of f plus the remainder of v and u from the short repeat was seen to run just below the normal position of f. The novel half molar long internal end fragment comprising the remainder of f and the remainder of v, calculated to be approximately $2.2x10^6$ daltons, is seen running below s and t.

Taken together the information from the different digests confirms a deletion of more than $6x10^6$ daltons of DNA which removes almost the entire internal long repeat region excluding 'a' sequences but does not extend further into U_L than the Hind III j/o site at 0.75m.c..

3.2c HG52X85/5

Restriction enzyme analysis of the plaque, HG52X85/5, obtained upon purification of HG52X85, revealed a genome structure which differed from the parental HG52 and from the variant HG52X85/4.

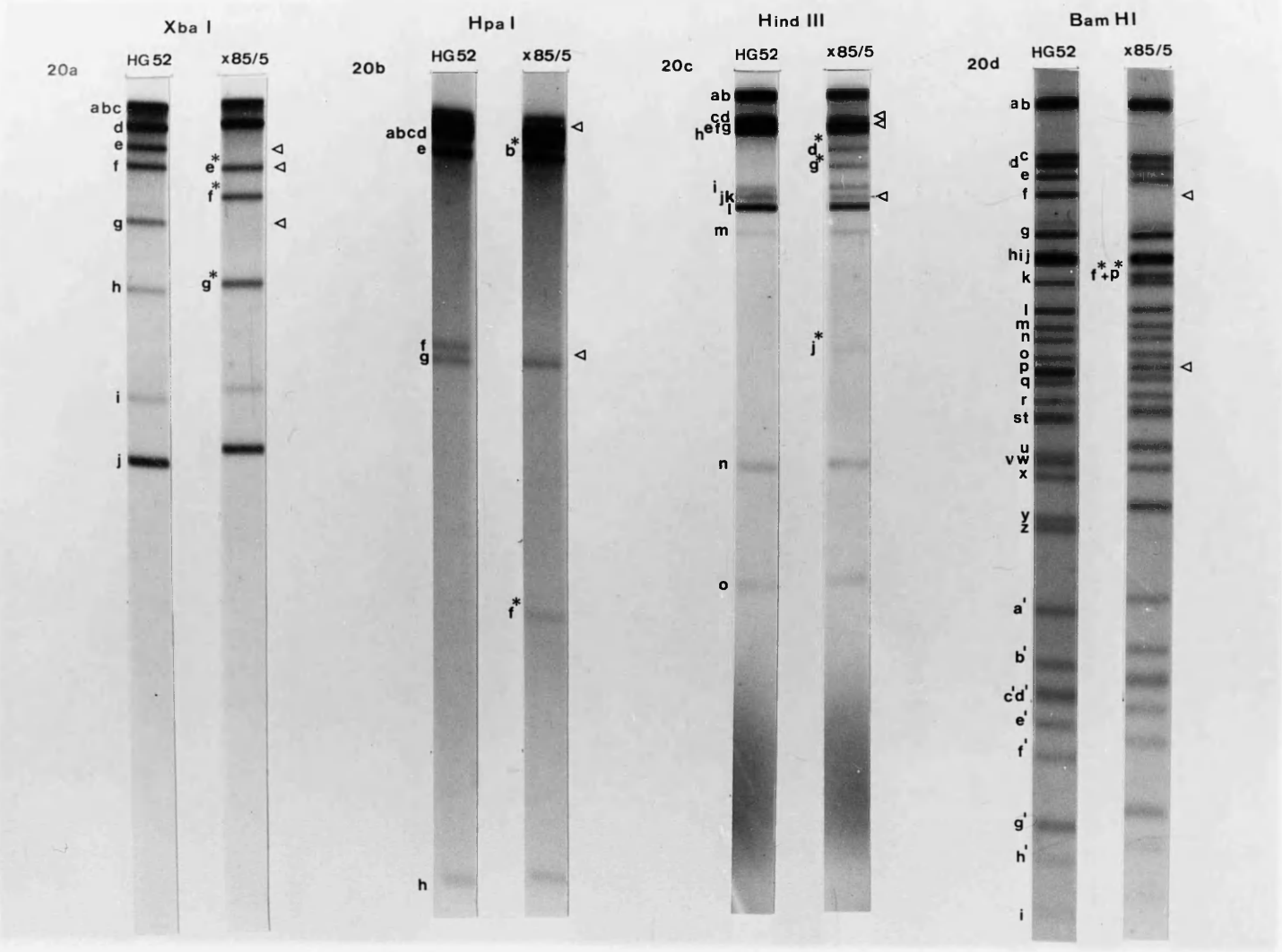
On digestion with Xba I (Fig. 20a) HG52X85/5 DNA exhibited a profile in which the g band was reduced in size to run with Xba I h indicating a deletion of approximately $4x10^6$ daltons. The g containing joints, e(g+h) and f(g+i), were reduced in size by the same amount to run below their normal positions.

The profile obtained on digestion with Hpa I (Fig. 20b) confirmed the deletion of information from IR_L. Hpa I f was missing and replaced by a novel fragment of approximately $3x10^6$ daltons running between Hpa I g and h. The normal b band (f+[S]) was lost and a half molar, novel b joint comprising the remainder of f and the short region was seen running between the c/d and e bands. Further confirmation of the deletion from IR_L was provided by the Hind III profile (Fig. 20c). The Hind III j fragment was seen to be deleted by approximately $3x10^6$ daltons to give a novel fragment which ran midway between the n and m bands. The joints containing j (ie d(j+k) and g(j+m)) were also reduced in size by the same amount and now ran between the i and h bands.

Figure 20(a-d)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* of HG52 and the second of the variants isolated from HG52X85, ie. HG52X85/5.
(a) Xba I digests, (b) Hpa I digests, (c) Hind III digests and (d) BamH I digests.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


Xba I
20a
HG52
x85/5
abc
d
e
f
g
h
i
j
e*
f*
g*
Hpa I
20b
HG52
x85/5
abcd
e
f
g
h
b*
f*
Hind III
20c
HG52
x85/5
ab
cd
h efg
i
jk
l
m
n
o
d*
g*
j*
Bam HI
20d
HG52
x85/5
ab
dc
e
f
g
hij
k
l
m
n
o
p
q
r
st
u
vw
x
yz
a'
b'
c'd'
e'
f'
g'
h'
i
f*+p*

Digestion with BamH I revealed a profile (Fig. 20d) in which f and one copy of p were missing although, unlike HG52X85/4, the BamH I v band and consequently the joint g(v+u) appeared normal. The fragment formed from the fused remnants of the deleted f and p fragments was seen as a molar band running just above k. Whereas the f and p fragments are normally $4.3x10^6$ and $2.6x10^6$ daltons respectively the novel band was about $3.2x10^6$ daltons which represents a deletion of approximately $3.7x10^6$ daltons. Variation is also apparent in the mobility of BamH I z. However, this is known to be variable due to different copy numbers of reiterated sequences in that region of the repeat (Rixon et al., 1984).

In HG52X85/5, therefore, the deleted sequence lies within the limits of the Hind III o/j site at approximately 0.75m.c. and the BamH I p/v site at 0.81m.c..

3.2d HG52X86

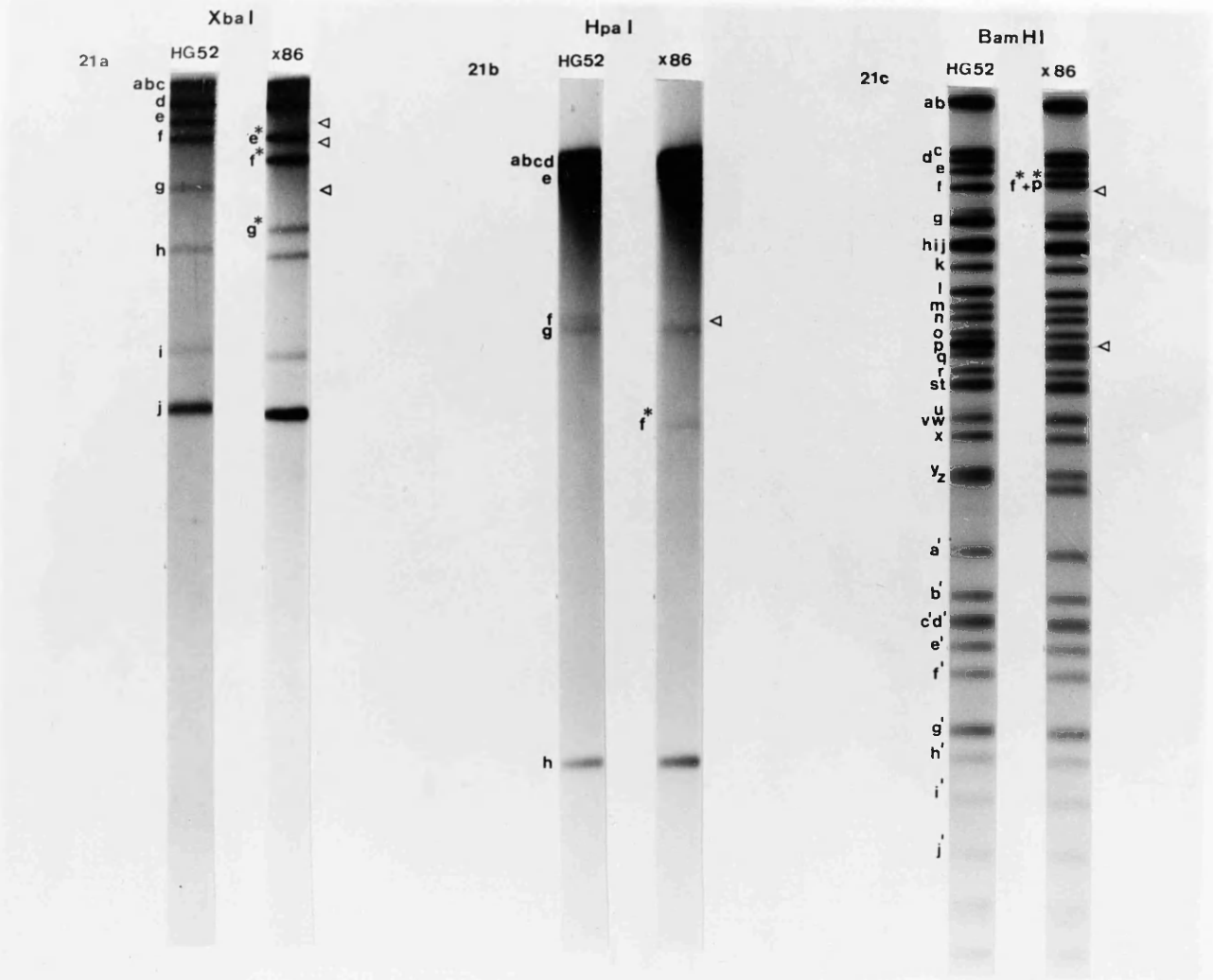
When screened with Xba I the isolate, HG52X86, exhibited an aberrant profile (Fig. 21a) in which the g band and its joints, e(g+h) and f(g+i), were reduced in size. The deleted g band was reduced in size by approximately $3x10^6$ daltons to run above h. The reduction in sizes of the e and f joints was consistent with the calculated deletion.

Digestion with Hpa I (Fig. 21b) showed that f was deleted to run below the g band. The mobility change due to a deletion of this size in the f containing joint, c(f+[S]), would be so small as to preclude detection under the gel conditions used.

Digestion with BamH I (Fig. 21c) gave a profile in which one copy of p was missing and the f band had been replaced

Figure 21(a-c)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* of HG52 and a variant isolated after enrichment selection of HG52 DNA, HG52X86. (a) Xba I digests, (b) Hpa I digests and (c) BamH I digests. Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


Xba I
21a
HG52
x86
abc
d
e
f
g
h
i
j
e*
f*
g*
Hpa I
21b
HG52
x86
abcd
e
f
g
h
f*
BamHI
21c
HG52
x 86
ab
d c
e
f
g
hi j
k
l
m
n
o
p
q
r
st
u
vw
x
y z
a'
b'
c' d'
e'
f'
g'
h'
i'
j'
f* + p*

by a larger band running above the normal position but below e. The alteration in the genome appeared to be confined to the BamH I p and f bands as the adjoining bands, k and v, were normal. As v comigrates with w it is difficult to detect. However, the intensity of the joint, g(v+u), did not appear to be affected and no new joints were apparent). The data were interpreted as indicating a deletion of information from the BamH I f and p fragments including the f/p site. The novel band below e was thought to be the product of the fusion of the remnants of the f and p bands and was calculated to be of approximately 4.4×10^6 daltons. Fusion of the normal f and p fragments would result in a band of 6.9×10^6 daltons indicating that the deletion must be of approximately 2.5×10^6 daltons.

Taking the information from all of the digests together, the deletion is approximately 2.5×10^6 daltons within the limits of the Hpa I e/f site at 0.75m.c. and the BamH I p/v site at 0.81m.c..

3.2e HG52X192

When screened for Xba I site loss, one of the isolates, HG52X192, obtained after transfection of Xba I digested HG52 DNA, exhibited an aberrant Xba I profile (Fig. 22a). The alteration in the genome was similar to those already described in that there was a deletion from Xba I g of approximately 1×10^6 daltons causing Xba I g and the two g containing joints, e(g+h) and f(g+i), to run below their normal positions.

When digested with Hpa I (Fig. 22b), however, not only was the expected deletion of 1×10^6 daltons from Hpa I f (0.76-0.82m.c.) seen, but a deletion of similar size was

Figure 22(a-d)

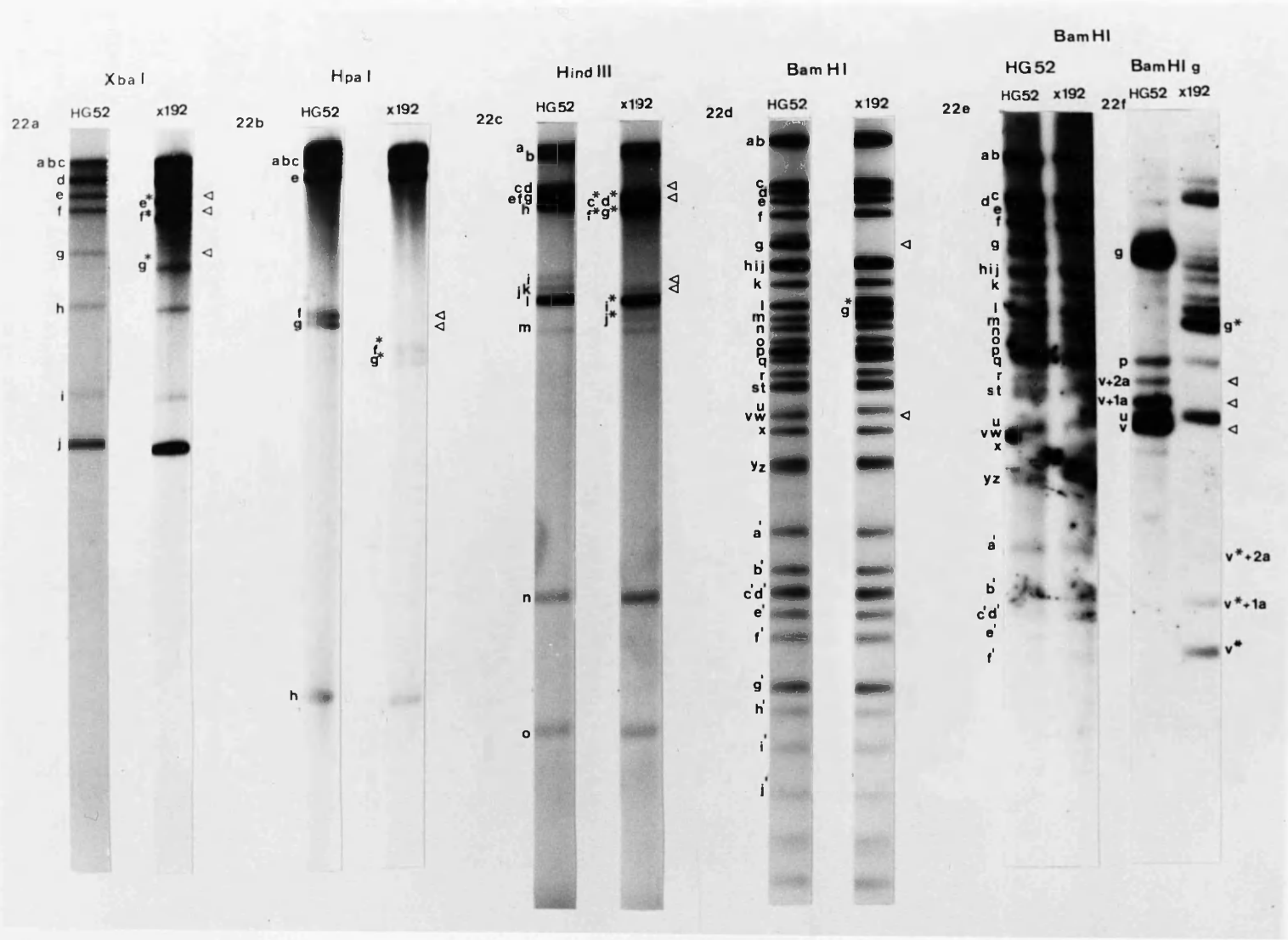
Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* of HG52 and a variant, HG52X192, isolated after enrichment selection of HG52 DNA. (a) Xba I digests, (b) Hpa I digests, (c) Hind III digests and (d) BamH I digests.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.

Figure 22(e+f)

Autoradiograph of nitrocellulose blot strips from a single gel containing BamH I restriction fragments of HG52 and HG52X192 to which nick-translated HSV-2 DNA probes have been hybridized. The probes were (e) HG52 and (f) the recombinant plasmid, pGZ1, containing BamH I g(v+u).

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment. Populations of v and v* are indicated containing the 'normal' one copy of the 'a' sequence and either one (+1a) or two (+2a) additional copies of the 'a' sequence.


Xba I
Hpa I
Hind III
Bam HI
Bam HI
HG52
Bam HI g
22a
22b
22c
22d
22e
22f
HG52
x192
abc
d
e
f
g
h
i
j
e*
f*
g*
Δ Δ
Δ
abc
e
l
g
h
r*
g*
ΔΔ
a
b
cd
e
f
g
h
j
k
l
m
n
o
c*
d*
g*
ΔΔ
ΔΔ
l*
k*
ab
c
e
f
g
hij
k
l
m
n
o
p
q
r
st
u
vw
x
yz
a'
b'
c'
d'
e'
f'
g'
h'
i'
j'
g*
Δ
Δ
ab
d
c
e
f
g
hij
k
l
m
n
o
p
q
r
st
u
vw
x
yz
a'
b'
c'
d'
e'
f'
g
p
v+2a
v+1a
u
v
g*
Δ
Δ
Δ
v*+2a
v*+1a
v*

evident from the long terminal Hpa I g fragment (0-0.065m.c.).

Confirmation of there being deletions from both long termini was obtained from a Hind III digest (Fig. 22c). The Hind III long terminal fragments, i and j, were both reduced by approximately $1x10^6$ daltons to run between the l and m bands. The joint fragments, c(i+k), d(j+k), f(i+m) and g(j+m) were all consequently reduced in size. The intensities of the joint and end fragments were normal in this stringently plaque purified stock indicating that 'a' sequences were present at the long terminus and at the L/S junction.

To locate the two deletions a BamH I digest was performed (Fig. 22d). As the two copies of BamH I p and the single copies of c and f ran in their normal positions, it was deduced that in each case the deletion must be confined to the BamH I v fragment and, indeed, the two half molar v bands are missing from their usual position. There is only one joint fragment, g, observed on digestion of HG52 with BamH I comprising v, present in both long repeats, plus u, present in both short repeats. However, differing copy numbers of 'a' sequences tend to create two major size classes of BamH I g. In HG52X192 the normal g band is missing and is replaced by two smaller bands running with l and m. The most likely explanations, therefore, are that (1) the two copies of BamH I v have been deleted by different amounts to give two new joint fragments, g_1* (v_1*+u) and g_2*(v_2*+u) or (2) the deletion from each end is the same and that the two observed novel joints are due to different numbers of 'a' sequences. Figure 22f shows a blot of BamH I digested HG52 and HG52X192 hybridized to BamH I g, compared

to the same samples probed with HG52 in Fig. 22e. There is, as expected, hybridization to the wild type g, v and u fragments. As previously stated, BamH I v and consequently g are heterogeneous in size due to different copy numbers of the 'a' sequence, and can be detected as 'ladders' of bands with decreasing intensity as the number of 'a' sequences increases. There is also apparent hybridization to the wild type BamH I p fragment which has been shown previously to be due to cross hybridization between the BamH I v and p fragments (Brown and Harland, 1984). BamH I g when hybridized to BamH I digested HG52Xl92 DNA revealed that the normal v bands were missing and were replaced by smaller bands running in the regions of a', c'/d' and f'. There was also positive hybridization to the bands comigrating with BamH I l and m confirming that these bands represent novel joint fragments g*(v*+u). The relative intensities of the two novel v* fragments and the joint fragments g* support the assumption that both copies of v have been deleted by the same amount and that the two size classes are simply due to the numbers of 'a' sequence repeats. There is also some hybridization seen in the region below the position of the wild type BamH I g and above BamH I c. As there is no indication of there being any band present in these regions in BamH I digests of HG52Xl92 (Fig. 22d) it seems probable that this is spurious hybridization due to partial digestion of the HG52Xl92 DNA.

The restriction enzyme and Southern blot analyses, therefore, show that HG52Xl92 is deleted by approximately $1x10^6$ daltons from both copies of BamH I v.

3.2f HG52X19

During the enrichment selection procedure involving transfection of partially Xba I digested HG52 DNA one isolate, HG52X19, exhibited an Xba I profile (Fig. 23a) with apparently aberrant g and g containing joints e(g+h) and f(g+i) plus a number of bands between g and f. Figure 23b shows an Xba I digest of the DNA of eleven plaques isolated after two further rounds of plaque purification. It is clear from the profiles that there is a high degree of instability. Because it appeared to be less variable, the isolate whose profile is shown in lane 4 was subjected to further rounds of plaque purification. Figure 23c shows an Xba I digest of the ^{32}P labelled DNA of six progeny plaques. These demonstrate less variability than some of the previously observed profiles. However, the isolate shown in lane 2 was clearly still unstable as demonstrated by the Xba I profiles of the DNA of a further ten progeny plaques (Fig. 23d). These demonstrate a variety of novel structures including revertants to the wild type profile (lanes 5 and 6).

It was obvious from the DNA profiles that there is an exceptional amount of variability in the DNA structures of the genomic population of HG52X19. Some indication of the possible alteration responsible for introducing the instability was given by comparing the profiles of Fig. 23c lanes 2 and 3. The relative abundance of the fragments Xba I h and i in the two lanes and the relative intensities of the two novel bands running just below and above the normal position of Xba I g indicated that these were novel L/S junction fragments e* and f*. In lane 2 the Xba I h band is relatively abundant, whereas, the i band is reduced in

Figure 23(a)

Autoradiograph of Xba I restriction digests of viral DNA ^{32}P labelled *in vivo* of HG52 and the variant, HG52X19, isolated after enrichment selection of HG52 DNA.

Letters refer to specific fragments, $\uparrow_{*}$ indicates a ladder of novel fragments.

Figure 23(b)

Autoradiograph of Xba I restriction digests of viral DNA ^{32}P labelled *in vivo* of HG52 (lane 12) and eleven plaques of HG52X19 isolated after a further two rounds of plaque purification (lanes 1-11).

Letters refer to specific fragments, $\uparrow_{*}$ indicates a ladder of novel bands.

Figure 23(c)

Autoradiograph of Xba I restriction digests of ^{32}P labelled viral DNA of HG52 (lane 7) and six plaques isolated after a single round of plaque purification of the isolate seen in Fig. 23(b) lane 4.

Letters refer to specific fragments, ▷ denotes missing fragment, * denotes novel fragment.

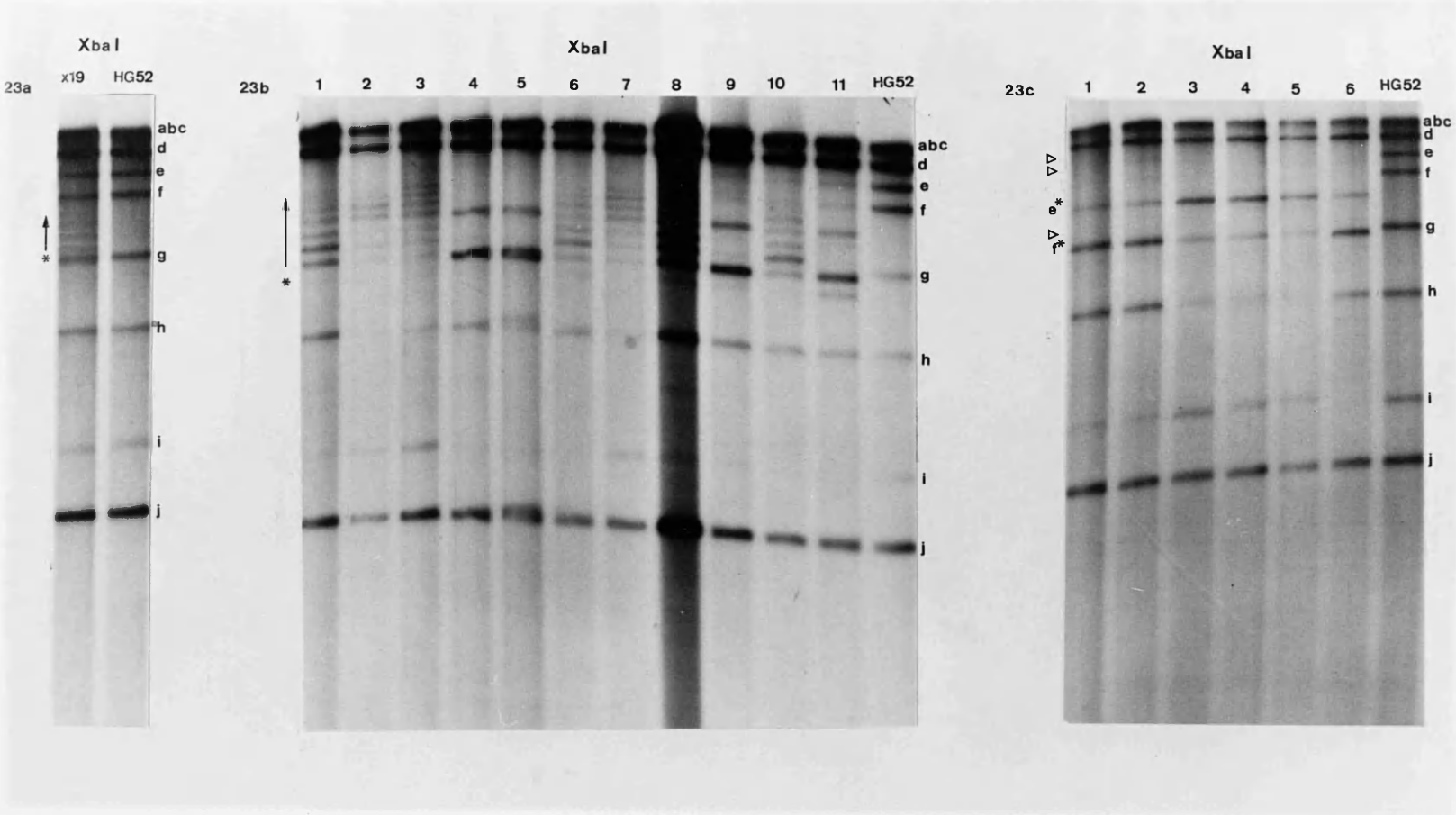

23a
XbaI
x19
HG52
abc
d
e
f
g
h
i
j
*
23b
XbaI
1
2
3
4
5
6
7
8
9
10
11
HG52
abc
d
e
f
g
h
i
j
*
23c
XbaI
1
2
3
4
5
6
HG52
abc
d
e
f
g
h
i
j
Δ Δ
* e
Δ *

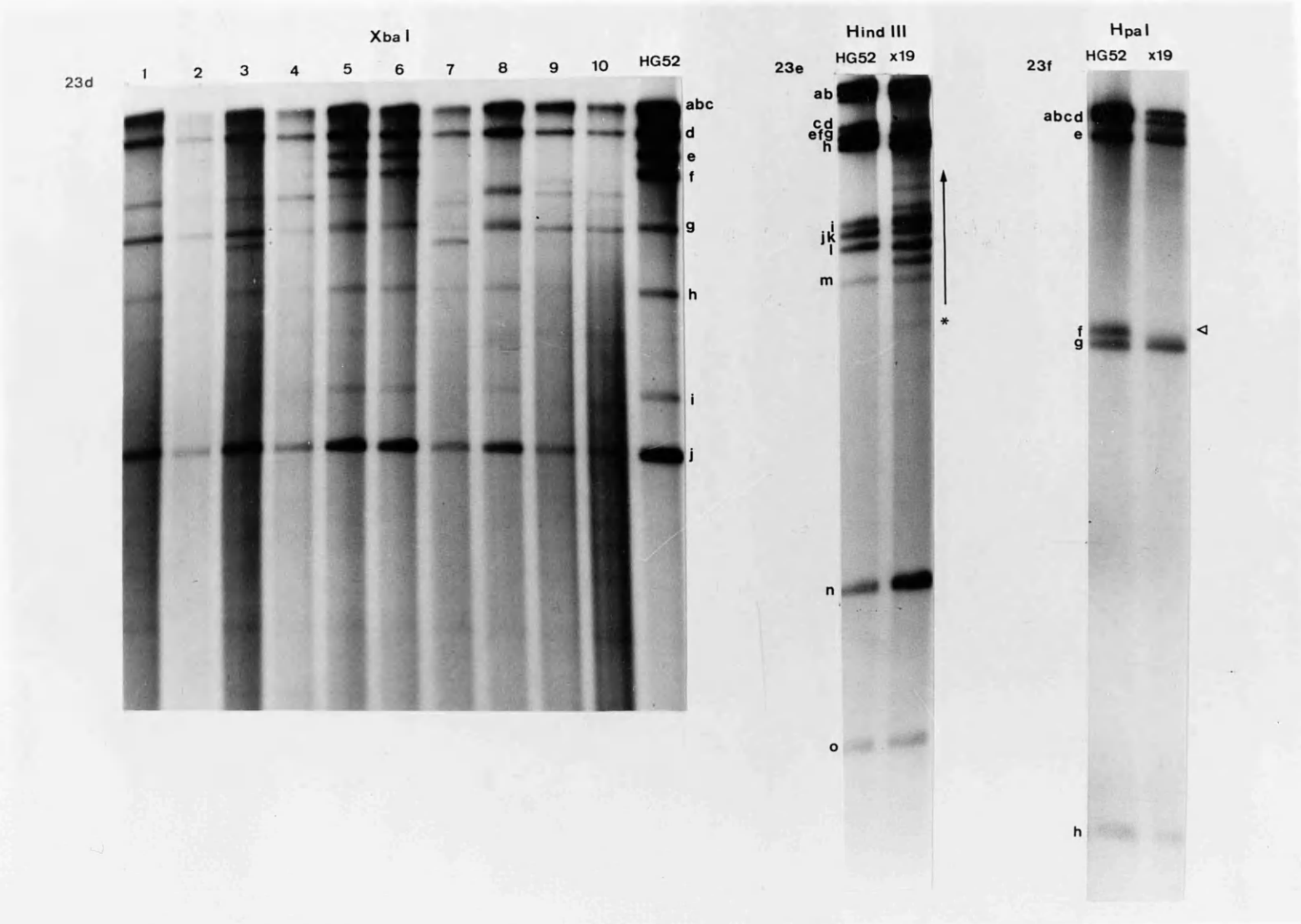
Figure 23(d)

Autoradiograph of viral DNA ^{32}P labelled in vivo of HG52 (lane 11) and ten plaques isolated after one round of purification of the sample seen in Fig. 23(c) lane 2. Letters refer to specific wild type fragments. Novel and missing fragments are not marked.

Figure 23(e+f)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled in vivo of HG52 and the isolate of HG52X19 seen in Fig. 23(b) lane 1.
(e) Hind III digests and (f) Hpa I digests.

Letters refer to specific fragments, ◁ denotes missing fragment, ↑* denotes ladder of novel fragments.


Xba I
23d
1
2
3
4
5
6
7
8
9
10
HG52
abc
d
e
f
g
h
i
j
Hind III
23e
HG52
x19
ab
cd
efg
h
i
jk
l
m
n
o
*
Hpa I
23f
HG52
x19
abcd
e
f
g
h
Δ

intensity. This is indicative of the short region being predominantly in the inverted orientation with the i fragment being more frequently in the L/S joint, f*(g*+i), and the h fragment more commonly as an end. In lane 3 the opposite picture is seen in which i is the more obvious of the two short end fragments and, therefore, the short region is predominantly in the opposite orientation (ie in the prototype orientation) with Xba I h more frequently in the L/S joint fragment e*(g*+h).

The implication from the Xba I digests is that the short region does not invert normally, although both orientations are present. The data also suggest a deletion from Xba I g to give novel joints e* and f*, each reduced by approximately $6x10^6$ daltons. The expected novel g* fragment which should be approximately half its normal size is not in its expected position above i. The absence of this band led to the proposal that the long segment is fixed in the prototype orientation so that g* is only present in the novel joint fragments e* and f* and never as an end. On digestion with Xba I the long terminal fragment, c, runs coincidently with its two joints a(c+h) and b(c+i) so that confirmation of the fixed orientation of the long region could not be obtained from digestion with Xba I.

Figure 23e shows a Hind III digest of a plaque isolate of HG52X19 demonstrating normal Hind III a and o bands which limits the deletion from Xba I g to the portion contained between 0.75m.c. and the right hand end of the long region (ie mostly within the repeat region). It can also be seen that ladders of different sized bands are evident on digestion with Hind III.

The missing Hpa I f band and the apparently normal Hpa I

e band (Fig. 23f) support the conclusion that there is a deletion from the long internal repeat which does not extend into the long unique region far beyond the U_L/IR_L junction, as the Hpa I e/f site is present. By extrapolation from the Xba I profiles it would be expected that on Hpa I digestion any ladder of novel joint fragments on Hpa I digestion would start just below Hpa I e. The poor separation of bands at the top of the Hpa I profile makes it difficult to tell if such ladders are present.

In order to study the relative abundance of end and joint fragments, analysis of encapsidated DNA was performed. This was done by extracting ^{32}P labelled viral DNA from DNase resistant (ie enveloped) virus and comparing the intensity of the end and L/S joint fragments of the variant and the wild type against total cellular DNA which would contain a proportion of concatameric DNA.

One of the isolates which exhibited pronounced ladders on digestion with Xba I and an Xba I profile indicative of the short region being predominantly in the inverted orientation was, unless otherwise stated, used in all subsequent experiments. This isolate on digestion with Xba I (Fig. 23g) showed no significant differences between the total cellular and encapsidated viral DNA samples.

Digestion with EcoR I (Fig. 23h) confirmed that the long region of HG52X19 is fixed in the prototype orientation. The long terminal fragment on digestion with EcoR I is f and the two f containing joints are b and d. HG52X19 packaged DNA, on digestion with EcoR I, exhibited slightly more f than normal but no b and d bands although both bands are present in concatameric DNA. The remaining joint fragments containing the internal long repeat fragment, EcoR I h, (ie

Figure 23(g+h)

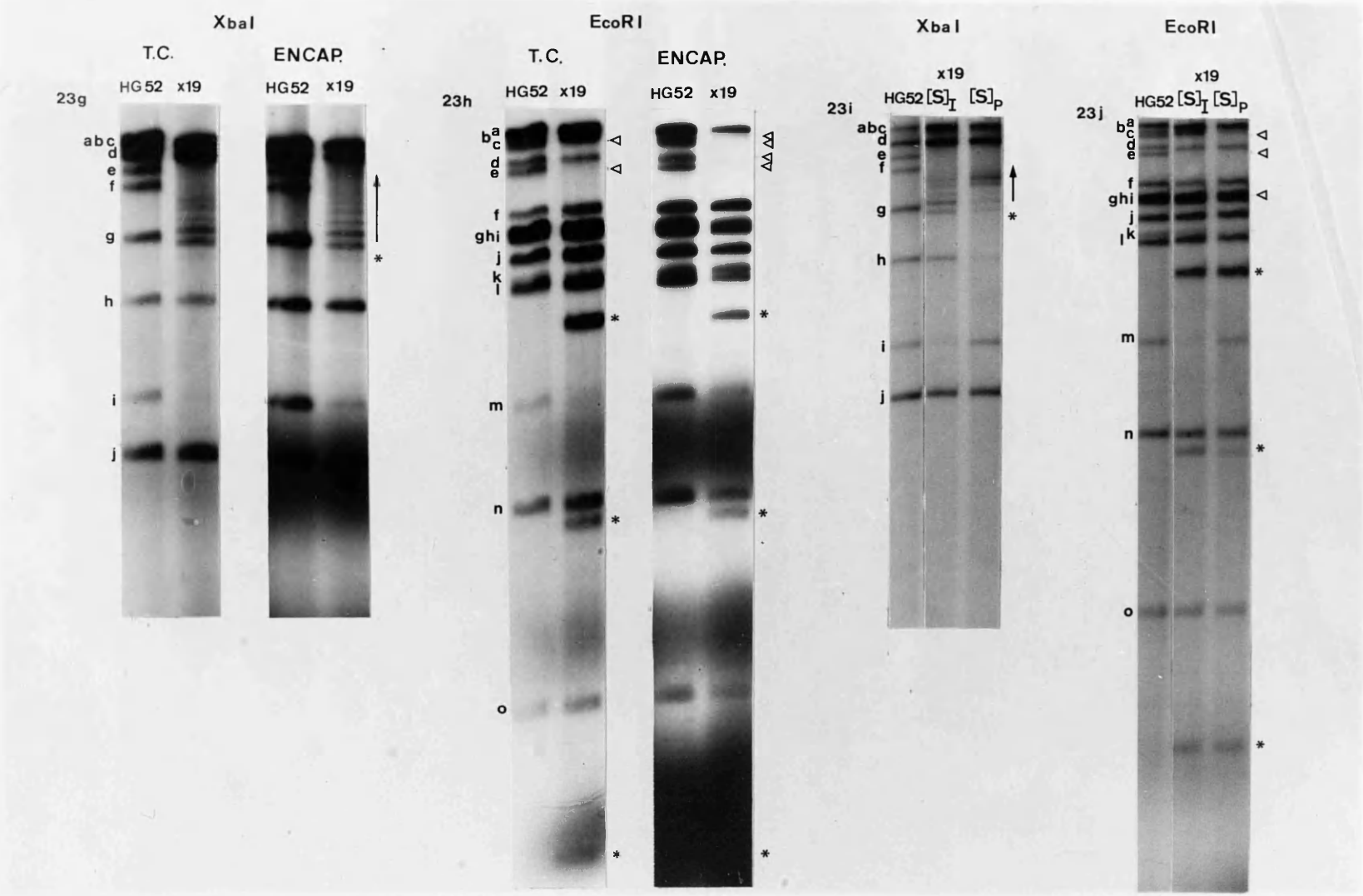
Autoradiographs of restriction endonuclease digests of total cellular (left hand pair) and encapsidated (right hand pair) viral DNA ^{32}P labelled *in vivo* of HG52 and the isolate of HG52X19 seen in Fig. 23(b) lane 1.
(g) Xba I digests and (h) EcoR I digests.

Letters refer to specific fragments, ↑* denotes a ladder of novel fragments, denotes missing fragment.

Figure 23(i+j)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* of HG52, an isolate of HG52X19 seen in Fig. 23(b) lane 1 with the short region predominantly in the inverted orientation and an isolate of HG52X19 seen in Fig. 23(b) lane 2 with the short region predominantly in the prototype orientation.
(i) Xba I digests and (j) EcoR I digests.

Letters refer to specific fragments, ◁ indicates missing fragment, * indicates novel fragment and ↑* denotes a ladder of novel fragments.


XbaI
T.C.
ENCAP.
23g
HG52 x19
HG52 x19
abc
d
e
f
g
h
i
j
*
EcoRI
T.C.
ENCAP.
23h
HG52 x19
HG52 x19
a
b
c
d
e
f
ghi
j
k
l
m
n
o
△
△
△△
*
XbaI
x19
23i
HG52 [S]I [S]P
abc
d
e
f
g
h
i
j
*
EcoRI
x19
23j
HG52 [S]I [S]P
a
b
c
d
e
f
ghi
j
k
l
m
n
o
△
△
△
*

c(h+k) and e(h+m)) were also absent from both encapsidated and total cellular DNA. This is due to the previously indicated deletion of sequences from the internal end of the long segment causing altered mobility of these joint fragments. As was shown from the Xba I digests, the short region is predominantly in the inverted orientation with EcoR I m being less and EcoR I k more than half molar. Unlike the Xba I profile, however, the EcoR I digest did not exhibit any laddering of different joint sizes but, three discrete novel bands : one of approximately $6x10^6$ daltons running between l and m; one of approximately $1.8x10^6$ daltons below n and one of approximately $0.6x10^6$ daltons below o.

To explain the ladders seen on digestion with Xba I and Hind III, it is proposed that in place of the deletion from IR_L, HG52X19 contains a sequence which is reiterated various numbers of times to give a heterogeneous genome population of different sizes depending on the numbers of reiterations. To account for the finding that digestion with EcoR I does not produce ladders of different sized fragments, the reiterated sequence must contain an EcoR I site. If the reiteration was of constant size then the EcoR I site would be repeated at regular intervals, thereby, forming fragments of constant size. Digestion with EcoR I would, therefore, create four novel bands: (1) comprising the remainder of the long internal fragment, EcoR I h, coupled with the reiterated portion up to the first EcoR I site; (2) a band containing only reiterated sequences; (3 and 4) the two remaining bands containing the portion of the reiterated sequence after the final EcoR I site plus either EcoR I m or less commonly EcoR I k. If this proposal is correct, then it

would follow that a different EcoR I profile would be seen in isolates with the short region in the prototype orientation. Figure 23i shows an Xba I digest of two isolates of HG52X19 compared with HG52. The first isolate $[S]_I$ which has already been described in detail has the short region predominantly in the inverted orientation (ie Xba I h is the major short end and i occurs less commonly as an end). The opposite orientation is demonstrated by the second HG52X19 isolate $[S]_P$ in which Xba I h is less commonly seen as an end compared to i. When digested with EcoR I (Fig. 23j) these two isolates show a number of bands with altered intensity. The isolate $[S]_I$ with the short region predominantly in the inverted orientation exhibited a pattern as described previously in which c and e and, therefore, presumably the h band, are all missing. The inverted orientation of the short region is confirmed by the presence of more EcoR I k than m. The EcoR I b(f+k) band is also increased, indicating the presence of a lot of concatameric DNA in this sample and, although it is hard to detect, it would be expected that the amount of d(f+m) would be reduced due to the inverted orientation of the short region. The three novel EcoR I bands described previously are also apparent (one of approximately $6x10^6$ daltons, one of approximately $1.8x10^6$ daltons and one of approximately $0.6x10^6$ daltons). The second isolate $[S]_P$ which has a short region predominantly in the prototype orientation, shows an EcoR I profile with reduced intensities of the b and k bands, whereas, m appears more intense. The d band would be expected to be increased in intensity although this is not detectable. The three novel bands described for the other isolate are all present. The $0.6x10^6$ and the $6x10^6$ dalton

bands are detectable in more or less the same quantities in both isolates, but the $1.8x10^6$ dalton band is clearly reduced in intensity in the second isolate $[S]_p$. The fact that this band is more intense in the isolate with a predominantly inverted short region suggests that it contains information from EcoR I m. If the band of $1.8x10^6$ daltons is a novel fragment composed of insert material plus EcoR I m, then it can be calculated that the size of the fourth proposed novel fragment composed of insert material plus EcoR I k should be approximately $5x10^6$ daltons and should be above m. The size of the proposed novel band composed of the remainder of EcoR I h plus insert sequences would also be expected to be in the region of $5x10^6$ daltons. As only one novel band is observed in this region, it is proposed that the band of $6x10^6$ daltons comprises two separate fragments; k plus insert and h plus insert. The novel band which is thought to be composed of EcoR I m plus insert is less than half the size of m, indicating that the deletion from IR_L must extend across the L/S junction to delete information from IR_S.

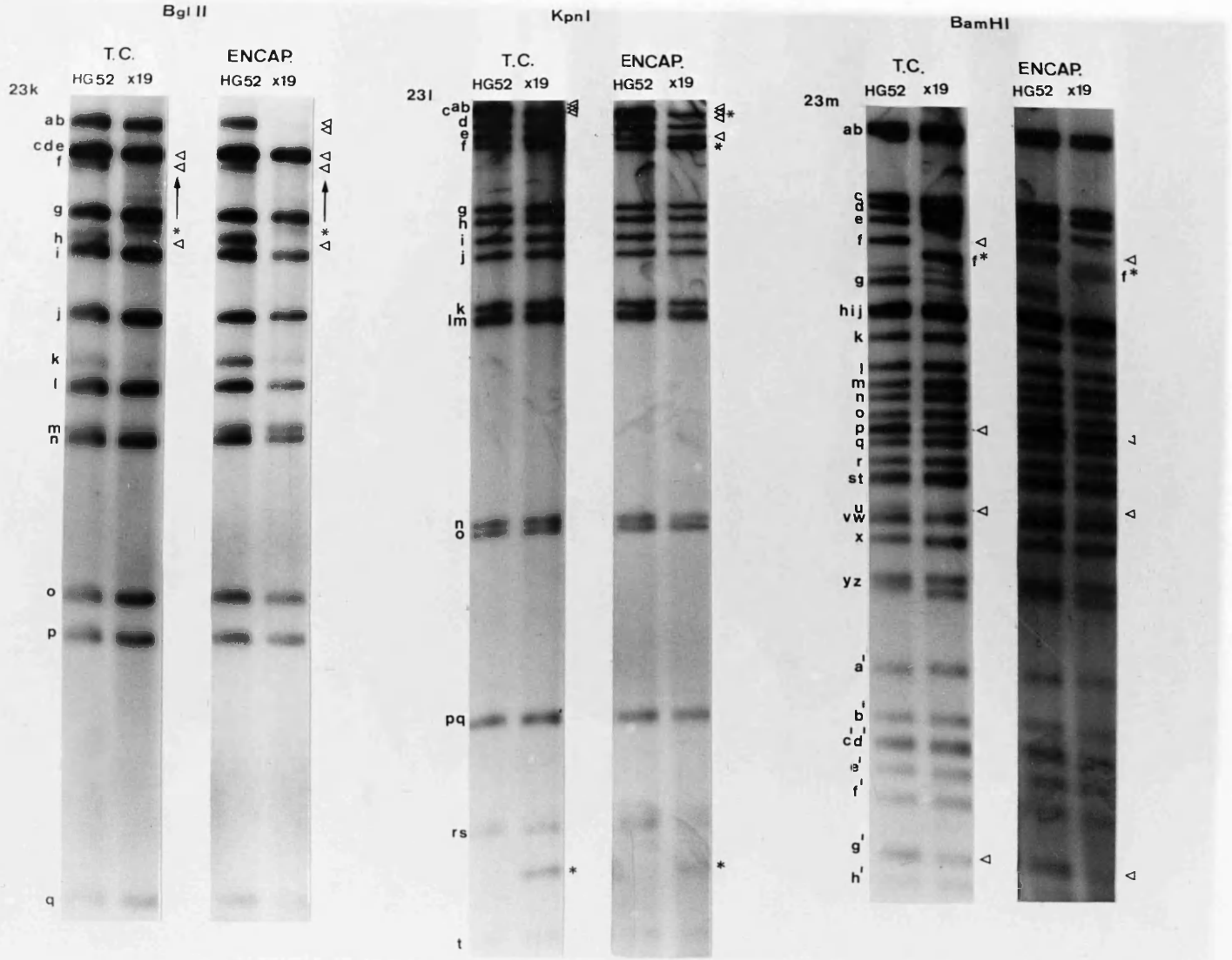
The data, therefore, indicate that HG52X19 has a deletion removing DNA from the internal repeat region spanning the IR_L/IR_S junction. Instability is introduced as the result of a compensatory insert of DNA sequences which are reiterated variable numbers of times to give genomes of differing sizes. The presence of ladders on digestion with Xba I (Fig. 23g) and with Hind III (Fig. 23e) but not with EcoR I (Fig. 23h) supports the view that the insert contains an EcoR I site but not an Xba I or Hind III site. The size of the Hpa I fragments (Fig. 23f) does not allow similar deductions to be made.

The Bgl II profile of HG52X19 DNA in Fig. 23k shows, as expected, that the long region is fixed in the prototype orientation. The two joint fragments containing the long terminal fragment d (ie a(d+k) and b(d+m)) are both absent from the encapsidated DNA sample, whereas, they are present in the total cellular, concatameric DNA. The joint fragment e(h+k) runs with c and d, so that the absence of this band is difficult to detect but it is apparent that the other h containing joint, f(h+m), is missing from both the total cellular and encapsidated DNA samples due to the deletion of information from IR_L. The deletion is confirmed by loss of the Bgl II h band. The predominantly inverted orientation of the short region is shown by the reduced intensity of the k band compared to the increased intensity of the m band. This indicates that the more common joint fragment will contain k. The novel ladder of bands, running from below Bgl II g, therefore, represents novel e(h+k) and less commonly f(h+m) joints containing information from deleted h and k or m fragments plus reiterated insert DNA. The presence of ladders indicates the absence of a Bgl II site in the reiterated sequences.

A Kpn I digest of HG52X19 is shown in Fig. 23l. Due to the deletion from IR_L it would be expected that c and its joint b(c+r) would be missing from both total cellular and encapsidated DNA samples and from the fixed orientation of the long region it would also be expected that e(f+r) would be present in concatameric but not unit length (encapsidated) DNA. The deletion from IR_S would remove the internal copy of Kpn I r and a small part of a and would, therefore, create a novel band of the remnants of c and a plus variable numbers of reiterated inserts. Although the

Figure 23(k-m)

Autoradiographs of restriction endonuclease digests of total cellular (left hand pair) and encapsidated (right hand pair) viral DNA ^{32}P labelled <u>in vivo</u> of HG52 and the isolate of HG52X19 seen in Fig. 23(b) lane 1.
(k) Bgl II digests, (l) Kpn I digests and (m) BamH I digests. Letters refer to specific fragments, ◁ indicates missing fragment, * denotes novel fragment and ↑* indicates a ladder of novel fragments.


Bgl II
T.C.
ENCAP.
HG52 x19
HG52 x19
23k
ab
cde
f
g
h
i
j
k
l
m
n
o
p
q
Kpn I
T.C.
ENCAP.
HG52 x19
HG52 x19
23l
cab
d
e
f
g
h
i
j
k
lm
no
pq
rs
t
BamHI
T.C.
ENCAP.
HG52 x19
HG52 x19
23m
ab
c
d
e
f
g
hij
k
l
m
n
o
p
q
r
st
u
vw
x
yz
a'
b'
c'
d'
e'
f'
g'
h'

size of these novel bands would probably preclude the detection of any laddering, they should be clearly seen at the top of the gel. The absence of such bands leads to the conclusion that there is a Kpn I site within the reiterated sequence. This conclusion is supported by the presence of an apparently molar, novel band of approximately $1x10^6$ daltons seen between s and t. There are, therefore, three novel fragments created: (1) composed of the remainder of c plus insert sequences up to the Kpn I site which is seen between where b and c would normally run. (2) composed entirely of reiterated insert of approximately $1x10^6$ daltons and which runs above t and (3) composed of insert material after the final Kpn I site plus the remainder of a which is seen running with Kpn I f.

A BamH I digest of HG52X19 provides additional information. It can be seen in Fig. 23m that BamH I f no longer runs in its normal position, one copy of p is missing as is one copy of g' and it is probable that one copy of BamH I u is also lost. Unfortunately, the m' fragments have run off the gel so it is not possible to determine if the deletion also removes one copy of m'. The change in the mobility of BamH I z is probably normal variation as it is a known variable band and if the deletion affects z then a' should also be deleted in the other orientation of the short, which is not apparent. The band above h, j and i in the total cellular DNA is not explained but may be due to partial digestion. The deletion removes at least part of f, all of p, v and u and all or part of g' and m'. It, therefore, removes between $7x10^6$ and $9x10^6$ daltons and even the apparently more stable structures seen in Fig. 23c contain an insert of at least $1x10^6$ daltons (ie at least one

copy of the insert).

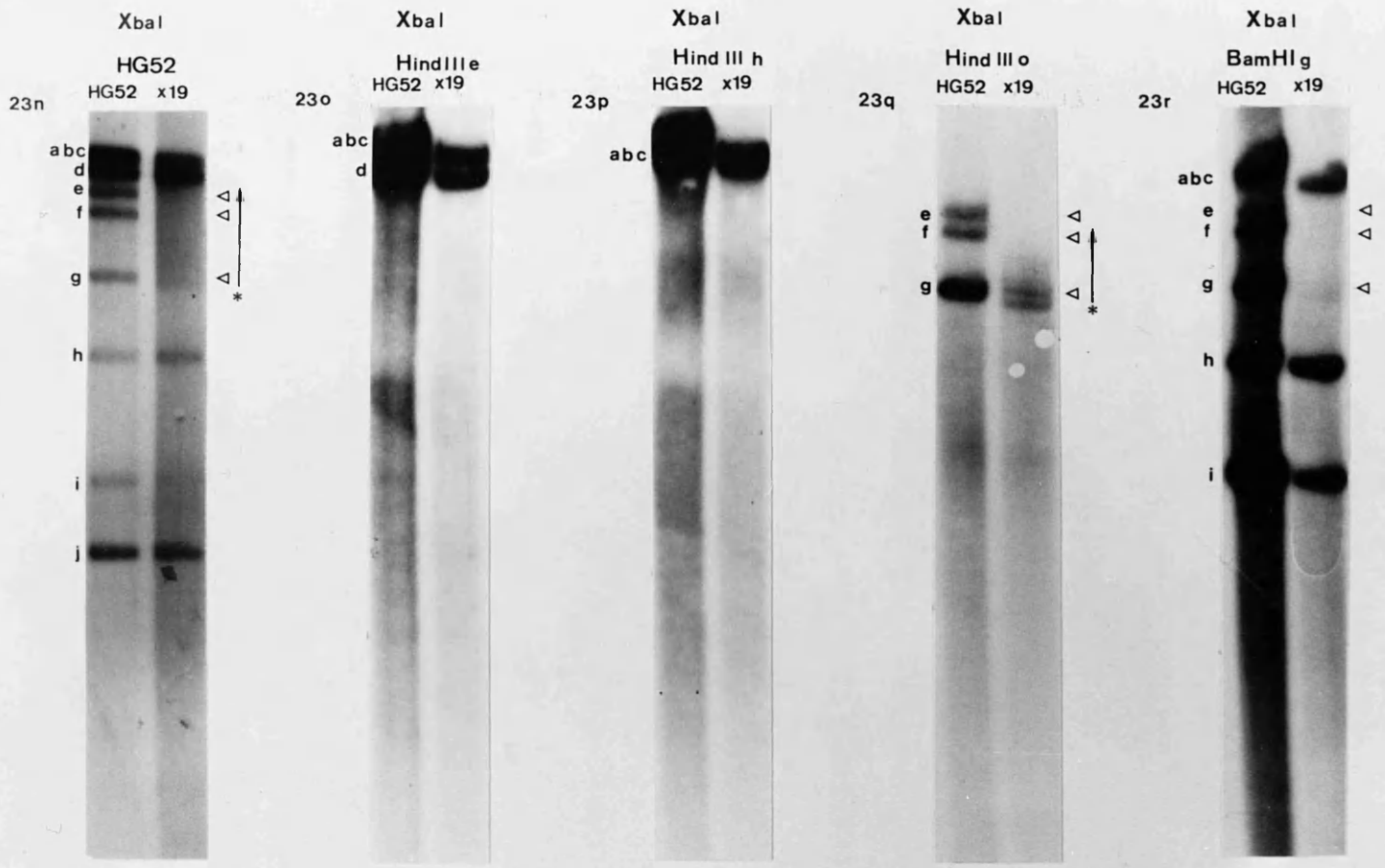
The large number of bands produced following BamH I digestion of HG52X19 means that it might be very difficult to detect the presence of a ladder of novel bands which would be generated if there were no BamH I site within the insert. If the insert DNA did contain a BamH I site digestion with BamH I should yield three novel bands, assuming that the deletion from IR_S is confined to the repeat region not extending beyond BamH I m'. The first would be composed of the remainder of f plus the insert sequences up to the first BamH I site, the second would be composed entirely of reiterated insert sequences and the third would contain insert sequences after the final BamH I site plus the remainder of the partially deleted BamH I g' or m' fragment. It is not possible to distinguish between these two possibilities on the basis of the BamH I digest.

Assuming that the insert was liable to be HSV DNA from another part of the genome, the obvious approach was to locate regions of the genome which, within a short stretch of DNA, contain an EcoR I and a Kpn I but no Hind III, Bgl II or Xba I sites. Only two regions, at 0.31m.c. and 0.42m.c., aparently fit the criteria. These regions which were used as probes in Southern blotting experiments are contained within Hind III h and e respectively. Figure 23o shows a Southern blot in which Xba I digested HG52 and HG52X19 DNA samples were probed with Hind III e and compared with Xba I digested HG52 and HG52X19 DNA probed with HG52 (Fig. 23n). Hind III e hybridizes to the wild type fragments c and d and the c containing joints a and b. The pattern of hybridization to HG52X19 is very similar although the quantities of a and b present depend on the proportion of

Figure 23(n-r)

Autoradiographs of nitrocellulose blot strips containing Xba I restriction fragments of HG52 and the isolate of HG52X19 seen in Fig. 23(b) lane 1 to which nick-translated HSV-2 DNA probes have been hybridized. The probes were (n) HG52, (o) a recombinant plasmid, pGZ14, containing Hind III e, (p) a recombinant plasmid, pGZ15 containing Hind III h, (q) a recombinant plasmid, pGZ13, containing Hind III o and (r) a recombinant plasmid pGZ1 containing BamH I g.

Letters refer to specific fragments, ◁ denotes missing fragment, ↑* indicates a ladder of novel fragments.


Xbal
HG52
23n
HG52 x19
abc
d
e
f
g
h
i
j
Xbal
Hind III e
23o
HG52 x19
abc
d
Xbal
Hind III h
23p
HG52 x19
abc
Xbal
Hind III o
23q
HG52 x19
e
f
g
Xbal
BamHI g
23r
HG52 x19
abc
e
f
g
h
i

concatameric DNA in the preparation. There is no evidence of hybridization of Hind III e to the novel Xba I joint fragments.

Figure 23p shows an Xba I digest of HG52 and HG52X19 probed with Hind III h. Hind III h only hybridizes to the wild type fragment c and its joints a and b. The pattern of hybridization of Hind III h to Xba I digested HG52X19 indicates that hybridization only occurs with Xba I c or a and b in concatameric DNA. There is no indication of any hybridization to the novel joint fragments.

Figure 23q shows an Xba I digest of HG52 and HG52X19 probed with Hind III o. Hind III o hybridizes to the wild type Xba I g and g containing joint fragments e and f. With the Xba I digest of HG52X19, however, there is positive hybridization only to the ladder of novel bands supporting the view that they contain information from the right hand end of U_L plus reiterated sequences.

Figure 23r shows an Xba I digest of HG52X19 and HG52 probed with BamH I g. BamH I g is a joint fragment comprising the long terminal repeat fragments v and the short terminal repeat fragments u. Therefore, it hybridizes to all the Xba I end fragments c, g, h and i and their joints a, b, e and f. The pattern on hybridization to HG52X19 is quite different. Because of the fixed orientation of the long segment, the two c containing joints, a(c+h) and b(c+i), are only present in concatameric DNA. The reduction in the quantities of a and b will be indirectly proportionate to the amount of c. However, as a, b and c comigrate the relative quantities of these fragments cannot be determined. The Xba I h and i bands in HG52X19 run in their normal positions although the i band, as expected, is

reduced in intensity due to the predominantly inverted orientation of the short region. The lack of any hybridization of BamH I g to the ladder of novel bands confirms that the sequences contained in BamH I v and u are not present in the novel Xba I joint fragments and must have been deleted. There must, however, be a more or less complete copy of the repeat present at the short terminus as the Xba I h and i fragments appear to be of normal size.

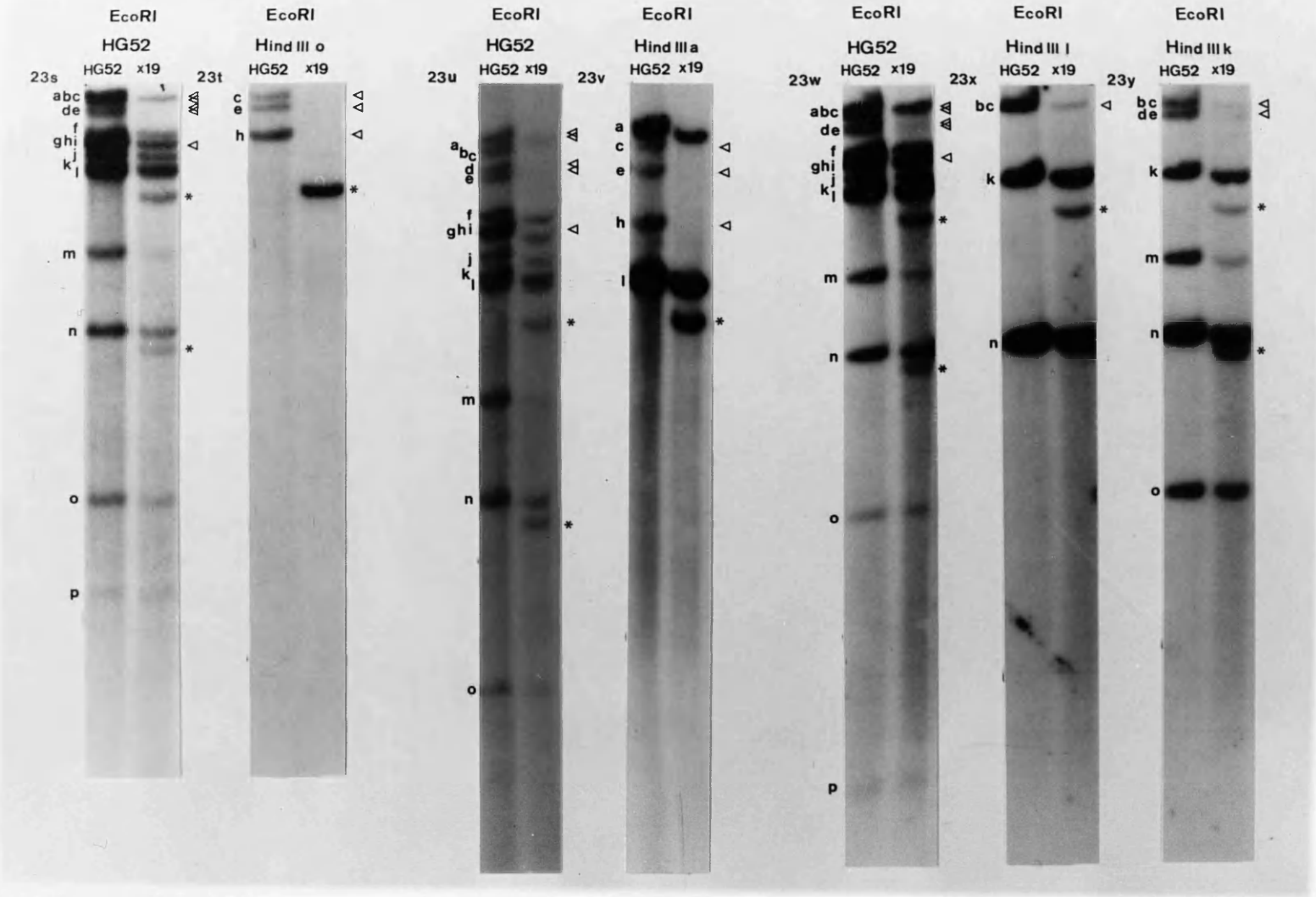
The data, therefore, whilst substantiating that the HG52X19 genome is deleted around the IR_L/IR_S junction and that there is an insert which is reiterated a variable number of times, ruled out the possibility that the reiterated sequence comes from either 0.31m.c. or 0.42m.c.. It was, therefore, decided to determine if the insert arose from another region of the HSV genome. Due to the limited availability of cloned HSV-2 fragments the available Hind III clones spanning most of the genome were used as probes. It has already been shown that there was no hybridization of Hind III e and h to the novel Xba I joint fragments. Hind III b also failed to hybridize (data not shown).

To ascertain the origin of the three novel EcoR I bands and because it was thought that hybridization would be easier to detect when no 'ladders' were present, the remaining Southern blots were performed using EcoR I digests of HG52 and HG52X19. Figure 23s shows EcoR I digests of HG52 and HG52X19 blotted onto nitrocellulose and probed with HG52. It has been demonstrated that Hind III o hybridizes to the novel Xba I joint fragments. Figure 23t shows the hybridization of Hind III o to EcoR I digested HG52 and HG52X19 samples. Hind III o hybridizes to the wild type

Figure 23(s-y)

Autoradiographs of nitrocellulose blot strips containing EcoR I restriction fragments of HG52 and the isolate of HG52X19 seen in Fig. 23(b) lane 1 to which nick-translated HSV-2 DNA probes have been hybridized. As the blots were from three different gels, tracks hybridized to HG52 probes are shown for each. The probes were (s) HG52, (t) the recombinant plasmid, pGZ13 containing Hind III o, (u) HG52, (v) the recombinant plasmid pGZ26 containing Hind III a, (w) HG52, (x) the recombinant plasmid, pGZ28 containing Hind III l and (y) the recombinant plasmid pGZ12 containing Hind III k.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


EcoRI
HG52
23s
HG52 x19
abc
de
f
ghi
j
k
l
m
n
o
p
EcoRI
Hind III o
23t
HG52 x19
c
e
h
EcoRI
HG52
23u
HG52 x19
a
bc
d
e
f
ghi
j
k
l
m
n
o
EcoRI
Hind III a
23v
HG52 x19
a
c
e
h
l
EcoRI
HG52
23w
HG52 x19
abc
de
f
ghi
j
k
l
m
n
o
p
EcoRI
Hind III l
23x
HG52 x19
bc
k
n
EcoRI
Hind III k
23y
HG52 x19
bc
de
k
m
n
o

EcoR I h fragment and its two joints c and e all of which are missing from HG52X19. There is hybridization, however, to the novel band of approximately $6x10^6$ daltons running below EcoR I l.

Figure 23u shows HG52 and HG52X19 digested with EcoR I and probed with HG52 (from the same experiment as the blot using the Hind III a probe). As can be seen in Fig. 23v Hind III a hybridizes to the wild type EcoR I bands a, l and h and to the h containing joints c and e (it should also hybridize to EcoR I p which has run off the gel). The HG52X19 profile shows normal hybridization to EcoR I l and a. As expected, however, the EcoR I h fragment and its joints c and e are not detected. In addition there is hybridization to the novel band of approximately $6x10^6$ daltons running below l.

The probes Hind III o and a, therefore, indicate the presence of information from the right hand end of the long unique region in the novel EcoR I band of approximately $6x10^6$ daltons.

Figure 23w shows a blot of HG52 and HG52X19 probed with HG52 (from the same experiment as the Hind III l and k blots). Figure 23x shows an EcoR I digest of HG52 and HG52X19 probed with Hind III l. Hind III l hybridizes to the wild type bands n and k and to the k containing joint fragments b and c. Hybridization to the HG52X19 EcoR I k and n bands is normal. EcoR I b is not generated in HG52X19 except in concatameric DNA and EcoR I c is not present due to the deletion of material from IR_L. However, there is hybridization to the novel band of approximately $6x10^6$ daltons below EcoR I l.

Figure 23y shows an EcoR I digest of HG52 and HG52X19

probed with Hind III k. Hind III k hybridizes to the wild type fragments EcoR I n, o and m and to k due to the inverted repeat sequences. Consequently, it also hybridizes to all four joint fragments b, c, d and e. The HG52X19 profile shows hybridization to o, n, m and k although m is reduced in intensity due to the predominantly inverted orientation of the short region. The two joint fragments b and d should only be present in concatameric DNA and are not detected. The two remaining joint fragments, c and e, are not seen due to the deletion of material from IR_L. There is hybridization to two novel EcoR I bands, one of approximately $6x10^6$ daltons below l and more strongly to the band of approximately $1.8x10^6$ daltons below n.

The fact that there is hybridization of probes Hind III a, o, l and k to the band of approximately $6x10^6$ daltons supports the restriction endonuclease analysis of HG52X19 isolates with both orientations of the short region (Fig. 23j) which indicated the presence of two fragments of approximately $6x10^6$ daltons, one composed of the remnant of EcoR I k plus insert and the other composed of the remnant of EcoR I h plus insert. The remnant of EcoR I h would hybridize to both Hind III a and o whilst both Hind III l and k would hybridize to the remnant of EcoR I k. The Southern blot analysis also showed that the fragment which hybridized to Hind III a and o is more abundant than that recognized by the Hind III l and k probes. This would be expected as the EcoR I h containing fragment, due to the fixed orientation of the long, would give a molar band, whereas, the EcoR I k fragment would be less than half molar due to the predominantly inverted orientation of the short region. Hybridization of Hind III k to the novel EcoR I band

of approximately $1.8x10^6$ daltons would be expected as it has been shown that this band is composed of the remnant of EcoR I m plus insert sequences and hence the information contained within the repeat would allow hybridization to the probe.

The lack of any positive hybridization to the $0.6x10^6$ dalton EcoR I fragment composed entirely of insert sequences, especially with the Hind III e and h probes, which from the available maps contain the correct combination of restriction enzyme sites, led to the conclusion that either the insert is non-HSV-2 in origin or that non-contiguous HSV DNA which is not recognized by the probes has been inserted.

The similarity of the observed 'ladders' to those seen in defective genomes (Kaerner et al., 1981) led to the hypothesis that the inserted sequences might contain an origin of replication. The region at 0.42m.c. which has the correct distribution of restriction enzyme sites to constitute the insert also contains ori_L. It is known that ori_L, a 144bp perfect palindrome, deletes out when cloned (Weller et al., 1985). This might have accounted for the failure of the cloned fragment, Hind III e (0.4-0.52m.c.) to detect insert material if it contained ori_L. This explanation seemed unlikely as the flanking sequences should still have had sufficient homology to allow hybridization. However, to test the hypothesis an EcoR I digest of HG52X19 was probed with an uncloned fragment of DNA containing ori_L. The fragment used was a Hind III/ Xba I e* fragment (0.4-0.45m.c.) which was cut and eluted from a gel. As it is known that such probes are prone to contamination from other fragments, the specificity of the probe was checked by

hybridization to a BamH I digest of HG52 and HG52X19. Figure 23z shows a BamH I digest of HG52 and HG52X19 probed with HG52. Figure 23a' shows the same digests probed with the Hind III/ Xba I e* fragment. The fragment should only hybridize to BamH I b, h', j' and o. It can be seen that, whilst the specificity is poor, the major constituent of the probe is confirmed as being the intended Hind III/ Xba I e* fragment. Figure 23b' shows an EcoR I digest of HG52 and HG52X19 with a wild type probe. The poor specificty of the Hind III/ Xba I e* probe was again demonstrated when it was hybridized to an EcoR I digest of HG52 and HG52X19 (Fig. 23c'). The probe should hybridize only to the wild type EcoR I a and g fragments but, whilst hybridization to these bands does constitute the major portion, there is hybridization to a number of other bands. The pattern of hybridization to HG52X19 was essentially the same in that the major bands detected were EcoR I a and g. There was no hybridization to the novel band of 0.6×10^6 daltons thought to be made up entirely of insert sequences. The small amount of hybridization to the novel bands of 6×10^6 and 1.8×10^6 daltons is probably not significant as there appears to be a low degree of cross reaction with the EcoR I k and m bands in the wild type track.

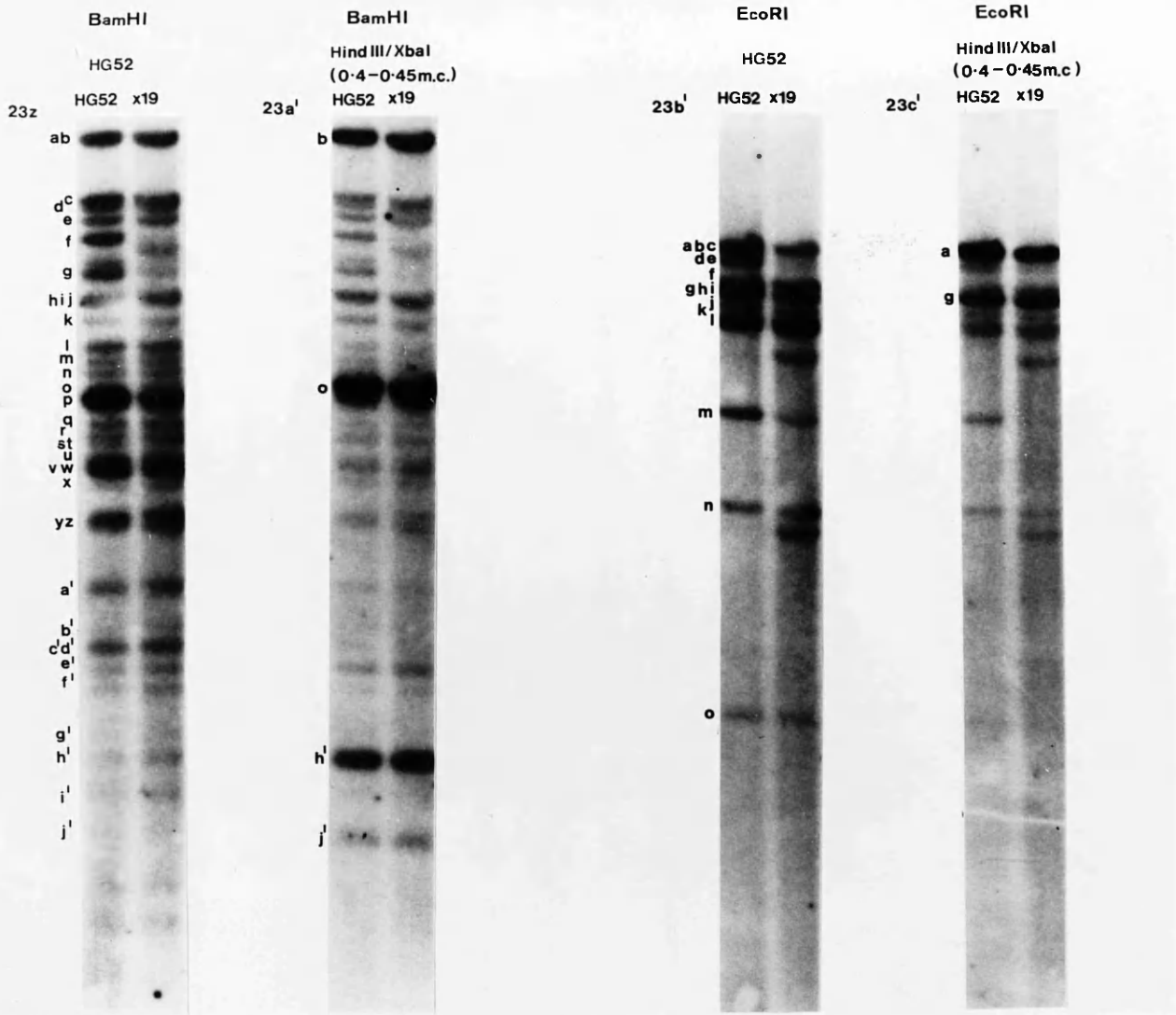
HG52X19 has been shown to have a deletion removing all of IR_L and at least half of IR_S. The deleted portion would, therefore, remove the internal copies of the 'a' sequence making inversion less efficient. The short region does invert, albeit inefficiently, probably by a mechanism involving the remaining inverted repeat sequences. The long region is only packaged in the prototype orientation indicating that all of the internal long repeat sequences

Figure 23(z-c')

Autoradiographs of nitrocellulose blot strips containing restriction endonuclease fragments of HG52 and the isolate of HG52X19 seen in Fig. 23(b) lane 1 to which nick-translated HSV-2 probes have been hybridized. (z and a') BamH I digests and (b' and c') EcoR I digests. The probes were (z and b') HG52 and (a' and c') a

Hind III/Xba I fragment spanning the region from the Hind III h/e site at 0.4m.c. to the Xba I c/d site at 0.45m.c.

Letters refer to specific fragments.


BamHI
HG52
23z
HG52 x19
ab
dc
e
f
g
hi j
k
l
m
n
o
p
q
r
st
u
v
w
x
yz
a'
b'
c'
d'
e'
f'
g'
h'
i'
j'
BamHI
Hind III/Xbal
(0·4–0·45m.c.)
23a'
HG52 x19
b
o
h'
j'
EcoRI
HG52
23b'
HG52 x19
abc
de
f
ghi
j
k
l
m
n
o
EcoRI
Hind III/Xbal
(0·4–0·45m.c)
23c'
HG52 x19
a
g

have been deleted, thereby ruling out even the less efficient inversion of the type seen in the short region. The data also show that in place of the deleted sequences there is material inserted which is reiterated a variable number of times to create genomes of different lengths (Fig. 23d'). The reiterated portion of DNA contains an EcoR I and a Kpn I site but no Hind III or Xba I site. A model showing the proposed derivation of the observed novel restriction enzyme fragments is shown in Fig. 23e'.

The Southern blotting data obtained using a range of Hind III probes covering almost the entire length of the genome, whilst supporting the proposed model of the structure of HG52X19, did not provide any information as to the origin of the insert sequences. Although the novel EcoR I band of approximately 0.6×10^6 daltons is clearly observed in EcoR I digests of ^{32}P labelled HG52X19 DNA (eg Fig. 23j) it was never seen to hybridize to any of the probes including intact HG52 in Southern blotting experiments. This suggests that the inserted DNA is not of HSV-2 origin or at least that the insert cannot be a simple insert of 0.6×10^6 daltons of normal HSV DNA.

To try and determine the origin of the insert sequences the 0.6×10^6 dalton EcoR I fragment of HG52X19, thought to be entirely insert DNA, was cut and eluted from a gel and used to make a nick-translated probe against a BamH I digest of HG52 and HG52X19 DNA.

Figure 23f' shows EcoR I digests of HG52 and HG52X19 probed with HG52. In Fig.14g' the same digests have been probed with nick-translated HG52X19 insert DNA (ie 0.6×10^6 dalton EcoR I fragment). Once again it can be seen that the HG52 probe fails to recognize the 0.6×10^6 dalton band of

Figure 23(d')

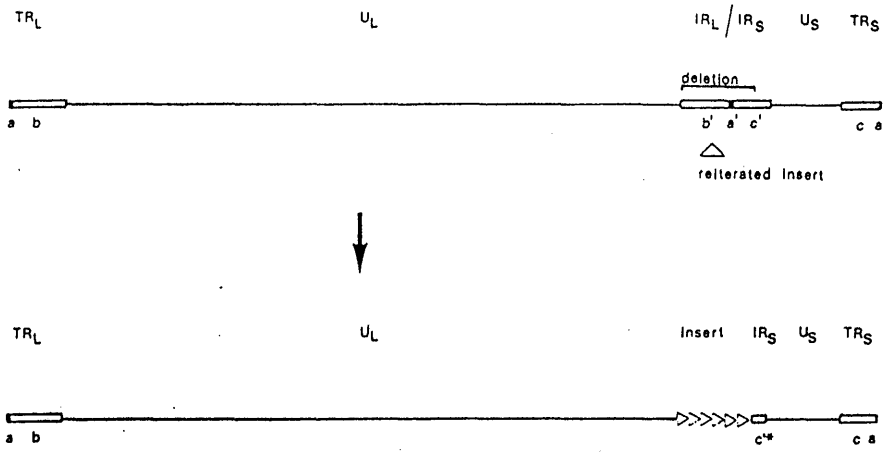
Diagram showing the proposed novel structure of HG52X19 due to the deletion of material from IR_L/IR_S and the accompanying reiterated insert. Only one example novel genome is depicted with six reiterations (indicated ▷) of the insert although it is thought that the number ranges between 1 and 14 copies giving genomes of approximately $6x10^6$ daltons greater or less than the wild type.

Figure 23(e')

Diagram showing the model proposed to account for the novel restriction endonuclease fragments. The left hand column shows the effect on specific restriction fragments of the deletion of IR_L and half of IR_S when the short region is in the prototype orientation. Novel fragments are marked * to denote the deletion of sequences. The variable number of inserts is represented in each case by a single example of six reiterations which are depicted as ▷. The presence of a restriction endonuclease site within the insert is represented by ▷. The second column shows an example of the range of fragments created by the presence of between one and six copies of the insert with their approximate molecular weights. In the cases where the insert contains a particular restriction endonuclease site the novel fragments created containing information from the deleted fragment plus insert DNA up to the restriction site are depicted as ending ▽ to signify the presence of part of the insert at the end of the fragment. When the insert containing a restriction site is reiterated a novel fragment is created which is composed entirely of insert DNA and which is the same size as the insert but which cannot be represented ▷ as it does not start and end at the same positions. These fragments are, therefore, represented ⋀.

The remaining two columns represent similar examples created when the short region is in the inverted orientation. In the case of Hpa I, Kpn I and BamH I the orientation of the short region does not make any difference to the size of the novel bands.

23d'


TRL
UL
IRL / IRS
US
TRS
deletion
a b
b' a' c'
c a
reiterated insert
TRL
UL
Insert
IRS
US
TRS
a b
c'*
c a

23e'

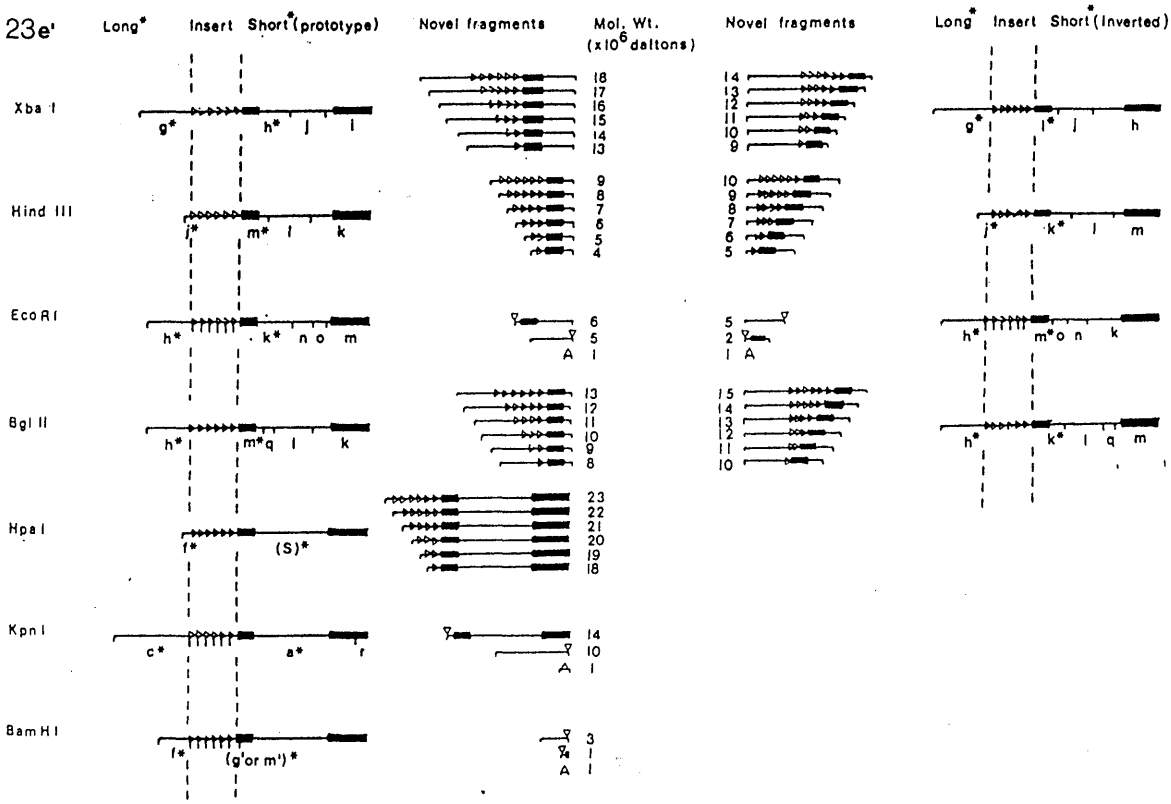

Long*
Insert
Short*(prototype)
Novel fragments
Mol. Wt. (x10^6 daltons)
Novel fragments
Long*
Insert
Short*(Inverted)
Xba I
Hind III
EcoRI
Bgl II
Hpa I
Kpn I
BamHI
(S)*
(g'or m')*

Figure 23(f'+g')

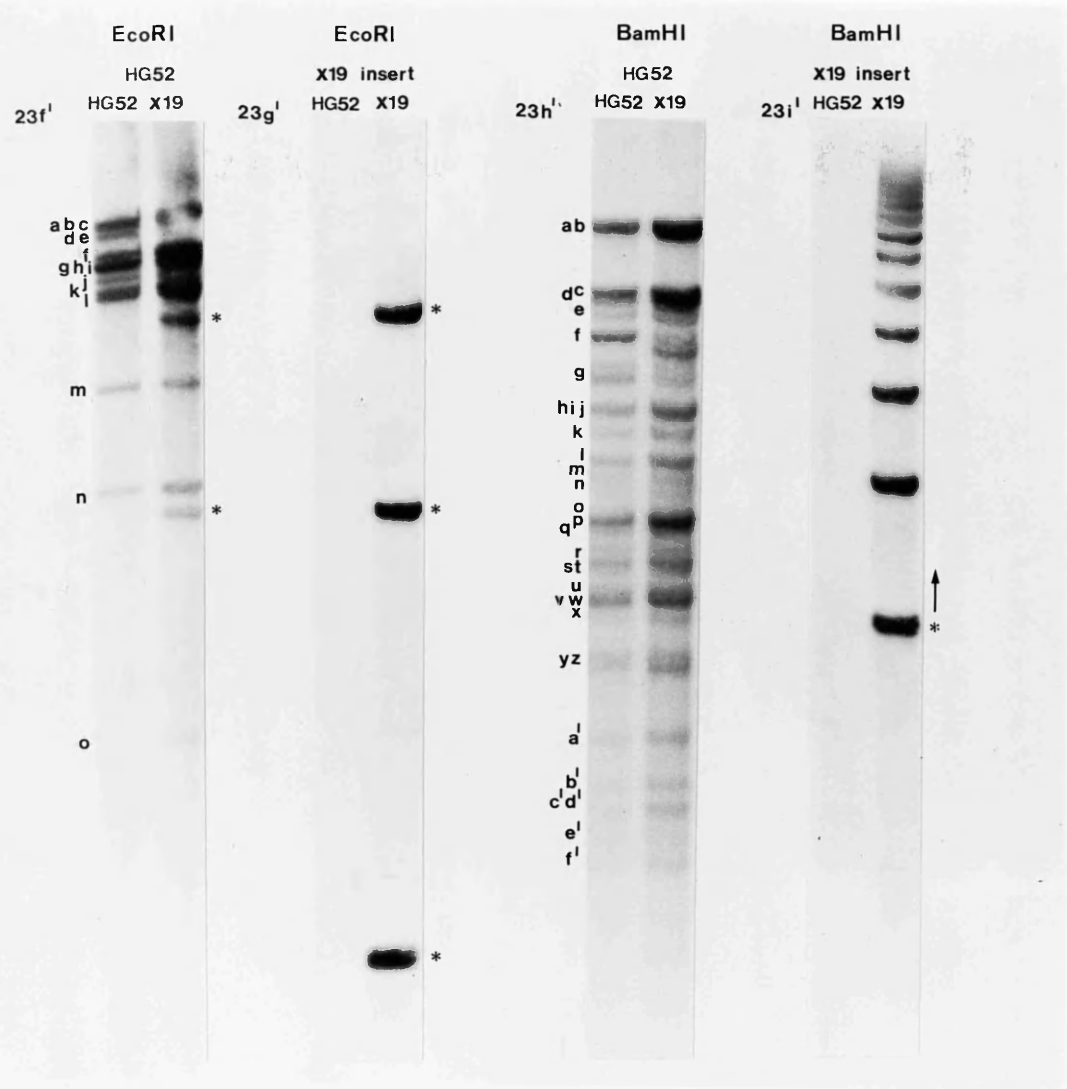
Autoradiographs of nitrocellulose blot strips containing EcoR I restriction fragments of HSV-2 HG52 and HG52X19 DNA to which DNA probes have been hybridized. The probes were HG52 (f') and the insert ($0.6x10^6$ dalton) EcoR I fragment of HG52X19 (g').

Letters refer to specific fragments, * indicates novel fragment.

Figure 23(h'+i')

Autoradiographs of nitrocellulose blot strips containing BamH I restriction fragments of HSV-2 HG52 and HG52X19 DNA to which DNA probes have been hybridized. The probes were HG52 (h') and the $0.6x10^6$ dalton EcoR I insert fragment of HG52X19 (i').

Letters refer to specific fragments, $\uparrow_{*}$ indicates a ladder of novel fragments.


EcoRI
HG52
23f'
HG52 X19
a b c d e f g h i j k l
m
n
o
EcoRI
X19 insert
23g'
HG52 X19
BamHI
HG52
23h'
HG52 X19
ab
dc e
f
g
hij
k
l m n
o p q
r st
u v w x
yz
a'
b' c' d'
e'
f'
BamHI
X19 insert
23i'
HG52 X19

EcoR I digested HG52X19 DNA indicating that the insert DNA is non-HSV-2 in origin. This conclusion is supported by the pattern of hybridization of the insert probe which htbridized to all three of the novel EcoR I bands of HG52X19 whilst failing to hybridize to any of the HG52 DNA fragments.

Figure 23h' shows BamH I digests of HG52 and HG52X19 probed with HG52. The large number of fragments means that it is difficult to tell if the insert in HG52X19 contains a BamH I site. The blot of the same digests probed with nick-translated HG52X19 $0.6x10^6$ dalton EcoR I insert fragment DNA (Fig. 23i') failed to show any hybridization to the wild type track supporting the conclusion that the insert is not composed of HSV-2 DNA. The ladder of bands observed when the insert probe was hybridized to BamH I digested HG52X19 DNA gave the first evidence that the insert DNA does not contain a BamH I site.

In order to eliminate the unlikely possibility that the insert was HSV-1 DNA a Southern blotting experiment was performed. EcoR I digested HSV-1, strain 17, and HG52X19 DNA were blotted onto nitrocellulose and probed with ^{32}P labelled nick-translated insert DNA (data not shown). There was hybridization only to the three EcoR I bands of HG52X19 containing insert material. The failure of the insert probe to hybridize to the digest of strain 17 DNA ruled out the possibility of the insert being HSV-1 in origin.

3.2g GROWTH CHARACTERISTICS OF VARIANTS ISOLATED AFTER ENRICHMENT SELECTION OF HG52

Single step growth experiments were performed comparing the growth of HG52X85/4, HG52X85/5, HG52X86, HG52X192 and

HG52X19 with the parental strain HG52 at 31°C, 37°C and 38.5°C (Fig. 24).

At 31°C all of the variants, except HG52X19, grew at a rate and to a final yield indistinguishable from the wild type strain. HG52X19 was significantly impaired both in terms of rate of growth and final yield.

At 37°C all the variants except HG52X19 grew at rates similar to HG52 and all gave yields at 24h which appeared to be slightly better than HG52. HG52X19 grew at a significantly slower rate and, due to a drop in titre after 12h, gave a 24h yield which was lower than the titre immediately post absorption.

At 38.5°C all of the variants except HG52X19 grew marginally better than HG52 which is itself slightly temperature sensitive. HG52X19 exhibited practically no growth at 38.5°C.

When an HG52X19 stock, grown at 31°C, was titrated at the same three temperatures there was no indication of temperature sensitivity.

	31°C Titre (pfu/ml)	37°C Titre (pfu/ml)	38.5°C Titre (pfu/ml)
HG52X19	5.3×10^7	7.0×10^7	5.4×10^7

It is evident that the virus produces plaques at 38.5°C although it grows very poorly at that temperature. The apparent drop in titre of HG52X19 between 12h and 24h especially at 37°C may indicate that the virus particles are more heat labile than the wild type.

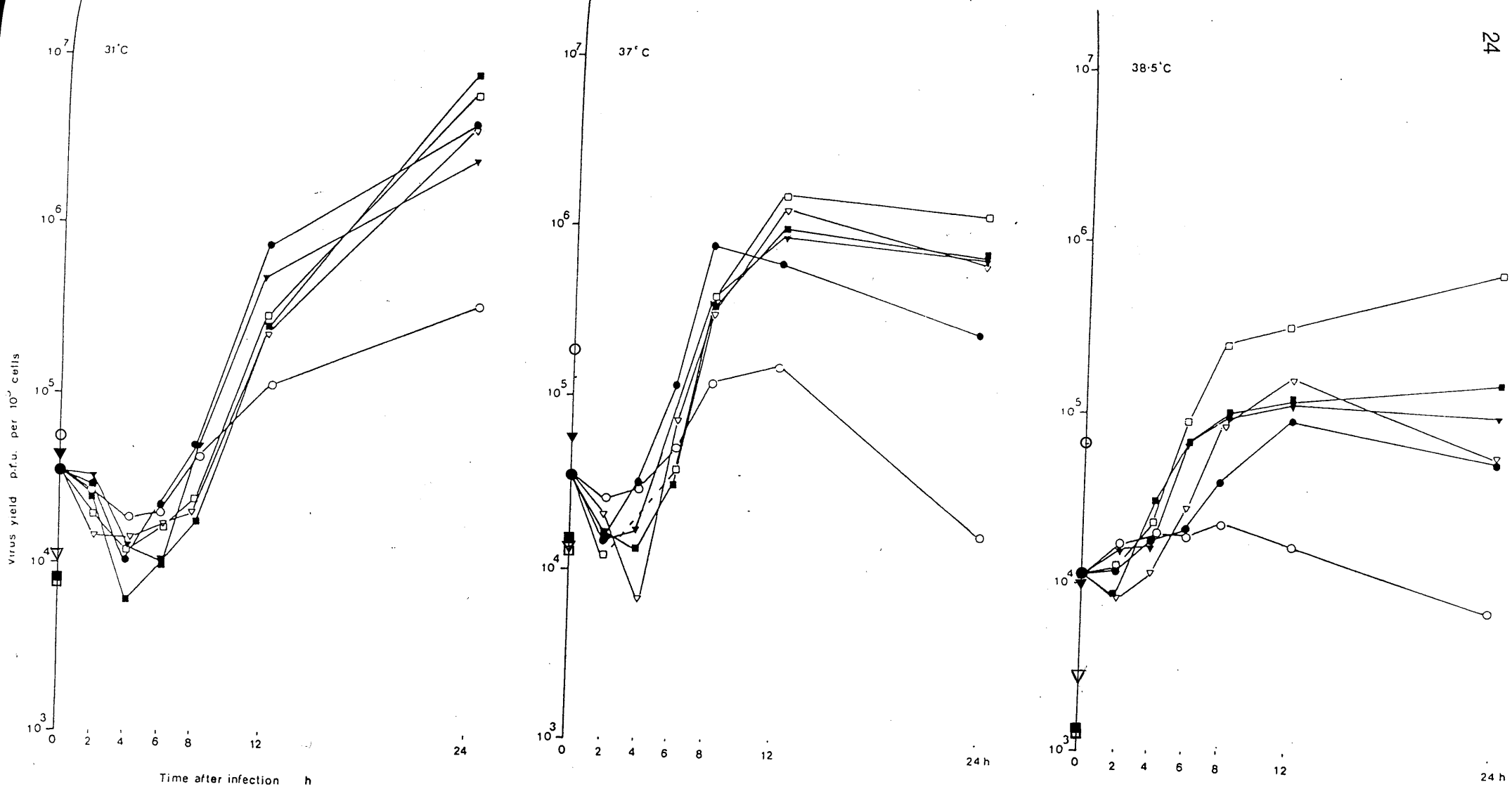
The particle: pfu ratios of all these variants are shown below:

HG52	1,263 : 1
HG52X85/4	332 : 1

Figure 24

One step growth curves of HSV-2, strain HG52●, HG52X19 ○, HG52X85/4 ■, HG52X85/5 □, HG52X86 ▼ and HG52X192 ▽ in BHK21/C13 cells at 31°, 37° and 38.5°C. Cells were infected at a MOI of 5pfu/cell. After absorption for 45min at the appropriate temperature, the monolayers were washed twice with phosphate-buffered saline, overlaid with ETC10% and incubated at the appropriate temperature. Cultures were harvested at the times indicated and virus titres measured by plaque assay on BHK21/C13 cells. To assist in comparisons of growth, the titres have all been standardized to the HG52 zero time titre.

To allow comparison zero hour samples were standardized to that of HG52. Actual zero hour values are marked on axis.


31°C
37°C
38·5°C
Virus yield p.f.u. per 10⁷ cells
Time after infection h
24 h

HG52X85/5	367 : 1
HG52X86	775 : 1
HG52X192	880 : 1
HG52X19	7,220 : 1

The particle: pfu ratio of the wild type stock was high but the equivalent figure for HG52X19 was almost six times as high. It is possible that the very high ratio of particles to infectious virus may have had some detrimental effect on the single cycle growth of the virus although the effect of this on the comparative growth curves might be expected to be minimized by the standardisation of the titres immediately post absorption (ie 0h). The very high particle: pfu ratio in HG52X19 is possibly more significant as a pointer to poor growth when the stock was prepared, another indication of generally poor replication. It was, however, possible to obtain reasonably high titre stocks of HG52X19 from low multiplicity infections at 31°C.

In conclusion, it is possible to obtain high titre stocks of all of these variants from low multiplicity infections in BHK21/C13 cells at 31°C. Furthermore, all of the variants with the exception of HG52X19 grew as well as or better than the parental HG52 in single cycle growth experiments at 31°C, 37°C and 38.5°C. HG52X19 appeared impaired in growth especially at the higher temperatures although it was not completely temperature sensitive as plaques were produced at 38.5°C.

3.2h INFECTED CELL POLYPEPTIDES INDUCED BY VARIANTS ISOLATED AFTER TRANSFECTION OF Xba I TREATED HG52 DNA (ie. HG52X85/4, HG52X85/5, HG52X86, HG52X192 and HG52X19).

Figure 25a shows general infected cell polypeptide

Figure 25a

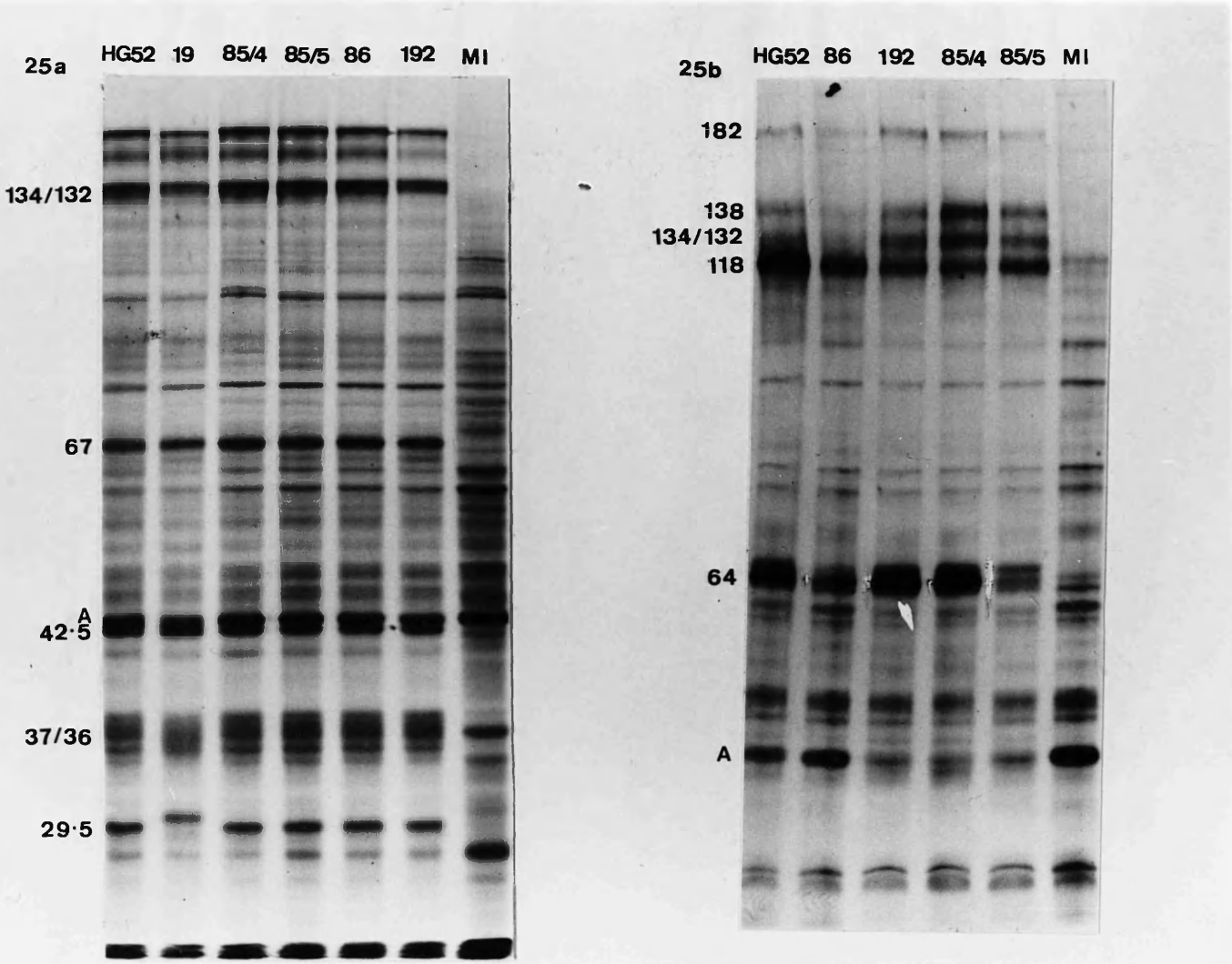
Autoradiograph of infected cell polypeptides induced in BHK21/C13 cells and separated by SDS-PAGE. Cells were infected at 38.5^{o}C, ^{35}S methionine labelled (3-20h) and polypeptides analysed on a 7.5% acrylamide gel. Lane 1 HG52, lane 2 HG52X19, lane 3 HG52X85/4, lane 4 HG52X85/5, lane 5 HG52X86, lane 6 HG52X192 and lane 7 mock infected.

Numbers show the apparent molecular weight (x10^{3} daltons) of HSV-2 infected cell polypeptides. A represents actin.

Figure 25b

Autoradiograph of immediate early infected cell polypeptides induced in BHK21/C13 cells at 38^{o}C. Cells were pretreated with cyclohexamide and RNA synthesis blocked at 5h PI by the addition of actinomycin D. Extracts were labelled with ^{35}S methionine for 1h at 5h PI and separated by SDS-PAGE on a 7.5% single concentration gel. Lane 1 HG52, Lane2 HG52X86, Lane 3 HG52X192, Lane 4 HG52X85/4, Lane 5 HG52X85/5 and Lane 6 mock infected control.

Numbers show the apparent molecular weight (x10^{3} daltons) of HSV-2 infected cell polypeptides. A represents actin.


25a
HG52
19
85/4
85/5
86
192
MI
134/132
67
A
42·5
37/36
29·5
25b
HG52
86
192
85/4
85/5
MI
182
138
134/132
118
64
A

extracts labelled with ^{35}S-methionine run on a denaturing gel. Under these conditions there is no apparent difference between the polypeptides induced by the variants HG52X85/4, HG52X85/5, HG52X96 and HG52Xl92 compared to the wild type HG52. Only one variant, HG52Xl9, differed noticably from the wild type in this analysis. HG52Xl9 exhibits alteration in the mobility of bands in the 36/37 kd molecular weight region and an apparent decrease in the mobility of the 29.5kd band.

Figure 25b shows ^{35}S-methionine labelled immediate early extracts of the variants HG52X86, HG52Xl92, HG52X85/4 and HG52X85/5 compared to the wild type HG52. HG52X85/4, HG52X85/5 and HG52X86 all have deletions affecting one copy of IEl. HG52Xl92 has deletions from both copies of the long repeat which do not extend to IEl and are in a region for which no coding has been convincingly identified.

The infections with HG52 and HG52X86 appear to be slightly poorer than those of HG52Xl92, HG52X85/4 and HG52X85/5 exhibiting less virus specific protein and more host cell protein (notably actin). The apparent variability in infection makes interpretation of the data difficult. For example, the quantities of VmwIEll8 of the three variants HG52X85/4, HG52X85/5 and HG52X86 are reduced as would be expected. However, the level of VmwIEll8 in HG52Xl92 which should produce a similar amount to HG52 also appears reduced. The most obvious alteration in the variant profiles is apparent in the levels of the IE gene 2 product VmwIE64. HG52Xl92 and HG52X85/4 both produce wild type amounts of VmwIE64 whilst HG52X85/5 and HG52X86 both exhibit a marked decrease in the quantity of the polypeptide.

3.3 VARIANTS ISOLATED FROM UNTREATED HSV

Variants with deletions from the long repeat regions occurred at a frequency of 3.5% after the first round of enrichment selection (ie 7 out of 200). This figure is probably an under estimate as the method of screening for site loss only involved analysis of Xba I digests which would preclude detection of deletions from TR_L. Although some (25%) were screened with EcoR I which would allow the detection of deletions from TR_L none was found. The Xba I c fragment is so large ($40x10^6$ daltons) that only very large deletions would cause appreciable changes in mobility. The structures of four of the seven deletion variants (ie HG52X19, HG52X85, HG52X86 and HG52X192) have been described in detail. The remainder all had smaller deletions from IR_L and were not analysed further.

To determine if the Xba I treatment of the DNA and its subsequent transfection was causing the observed high frequency of variation, the genome structures of individual plaque stocks of untreated HG52 and other HSV-2 and HSV-1 strains were analysed for alterations.

The HG52 field isolate was stringently plaque purified when originally isolated and subsequently passaged four times in BHK21/C13 cells (Timbury, 1971). The departmental elite stock (P4) was grown up once to give a working stock (P5). This stock was titrated on BHK21/C13 cells in medium containing 5% human serum and fifty well separated plaques were picked (P6). Virus from the individual plaques was grown on 50mm confluent monolayers of BHK21/C13 cells to give plate harvests (P7). The fifty recloned stocks were used to infect $5x10^5$ BHK21/C13 cells in the presence of ^{32}P for restriction enzyme analyses of their DNA (P8).

Recloned stocks of HSV-2 strains 333 and 186 and the HSV-1 strains Glasgow 17 and KOS were prepared in a similar manner except that plaques were picked from titrations of departmental elite stocks thereby omitting one passage. Fifty plaques each of strains 186 (P elite+3), 333 (P elite+3) and KOS (P elite+3) were analysed plus one hundred of Glasgow strain 17 (P elite+3).

Analyses were performed using Xba I and EcoR I to allow detection of deletions from both TR_L and IR_L. Of the fifty HG52 (P8) plaques analysed, twelve showed alterations compared to the archetypal profile. The twelve variants fell into two groups: (1) five, apparently identical, isolates represented by HG52 clone 5 (HG52/5) and (2) seven isolates which all appeared to be the same represented by HG52 clone 10 (HG52/10).

The variant HG52/5 (Fig. 26a) showed altered mobilities of the Xba I g, e and f fragments and (Fig. 26b) the EcoR I h containing joints c and e, indicative of a deletion from EcoR I h of $2\text{-}3 \times 10^6$ daltons. The deletion was confirmed by a BamH I digest (Fig. 26c) which showed a missing f fragment, only one copy of p and a novel fragment of approximately 4.4×10^6 daltons running above f. Overall, the data indicate a deletion of 2.5×10^6 daltons spanning the BamH I f/p site and possibly crossing the U_L/IR_L junction. The deletion appears, therefore, to be the same as that described for HG52X86 isolated following enrichment selection.

The variant HG52/10 (Fig. 27a) showed Xba I g and its joints e and f to be reduced in size consistent with a deletion of 1×10^6 daltons from Xba I g. The EcoR I digest (Fig. 27b) supported this, showing novel EcoR I h containing joints c and e reduced by similar amounts. However, the

Figure 26(a-c)

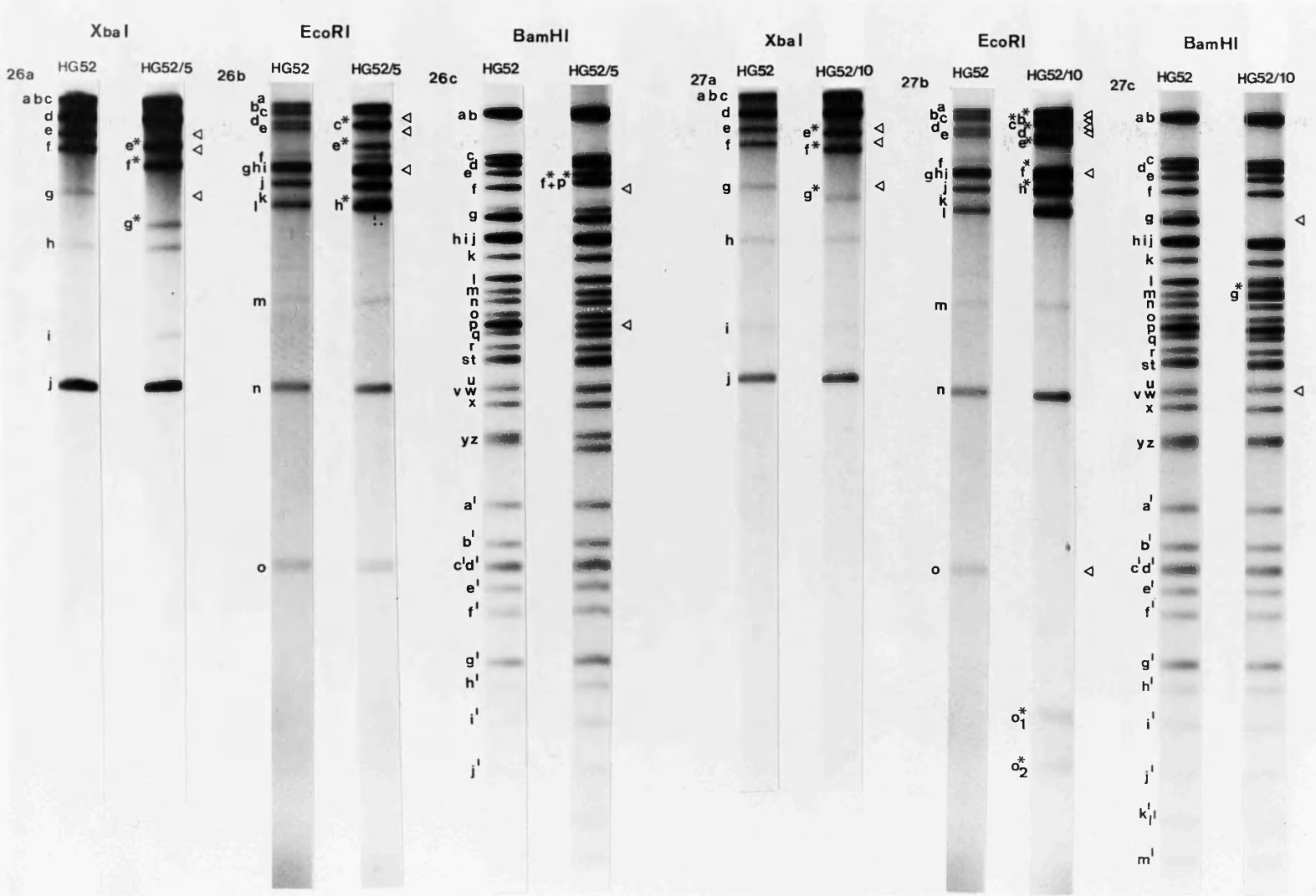
Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled in vivo of the HSV-2 wild type strain HG52 and the variant HG52/5 isolated at a frequency of 10% by plaque purification of untreated HG52 virus stock at passage eight. (cf. HG52X86 Fig. 21).
(a) Xba I digests, (b) EcoR I digests and (c) BamH I digests.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.

Figure 27(a-c)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled in vivo of the HSV-2 wild type strain HG52 and the variant HG52/10 isolated at a frequency of 14% by plaque purification of untreated HG52 virus stock at passage eight (cf. HG52X192 Fig. 22).
(a) Xba I digests, (b) EcoR I digests and (c) BamH I digests.

Letters refer to specific fragments, ◁ denotes missing fragment and * denotes novel fragment. The novel fragments created by cleavage of EcoR I o by the new EcoR I site are given a subscript (1) denoting the higher molecular weight and (2) the smaller.


XbaI
EcoRI
BamHI
XbaI
EcoRI
BamHI
26a
HG52
HG52/5
26b
HG52
HG52/5
26c
HG52
HG52/5
27a
HG52
HG52/10
27b
HG52
HG52/10
27c
HG52
HG52/10

EcoR I f fragment and its joints b and d were also reduced by a similar amount indicating deletions from both long terminal fragments. A presumably unrelated alteration was observed with the loss of the EcoR I o fragment and the appearance of two novel smaller molecular weight fragments in its place. This is probably due to the creation of a novel EcoR I restriction enzyme site within the EcoR I o fragment causing it to be bisected to give two molar bands of similar size. The BamH I profile (Fig. 27c) was consistent with there being a deletion of information from both copies of BamH I v so that both copies of the joint BamH I g run lower (with l and m). The BamH I b' and k' fragments spanning EcoR I o showed no alterations indicating that the change observed in that fragment is probably due to a base change creating a novel EcoR I site. The variant HG52/10, therefore, was indistinguishable from HG52X192 isolated after enrichment selection. The fact that all of the seven isolates and HG52X192 which were deleted by approximately $1x10^6$ daltons from both long inverted repeats also had a novel EcoR I site in the short unique region (data not shown) is strong evidence that all of these isolates are clonally related.

It was apparent that at least two of the variants isolated after enrichment selection were present at high frequency in untreated stock; HG52X86 (HG52/5) at a frequency of 10% and HG52X192 (HG52/10) at a frequency of 14%. Although detected at pass 8 (ie at the restriction analysis stage) presumably the variants were present at these frequencies by pass 6 as it seems highly unlikely that the same deletions would have arisen spontaneously in separate stocks, after the isolation of the individual

plaques.

Apart from inherent variability in TR_S, only one isolate of fifty plaques of HSV-2 strain 186 (P elite+3) analysed, exhibited a deletion of approximately $1x10^6$ daltons from Xba I g (data not shown).

The fifty plaques of HSV-2 strain 333 (P elite+3) analysed, showed no DNA differences except variability in the short terminal fragments due presumably to variable copy numbers of tandemly repeated sequences.

An HG52 stock grown from a plaque which was shown to have a normal wild type profile (P1) was titrated and fifty plaques picked (P2). The plaques were in turn grown up (P3) and $5x10^6$ BHK21/C13 cells were infected (P4) in the presence of ^{32}P to yield labelled viral DNA for analysis. All fifty plaques showed normal wild type Xba I and EcoR I DNA profiles with no detectable deletions. The frequency of occurrence of deletions was, therefore, less than 2% after four passages.

The temperature sensitive mutant of HG52, ts1, was chosen as essentially a plaque purified stock of HG52, treated as previous stocks and analysed for deletions. Fifty isolates whose DNAs were analysed with Xba I and EcoR I revealed no variants, indicating a frequency of deletions of less than 2% after eight passages since plaque purification.

As it had been shown that spontaneous variation was occurring in HG52 stocks at high frequency by six passes after plaque purification it was decided to examine the DNA profiles of individual plaques of three HSV-1 wild type strains. Plaques were picked from departmental elite stocks of Glasgow strain 17, strain KOS and strain McKrae obtained from Dr.S. Cook. The virus was grown up and the viral DNA

labelled with ^{32}P and subjected to analysis with Xba I and Hpa I. Of one hundred plaques of strain 17 (P elite+3) and fifty plaques of strain KOS (P elite+3) no deletion was detected indicating frequencies of occurrence of deletions of less than 1% and 2% respectively at pass numbers at least as high as that for HG52 and tsl.

The fifty plaques of HSV-1 strain McKrae (passage number unknown) exhibited a high degree of variability especially in the EcoR I j and k long and short terminal fragments (data not shown). The remaining terminal fragment, EcoR I e, is masked by the presence of other bands. In addition to the small scale variations observed in the terminal fragments there were four isolates which exhibited novel bands (not shown). Two of the isolates exhibited half molar bands of different sizes which ran above EcoR I k. As the j band appeared normal and the e fragment large, the most likely explanation for the novel half molar terminal bands is the insertion of sequences into one copy of EcoR I k. The inserts would be of approximately 2.5×10^6 and 0.8×10^6 daltons each. The third variant exhibited a novel half molar band running below EcoR I l. This is probably due to a deletion of approximately 0.5×10^6 daltons from one copy of EcoR I k. The fourth variant showed an extra molar band running above EcoR I k for which the most likely explanation is a novel EcoR I site within one of the large fragments. The novel band would be composed of two fragments caused by bisecting a band of approximately 11×10^6 daltons in the c, d, e, f and g group or by a new EcoR I site in the a or b fragments to give the observed novel band plus a band of approximately 10×10^6 daltons which would run with the c, d, e, f and g group. Although further restriction analyses

would have clarified the situation, it was decided not to pursue it further as the data had demonstrated that variation occurs in type 1 as well as type 2 HSV strains. The lack of information regarding the history of the McKrae stock meant that further interpretation would be of limited value.

3.4 VARIANTS ISOLATED AFTER Xba I TREATMENT OF HG52X163 DNA

After the first round of enrichment selection in which HG52 DNA was partially digested with Xba I and transfected, a number of spontaneously generated variants was isolated. As previously described these variants mostly showed deletions of sequences from IR_L. However, from the second round of enrichment selection in which HG53X163 DNA (lacking the 0.7m.c. Xba I site) was partially Xba I digested and transfected, a number of variants which all had deletions from the short region were isolated.

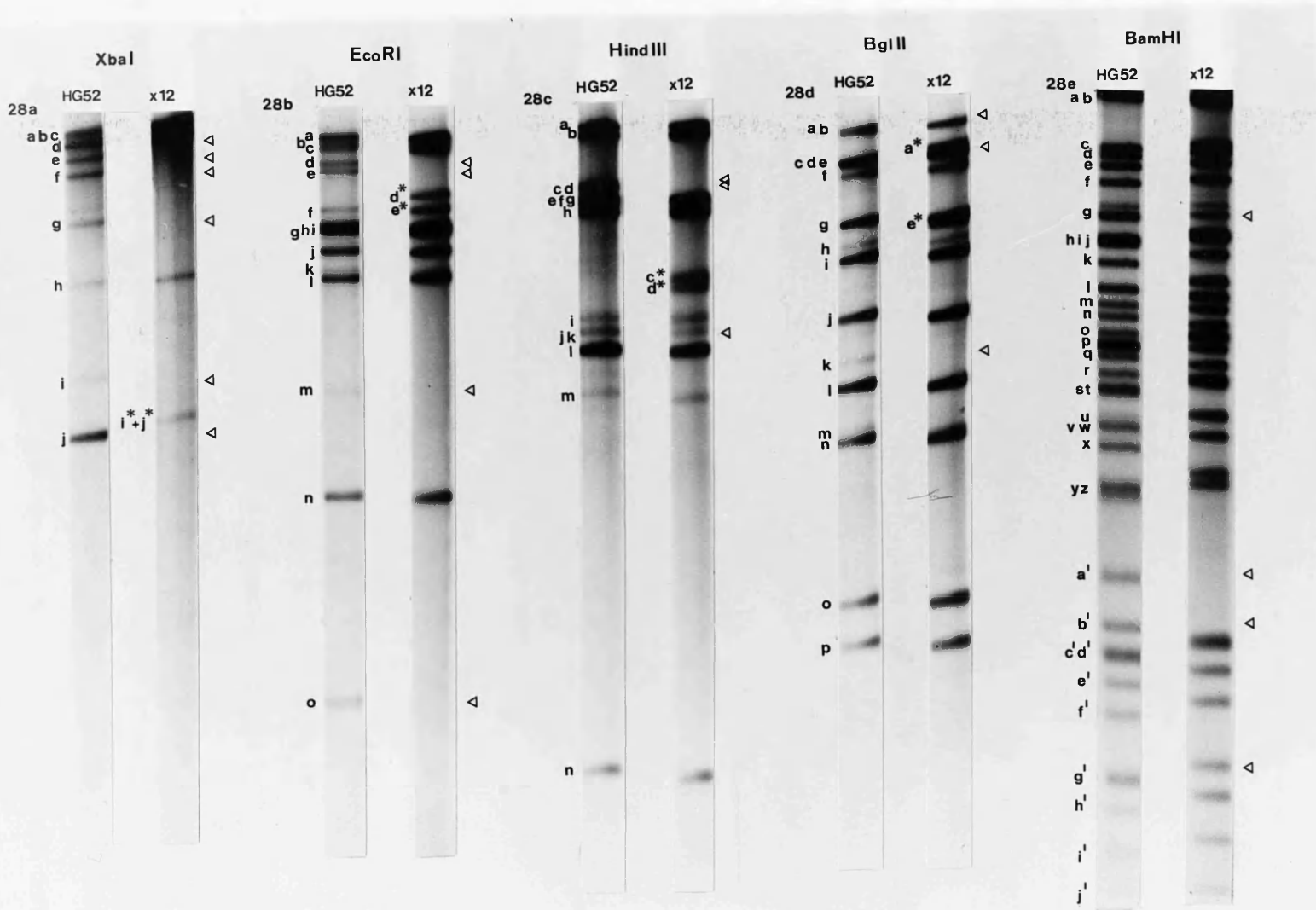
3.4a HG52X163X12

An Xba I digest of this variant is shown in Fig. 28a. The parental genome HG52X163 lacks the Xba I g/d site at 0.7m.c. due to a small insertion and consequently its Xba I profile has missing g, d, e and f bands while the novel end and joint fragments all run together at the top of the gel. HG52X163X12, as well as lacking the g, d, e and f bands is also missing the half molar Xba I i and the one molar Xba I j bands while there is a novel half molar band of approximately $5x10^6$ daltons running just above the normal position of Xba I j. The fact that this band is half molar demonstrates that it is an end fragment. As j is missing completely and the novel band runs below Xba I i a deletion

Figure 28(a-e)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* of the HSV-2 wild type strain HG52 and a variant HG52Xl63Xl2 isolated after transfection of partially Xba I digested HG52Xl63 DNA.
(a) Xba I digests, (b) EcoR I digests, (c) Hind III digests, (d) Bgl II digests and (e) BamH I digests.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


XbaI
EcoRI
HindIII
BglII
BamHI
28a
28b
28c
28d
28e
HG52
x12

of approximately $5x10^6$ daltons removing the 0.94m.c. Xba I site is indicated. The presence of a half molar Xba I h band and a half molar novel end fragment demonstrates that the short region inverts normally and, therefore, that the 'a' sequence of the terminal short repeat is not deleted.

An EcoR I digest (Fig. 28b) of HG52X163X12 supported the finding of the Xba I digest that there is a deletion from the short region of the genome. EcoR I n appeared as a normal one molar band, however, both the m and o bands were missing. The two m containing joints, d and e, were absent and apparently replaced by two smaller bands, d* running just above f and e* running with f. The sizes of the novel joint fragments indicate an end fragment of approximately $1x10^6$ daltons but as it has apparently run off the gel it must be less than $0.7x10^6$ daltons. The size of this novel end fragment composed of the remnants of EcoR I o and m indicates that the normal o+m has a deletion of more than $4x10^6$ daltons which removes DNA from both U_S and TR_S whilst leaving at least the terminal 'a' sequence.

On digestion with Hind III (Fig. 28c) the two k containing joints, c and d, are deleted and are running above i. As expected Hind III l is unaffected, indicating that the deletion is confined to the k fragment. The size of the deletion, calculated from the change in mobility of the c and d joint fragments, would be approximately $5x10^6$ daltons. Unfortunately the novel k fragment is not seen and, therefore, must be either comigrating with or smaller than EcoR I n ($1.5x10^6$ daltons) confirming a deletion of at least $4.8x10^6$ daltons.

The Bgl II profile (Fig. 28d) confirmed the previous analyses in that the Bgl II k band was missing and the novel

k* fragment had apparently run off the gel. The two k containing joint fragments, a and e, were also reduced in size to run just above c/d and just below g respectively. The deletion, calculated from the change in mobility of a and e, would be approximately $5x10^6$ daltons. The presence of the Bgl II l band delimits the left hand end of the deletion which removes the Xba I i/j site at 0.94m.c. but does not remove the Bgl II l/k site approximately 3kb to its left.

When digested with BamH I (Fig. 28e) it was clear that the a' and b' fragments were missing. There was also a suggestion that one copy of BamH I g' was missing and that the molarity of the BamH I g(u+v) joint fragment was also reduced. The simplest explanation is that the deletion starts in BamH I b' and extends into u; correspondingly b', k', a' and one copy each of m', g' and u would be missing and the joint fragment, g, would be half molar. There should be two novel bands; one half molar composed of the remnants of b' and u and the other, the half molar joint of this novel end fragment plus v. It was not possible to detect either of the proposed novel bands on this gel.

To confirm the conclusions drawn from the restriction enzyme analyses and to ensure that the deleted sequences were not inserted elsewhere in the genome, a number of Southern blotting experiments was performed using probes from within and flanking the proposed deleted region. Figure 28g shows an EcoR I digest of HG52 and HG52X163X12 hybridized to BamH I a'. The control HG52 probe is shown in Fig. 28f. BamH I a' hybridizes to EcoR I m and, due to the inverted repeat sequences, to k and consequently to all four joint fragments b, c, d and e of HG52. The pattern with HG52X163X12 DNA shows positive hybridization to k and its

Figure 28(f-i)

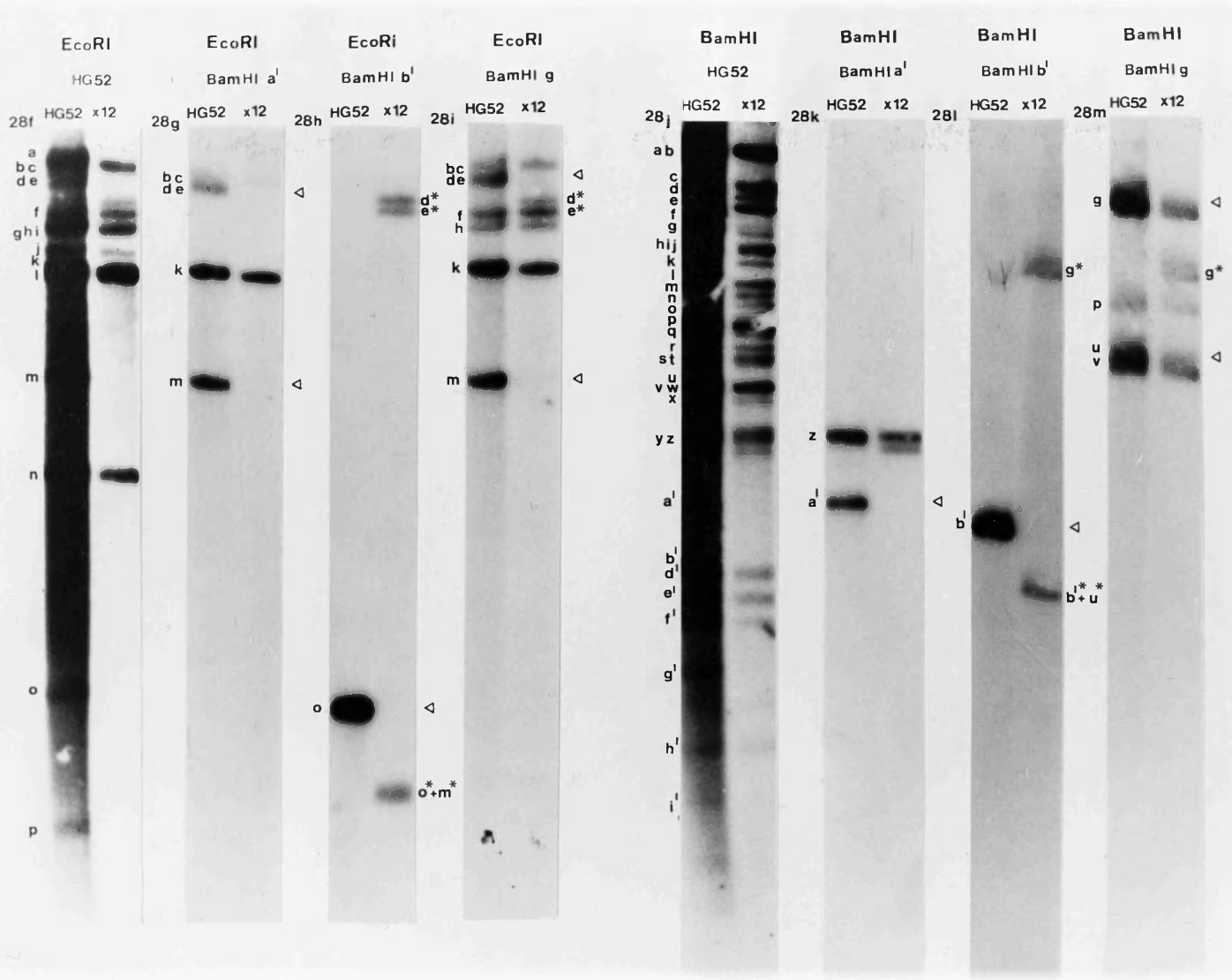
Autoradiographs of nitrocellulose blot strips from a single gel containing EcoR I restriction fragments of HG52 and HG52X163X12 to which nick-translated HSV-2 DNA probes have been hybridized. The probes were (f) HG52, (g) the recombinant plasmid pGZ66 containing BamH I a', (h) the recombinant plasmid pGZ67 containing BamH I b' and (i) the recombinant plasmid pGZ1 containing BamH I g.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.

Figure 28(j-m)

Autoradiographs of nitrocellulose blot strips from a single gel containing BamH I restriction fragments of HG52 and HG52X163X12 to which nick-translated HSV-2 probes have been hybridized. The probes were (j) HG52,(k) the recombinant plasmid pGZ66 containing BamH I a', (l) the recombinant plasmid pGZ67 containing BamH I b' and (m) the recombinant plasmid pGZ1 containing BamH I g.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


EcoRI
HG52
28f
HG52 x12
a
b c
d e
f
g h i
j
k
l
m
n
o
p
EcoRI
BamHI a'
28g
HG52 x12
b c
d e
k
m
EcoRi
BamHI b'
28h
HG52 x12
d*
e*
o
o*+m*
EcoRI
BamHI g
28i
HG52 x12
b c
d e
f
h
d*
e*
k
m
BamHI
HG52
28j
HG52 x12
a b
c
d
e
f
g
h i
j
k
l
m
n
o
p
q
r
s t
u
v
w
x
y z
a'
b'
d'
e'
f'
g'
h'
i'
BamHI
BamHI a'
28k
HG52 x12
z
a'
BamHI
BamHI b'
28l
HG52 x12
g*
b'
b'* u'*
BamHI
BamHI g
28m
HG52 x12
g
p
u
v
g*

joints b and c, m is missing and there is no hybridization to the novel end or joint fragments demonstrating that BamH I a' has been completely deleted.

Figure 28h shows the hybridization pattern of BamH I b' to EcoR I digested HG52 and HG52X163X12 DNA. The probe, when hybridized to the wild type DNA, recognized only EcoR I o. HG52X163X12 does not produce an EcoR I o band but there was hybridization to the band running below o and to two bands near the top of the gel. It is assumed that these novel bands represent the novel end fragment composed of the remainder of the EcoR I o and m fragments and the two novel joint fragments d* and e*. The fact that BamH I b' hybridized to these bands indicates that the deletion starts within the BamH I b' fragment and that the novel end fragment contains enough b' information to allow hybridization.

Figure 28i shows an EcoR I digest of HG52 and HG52X163X12 hybridized to BamH I g. BamH I g hybridized to all the end and joint fragments of the wild type track (ie. EcoR I f, h, k, m, b, c, d and e). That the two bands running above and with EcoR I f of HG52X163X12 are novel joints is confirmed by the positive hybridization of BamH I g to these bands. The novel end fragment composed of the remainders of m plus o was not detected. As it was present in the adjacent track, the poor hybridization was thought to be due to the limited amount of BamH I u information remaining in the novel end fragment.

Finally, the same probes were used in blotting experiments with BamH I digests of HG52 and HG52X163X12. Figure 28j shows the pattern of hybridization of BamH I digested HG52 and HG52X163X12 with an HG52 probe. Figure 28k

shows the hybridization pattern of BamH I a' to the same digests. BamH I a' hybridized to the wild type a' and, due to the inverted repeat sequences, also to z. The fact that the only hybridization was to z in HG52X163X12 supports the EcoR I data that all of the BamH I a' fragment has been deleted. The variability in the z band is probably due to different copy numbers of a reiterated sequence.

In Fig. 28l it can be seen that BamH I b' hybridized only to the equivalent band of BamH I digested HG52. The BamH I b' band was missing from HG52X163X12 but there was hybridization to three bands representing the novel end fragment and its joints with v. The blot shows that the end fragment comprising the remainders of b' and u ran in the region e' or f' and that the novel joints ran with p and q. The sizes of the novel bands demonstrated a deletion of 5.4×10^6 daltons.

The BamH I g probe (Fig. 28m) hybridized to the BamH I HG52 bands u, v and g and there was also limited cross-hybridization to the p fragment which is adjacent to v. HG52X163X12 also showed hybridization to the p, u, v and g fragments although the g and u fragments appeared less intense. There was hybridization to the novel joint fragment running in the region of l and m. However, the novel end fragment composed of the remainders of b' and u was not detected although it was seen hybridizing to the BamH I b' probe. It is concluded that the novel terminal fragment contains little BamH I u DNA.

The Southern blotting data, therefore, substantiated the restriction enzyme analyses indicating a deletion removing approximately 5×10^6 daltons extending from 0.94-0.99m.c.

3.4b HG52Xl63Xl4

The second variant with a deletion from the short region was HG52Xl63Xl4 which was first detected because of an aberrant Xba I profile (Fig. 29a). As previously described, the parental genome HG52Xl63 lacks the 0.7m.c. Xba I g/d site. The novel fragment g+d and its joints g+d+h and g+d+i all run at the top of the gel with a, b and c and consequently the g, d, e(g+h) and f(g+i) bands are missing. The Xba I profile of HG52Xl63Xl4 was similar to that of the parental HG52Xl63 except for the absence of the Xba I i band. As both the h and j bands were present, this was not due to the loss of an Xba I restriction site and as no novel i band could be seen, the probable explanation was a large deletion of information from Xba I i.

An EcoR I digest (Fig. 29b) of HG52Xl63Xl4 DNA exhibited missing o and m bands and m containing joints d and e. Unlike HG52Xl63Xl2, however, there was no evidence of any novel d and e joints. There was a novel, apparently one molar, band of approximately 0.5×10^6 daltons running at the base of the gel.

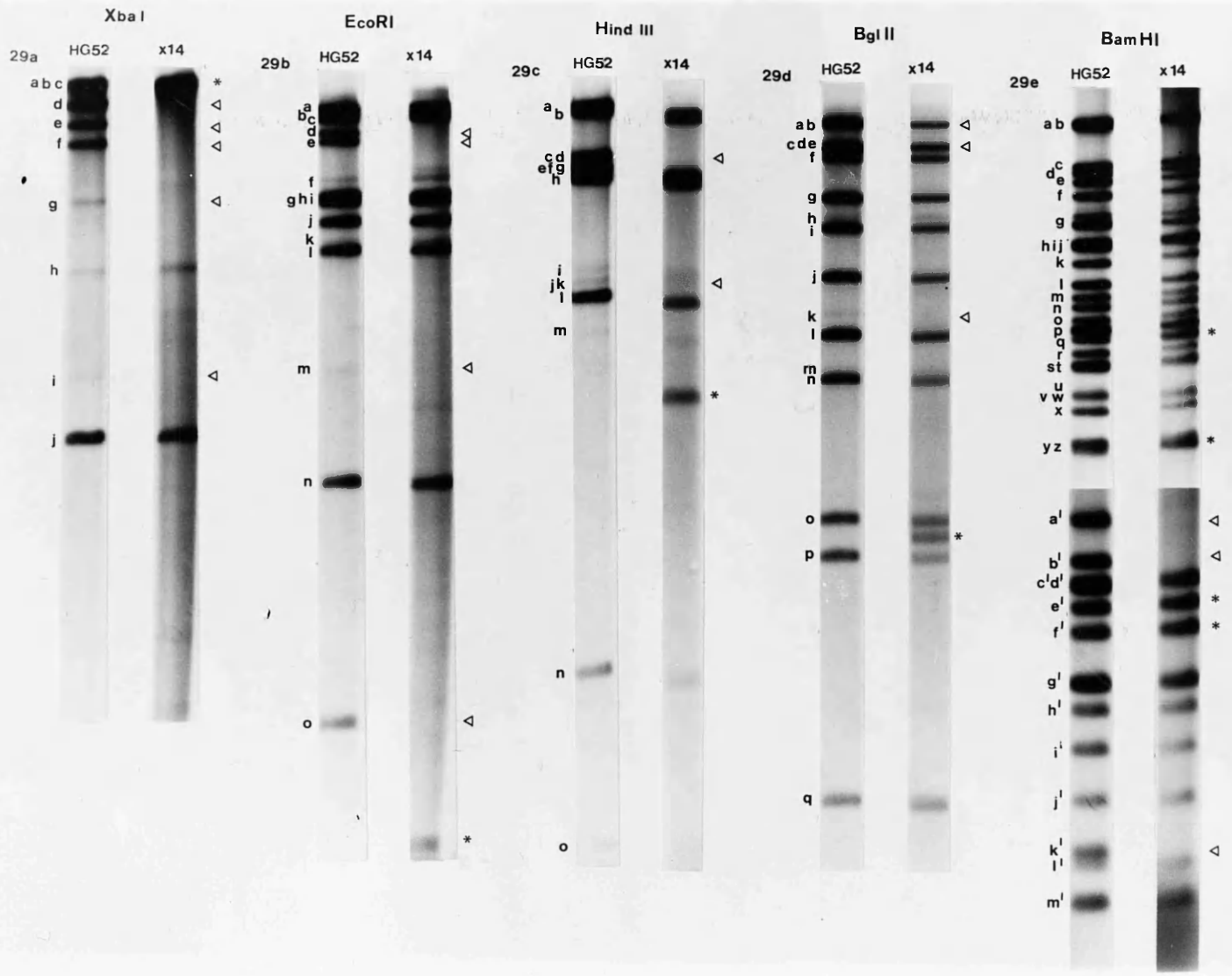
A Hind III digest of HG52Xl63Xl4 (Fig. 29c) confirmed the apparent deletion from the short terminus. As k and j run together it was hard to see but the Hind III k band was probably missing as the two k containing joints, c and d, were no longer present. There was no indication of novel c and d joints, the only novel band being an apparently one molar band of approximately 4×10^6 daltons below m.

A Bgl II digest of HG52Xl63Xl4 (Fig. 29d) indicated a loss of information from the short terminal fragment k. Bgl II k and its two joints, a and e, were all missing with no detectable novel end or joint fragment. There was also a

Figure 29 (a-e)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* of the HSV-2 wild type strain HG52 and a variant HG52X163X14 isolated after enrichment selection of HG52X163 DNA.
(a) Xba I digests, (b) EcoR I digests,(c) Hind III digests, (d) Bgl II digests and (e) BamH I digests.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment. The BamH I digests shown are composite figures using two exposures of the same gel to increase the intensity of bands at the bottom of the gel.


Xba I
EcoRI
Hind III
Bgl II
Bam HI
29a
29b
29c
29d
29e
HG52
x14

novel one molar band of approximately $2.7x10^6$ daltons seen between o and p.

The BamH I profile of HG52X163X14 (Fig. 29e) gave support to the theory of a deletion from the right hand end of the short region. The BamH I a', b' and k' fragments were absent while the molarities of the e', f' z and q bands were apparently increased. This led to the conclusion that instead of a straight forward deletion there has been a substitution of information from the left hand end for information from the right hand end of the short region. The apparent over representation of q was probably due to a coincident novel band rather than to two copies of q. The small increase in the mobility of one copy of BamH I u was unrelated to the substitution as the same alteration was noted in the parental HG52X163 genome.

A range of Southern blotting experiments was performed on HG52X163X14. Figure 29f shows an EcoR I digest of HG52 and HG52X163X14 probed with HG52. An EcoR I digested wild type sample of similar separation is also shown, as the probe did not give good hybridization. As the deletion appeared to extend into BamH I b', it was used as a probe. When hybridized to an EcoR I digest of HG52 (Fig. 29g), it recognized only o. HG52X163X14, as has already been shown, lacks a normal o band and consequently there was no hybridization to this region. There was, however, hybridization to the novel band of approximately $0.5x10^6$ daltons running below o.

Figure 29h shows a Hind III digest of HG52 and HG52X163X14 probed with HG52. A labelled sample of Hind III digested HG52 DNA is also shown as the wild type probe was poor. Figure 29i shows a Hind III digest of HG52 and

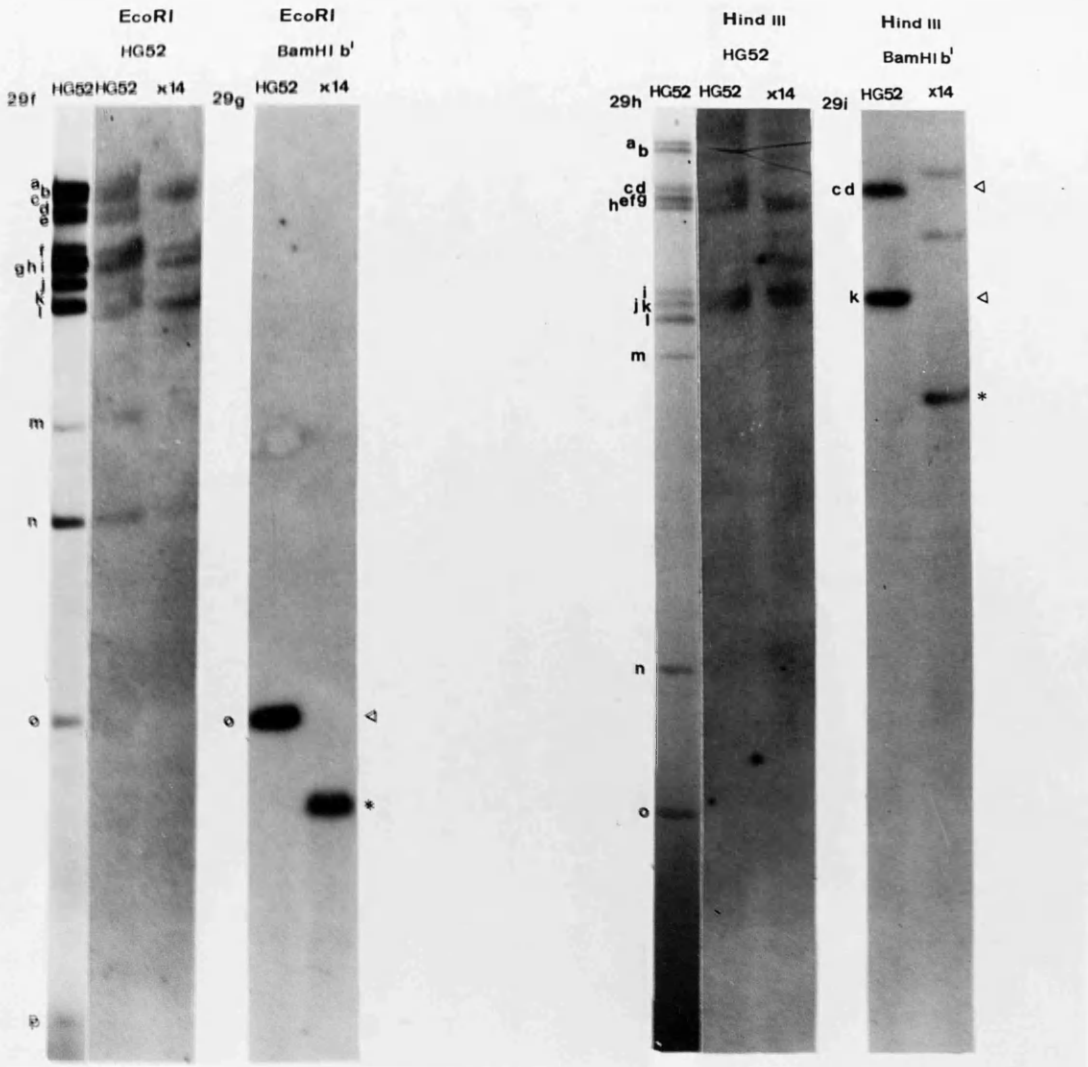
Figure 29(f+g)

Autoradiographs of nitrocellulose blot strips from a single gel containing EcoR I restriction fragments of HG52 and HG52X163X14 to which nick translated HSV-2 DNA probes have been hybridized. As the wild type probe was poor ((f) lanes 2 and 3) an autoradiograph of ^{32}P labelled HG52 DNA digested with EcoR I with similar distribution is shown in lane 1 to help with identification of bands. The probe in (g) was the recombinant plasmid pGZ67 containing BamH I b'. Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.

Figure 29(h+i)

Autoradiographs of nitrocellulose blot strips from a single gel containing Hind III restriction fragments of HG52 and a variant HG52X163X14 to which nick-translated HSV-2 DNA probes have been hybridized. As the wild type probe was poor ((h) lanes 2 and 3) an autoradiograph of ^{32}P labelled HG52 digested with Hind III with similar separation is shown in lane 1 to facilitate identification of bands. The probe in (i) was the recombinant plasmid pGZ67 containing BamH I b'.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


EcoRI
HG52
29f
HG52
HG52
x14
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
EcoRI
BamHI b'
29g
HG52
x14
o
Δ
*
Hind III
HG52
29h
HG52
HG52
x14
a
b
cd
h
efg
i
j
k
l
m
n
o
Hind III
BamHI b'
29i
HG52
x14
cd
k
Δ
Δ
*

HG52X163X14 probed with BamH I b'. BamH I b' hybridized to the wild type k fragment and the two k containing joints, c and d, all of which were missing from HG52X163X14. There was, however, hybridization to the novel one molar band of approximately $4x10^6$ daltons running below m. (Due to a proportion of partially digested DNA there was also spurious hybridization at the top of the gel to bands which do not appear in the fully digested sample (Fig. 29c)).

Figure 29j shows a Bgl II digest of HG52 and HG52X163X14 hybridized to a wild type probe. Figure 29k shows the same digests probed with BamH I b'. BamH I b' hybridized to the wild type fragment Bgl II k and to the two k containing joint fragments, a and e, all of which were missing from HG52X163X14. The only fragment detected, was the novel one molar band of approximately $2.7x10^6$ daltons below o.

Finally when hybridized to a BamH I digest of HG52 (Fig. 29o) the BamH I b' probe, as expected, hybridized solely to BamH I b' confirmed by comparison with Fig. 29n which shows HG52 and HG52X163X14 probed with HG52. It was previously demonstrated that HG52X163X14 has no BamH I b' fragment and this was confirmed by the lack of hybridization in that region. However, there was hybridization to the novel band running with g.

From these data it is concluded that some BamH I b' DNA is still present in the novel molar fragments. For EcoR I, Hind III and Bgl II the novel bands were smaller than the original BamH I b' containing fragment but in the case of the BamH I digest the novel fragment was larger than BamH I b' itself.

As the restriction endonuclease analyses had suggested that information contained within BamH I a' was missing

Figure 29(j-m)

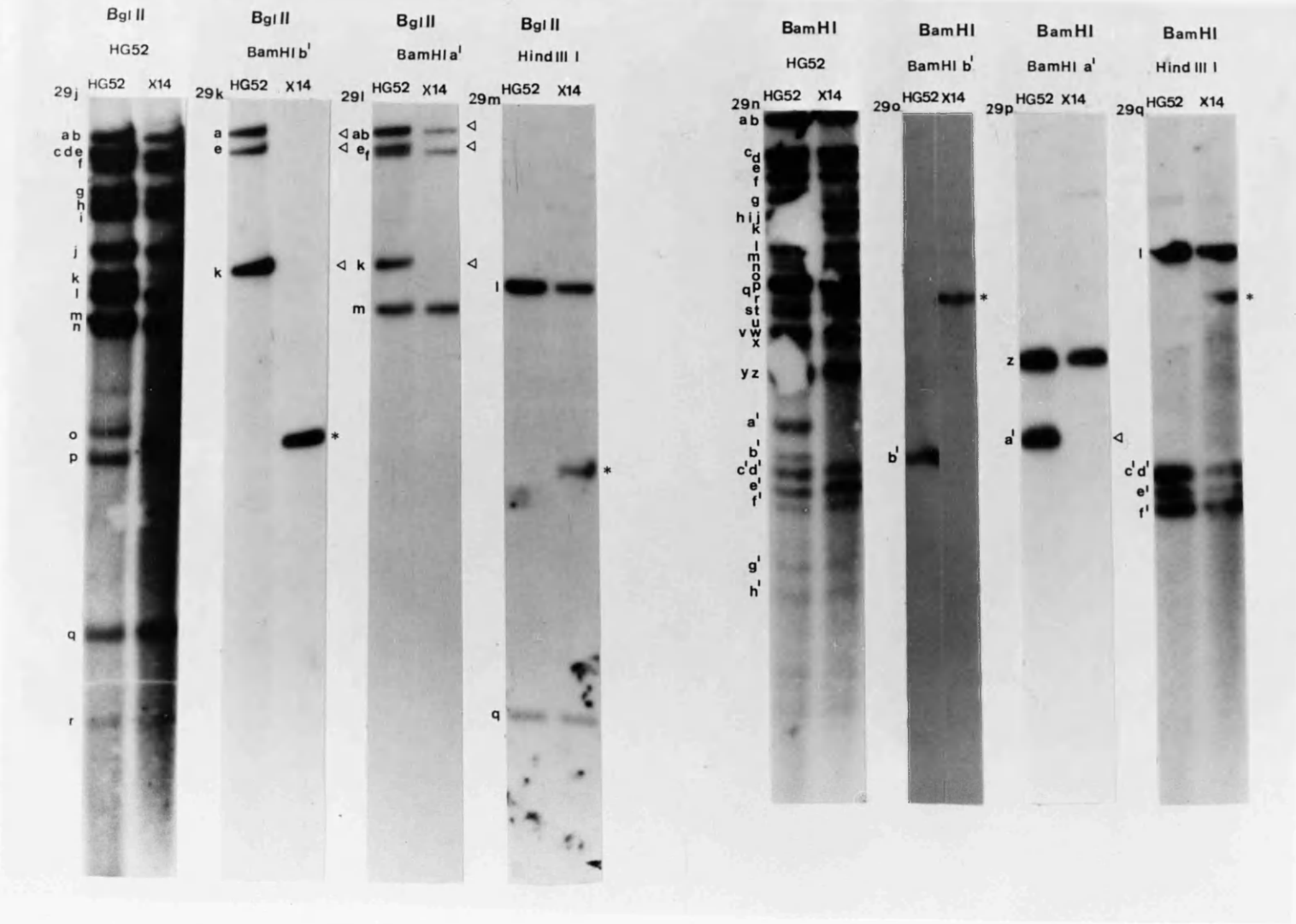
Autoradiographs of nitrocellulose blot strips containing Bgl II restriction fragments of HG52 and HG52Xl63Xl4 to which nick-translated HSV-2 DNA probes have been hybridized. The probes were (j) HG52, (k) the recombinant plasmid pGZ67 containing BamH I b', (l) the recombinant pGZ66 containing BamH I a' and (m) the recombinant plasmid pGZ28 containing Hind III l. The samples shown in (m) are from a different gel than the samples in (j), (k) and (l) and show slightly different separation.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.

Figure 29(n-q)

Autoradiographs of nitrocellulose blot strips from a single gel containing BamH I restriction fragments of HG52 and HG52Xl63Xl4 to which nick-translated HSV-2 DNA probes have been hybridized. The probes were (n) HG52, (o) the recombinant plasmid pGZ67 containing BamH I b', (p) the recombinant plasmid pGZ66 containing BamH I a' and (q) the recombinant plasmid pGZ28 containing Hind III l.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


Bgl II
HG52
29j HG52 X14
ab
cde
f
g
h
i
j
k
l
m
n
o
p
q
r
Bgl II
BamHI b'
29k HG52 X14
a
e
k
*
Bgl II
BamHI a'
29l HG52 X14
ab
ef
k
m
Bgl II
Hind III 1
29m HG52 X14
l
*
q
BamHI
HG52
29n HG52 X14
ab
cde
f
g
hijk
lmnopqrstuvwx
yz
a'
b'
c'd'e'f'
g'
h'
BamHI
BamHI b'
29o HG52 X14
*
b'
BamHI
BamHI a'
29p HG52 X14
z
a'
BamHI
Hind III 1
29q HG52 X14
l
*
c'd'
e'
f'

entirely, a couple of Southern blotting experiments were performed using this fragment as probe. When BamH I a' was hybridized to a Bgl II digest of HG52 (Fig. 29l), the k fragment and the two k containing joints, a and e, were detected. Because BamH I a' contains information from the short repeat region there was also hybridization to Bgl II m and to the two m containing joints, b and f. The Bgl II digest of HG52X163X14 still showed positive hybridization of BamH I a' to the m, b and f bands but there was no hybridization where k, a and e normally run.

A BamH I a' probe when directed against a BamH I digest of HG52 and HG52X163X14 (Fig. 29p) showed hybridization to the wild type BamH I a' and to the BamH I z fragment which contains the same region of inverted repeat. When directed against the BamH I digest of HG52X163X14, however, only the BamH I z fragment was detected. These two blots, therefore, confirm that the unique portion of BamH I a' has been completely lost from HG52X163X14.

The blotting data confirm that there is a deletion from HG52X163X14 removing DNA from the right hand end of the short region including part of BamH I b' and the unique portion of a'. To confirm that in place of the deleted information there is a second copy of a DNA segment normally present only at the left hand end of the short unique region, two Southern blotting experiments were performed using Hind III l as the probe. Figure 29m shows the hybridization of Hind III l to a Bgl II digest of HG52 and HG52X163X14. Hind III l hybridized to the wild type fragments Bgl II q and l. In HG52X163X14 both of these fragments were detected. However, there was also hybridization to the novel Bgl II band of approximately

$2.7x10^6$ daltons below o.

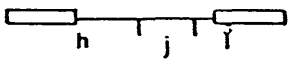
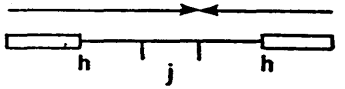
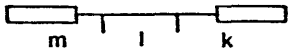
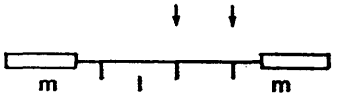
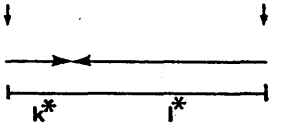
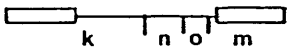
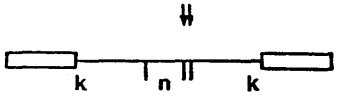
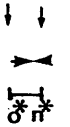
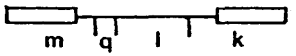
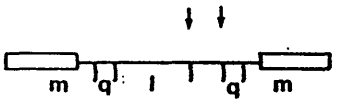
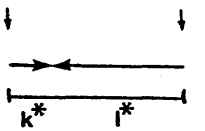
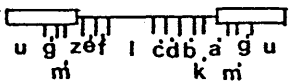
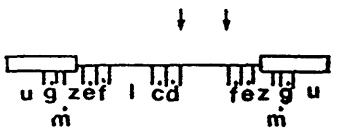
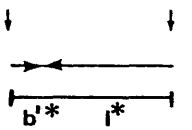
When used against a BamH I digest of HG52 (Fig. 29q) the Hind III l probe detected e', f', l, c' and d'. In HG52Xl63Xl4 the same fragments were all detected but in addition there was hybridization to the novel band with q. These experiments confirmed that information from the left hand end of the short unique region has transferred to the right hand end of the genome. The novel molar Bgl II and BamH I fragments contain information from both BamH I b' and Hind III l which would not normally occur within a single fragment. Figure 29r shows a diagram explaining the proposed origin of the novel restriction enzyme fragments observed in HG52Xl63Xl4. The data from the BamH I digest of HG52Xl63Xl4 showed that DNA from the internal copy of BamH I u to some point within BamH I l (0.83-0.9m.c.) is repeated in the opposite orientation replacing DNA from within BamH I b' to the terminal copy of BamH I u (0.95-1.00m.c.).

It would be expected from the BamH I data that the novel terminal Xba I fragment would be approximately the same size as Xba I h. As no new Xba I fragment was observed (Fig. 29a), it is concluded that the novel band ran coincidently with Xba I h. The implication is that all of Xba I i (0.94-1.0m.c.) has been deleted and replaced by Xba I h (0.83-0.94m.c.) in inverted orientation to give a short region composed of Xba I h/j/h thereby increasing the genome size by 3kb.

The deletion from HG52Xl63Xl4, therefore, removes the genes US 10, 11, 12 and 9 whilst gaining a second copy of US 1, 2, 3 and 4 and the coding region of US5.

Figure 29(r)

Proposed model for the structure of HG52X163X14 compared to HG52 showing the positions of the various restriction endonuclease sites. The proposed novel fragments are indicated by vertical arrows and their composition and expected molecular weights are shown on the right.

	HG 52	HG 52 X 163 X 14	M.W. x10^6	
Xba I				
Hind III			4·0	
EcoR I			0·5	
Bgl II			2·7	
BamH I			2·5	

3.4c HG52Xl63X21

The final variant with a deletion from the short region was HG52Xl63X21 which again was detected because of an aberrant Xba I profile (Fig. 30a). The one molar j and the half molar i bands were missing whilst h appeared normal. There was, in addition, a novel half molar band of apparent molecular weight 12-13x10^6 daltons running above the normal position of Xba I g. The novel band was too large to be accounted for by postulating the loss of the 0.94m.c. Xba I site. It ran, however, in the same position as the h+j fusion fragment seen on Xba I digestion of HG52Xl63X3 (Fig. 13a). The apparent anomaly that Xba I h was present both as a normal half molar end fragment and fused to Xba I j to give a novel half molar j+h end fragment led to a hypothesis similar to that proposed for HG52Xl63Xl4 in which information from Xba I i was lost and replaced by information from Xba I h. This is supported by the EcoR I profile of HG52Xl63X21 shown in Fig. 30b. The profile was very similar to that of HG52Xl63Xl4 in that the m and o bands were missing as were the d and e joints containing m; also there was a novel one molar band running below o. In this case, however, it was slightly smaller being approximately 0.4x10^6 daltons.

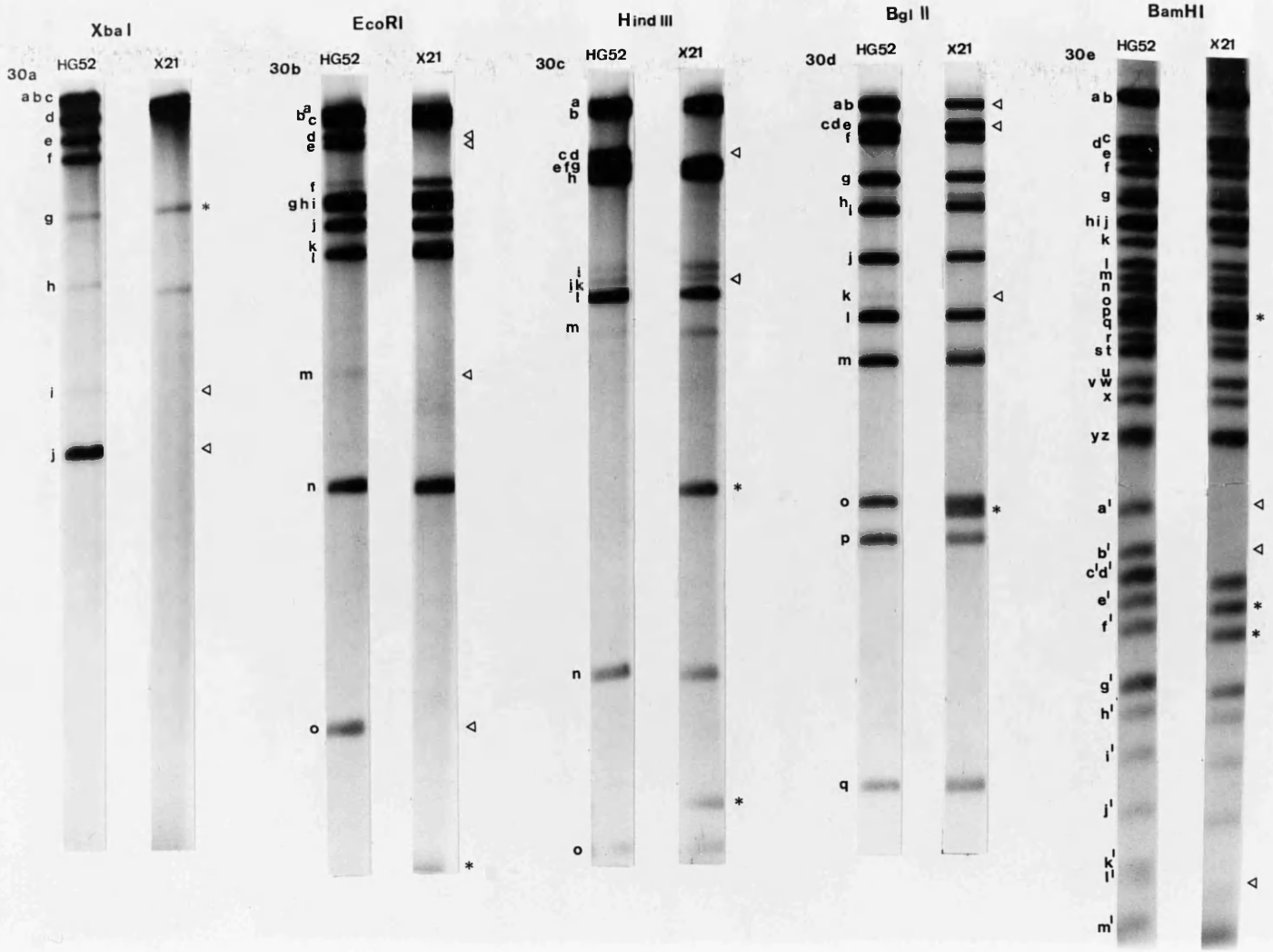
A Hind III digest of HG52Xl63X21 (Fig. 30c), again showed a profile similar to that of HG52Xl63Xl4 with the Hind III k joints c and d missing. Because Hind III k runs with j it was difficult to tell if it was absent. The Hind III l/k site is unaffected as shown by the presence of a normal l band. Unlike HG52Xl63Xl4 this variant exhibited two novel molar Hind III bands; one of 3x10^6 daltons between n and m and the other of 1x10^6 daltons above o.

Figure 30(a-e)

Autoradiographs of restriction endonuclease digests of viral DNA ^{32}P labelled *in vivo* of the HSV-2 wild type HG52 and variant HG52X163X21 isolated after enrichment selection of HG52X163 DNA.

(a) Xba I digests, (b) EcoR I digests, (c) Hind III digests, (d) Bgl II digests and (e) BamH I digests.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


Xba I
30a
HG52
X21
EcoRI
30b
HG52
X21
Hind III
30c
HG52
X21
Bgl II
30d
HG52
X21
BamHI
30e
HG52
X21

A Bgl II digest of HG52X163X21 (Fig. 30d) again showed a striking resemblance to that of HG52X163X14 with the Bgl II k fragment and its two joints, a and e, all missing. Like HG52X163X14 the only novel band observed was a molar band running between o and p which in this case was marginally larger (2.8×10^6 daltons) than in HG52X163X14 (2.7×10^6 daltons).

Finally a BamH I digest of HG52X163X21 (Fig. 30e) produced a profile almost indistinguishable fron HG52X163X14 with a', b' and k' missing while e' and f' were over represented. The only apparent difference between this variant and HG52X163X14 on BamH I digestion was that HG52X163X21 had a novel band running with BamH I p instead of q as in HG52X163X14 (a difference of approximately 0.1×10^6 daltons).

The restriction enzyme profiles described above indicate that both HG52X163X14 and HG52X163X21 have information deleted from the right hand end (0.94-1.00m.c.) of the short region which is replaced by sequences from the left hand end (0.83-0.91m.c.) to give a short region which on Xba I digestion gives h/j/h for HG52X163X14 and h+j/h for HG52X163X21. There were some apparently contradictory results in that the Bgl II and BamH I novel bands formed by HG52X163X14 were smaller by approximately 0.1×10^6 daltons than those seen with HG52X163X21 while the novel EcoR I band seen with HG52X163X14 was larger than that with HG52X163X21 by 0.1×10^6 daltons. The size of the two novel fragments created when HG52X163X21 was digested with Hind III (1×10^6 and 3×10^6 daltons) gave a total size which was the same as the single novel Hind III band of HG52X163X14 (4×10^6 daltons).

As HG52X163X21 appeared to be very similar to HG52X163X14, the range of Southern blotting experiments performed was the same. Figure 30f shows the pattern of hybridization observed when EcoR I digests of HG52 and HG52X163X21 were probed with HG52. As this gave poor hybridization an EcoR I digest of ^{32}P labelled HG52 DNA with comparable separation is shown. When an EcoR I digest of HG52X163X21 was probed with BamH I b' (Fig. 30g) EcoR I o was not seen but there was hybridization to a novel band of approximately $0.4x10^6$ daltons.

Figure 30h shows Hind III digested HG52 and HG52X163X21 samples probed with HG52. As the hybridization was poor a Hind III digest of ^{32}P labelled HG52 DNA with similar separation is shown. A Hind III digest of HG52X163X21 when probed with BamH I b' (Fig. 30i) showed loss of the k band and its joints c and d. There was hybridization to a novel band of approximately $1x10^6$ daltons.

Figure 30j shows Bgl II digests of HG52 and HG52X163X21 probed with HG52. A Bgl II digest of HG52X163X21 probed with BamH I b' (Fig. 30k) showed loss of the k fragment and its joints, a and e. There was novel hybridization to a fragment of $2.8x10^6$ daltons below o.

Figure 30n shows BamH I digests of HG52 and HG52X163X21 probed with HG52. A BamH I digest of HG52X163X21 when probed with BamH I b' (Fig. 30o) again showed loss of the b' band, seen in the wild type track, and hybridization to a larger novel fragment of approximately $2.6x10^6$ daltons running with BamH I p.

These blots of HG52X163X21 gave a similar pattern to HG52X163X14 in that the same fragments were missing. However, the novel fragments were of different sizes. Both

Figure 30(f+g)

Autoradiographs of nitrocellulose blot strips from a single gel containing EcoR I restriction fragments of HG52 and HG52X163X21 to which nick-translated HSV-2 DNA probes have been hybridized. As the wild type probe ((f) lanes 2 and 3) was poor, an autoradiograph of EcoR I digested ^{32}P labelled HG52 DNA with similar separation is shown in lane 1 to help with identification of the bands in Fig. 30(g). The probe in (g) was the recombinant plasmid pGZ67 containing BamH I b'.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.

Figure 30(h+i)

Autoradiographs of nitrocellulose blot strips from a single gel containing Hind III restriction fragments of HG52 and a variant HG52X163X21 to which nick-translated HSV-2 DNA probes have been hybridized. As the wild type probe was poor ((h) lanes 2 and 3) an autoradiograph of Hind III digested ^{32}P labelled HG52 DNA with similar separation is shown in lane 1 to help to identify the bands in (i). The probe in (i) was the recombinant plasmid pGZ67 containing BamH I b'.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.

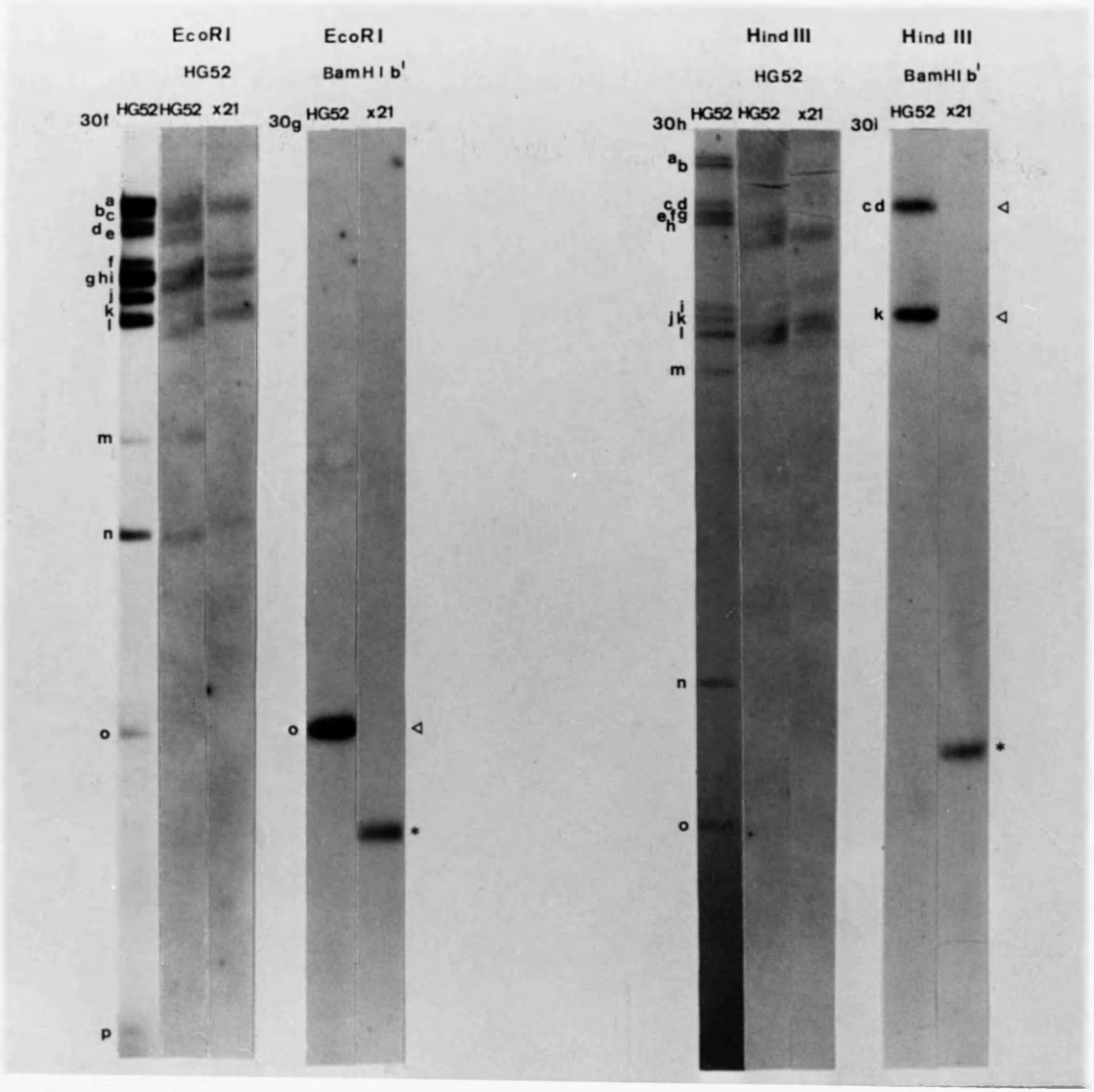

EcoRI
HG52
30f
HG52 HG52 x21
a
b c
d e
f
g h i
j
k
l
m
n
o
p
EcoRI
BamHI b'
30g
HG52 x21
o
◁
*
Hind III
HG52
30h
HG52 HG52 x21
a b
c d
e f g
h
i
j k
l
m
n
o
Hind III
BamHI b'
30i
HG52 x21
c d
k
◁
◁
*

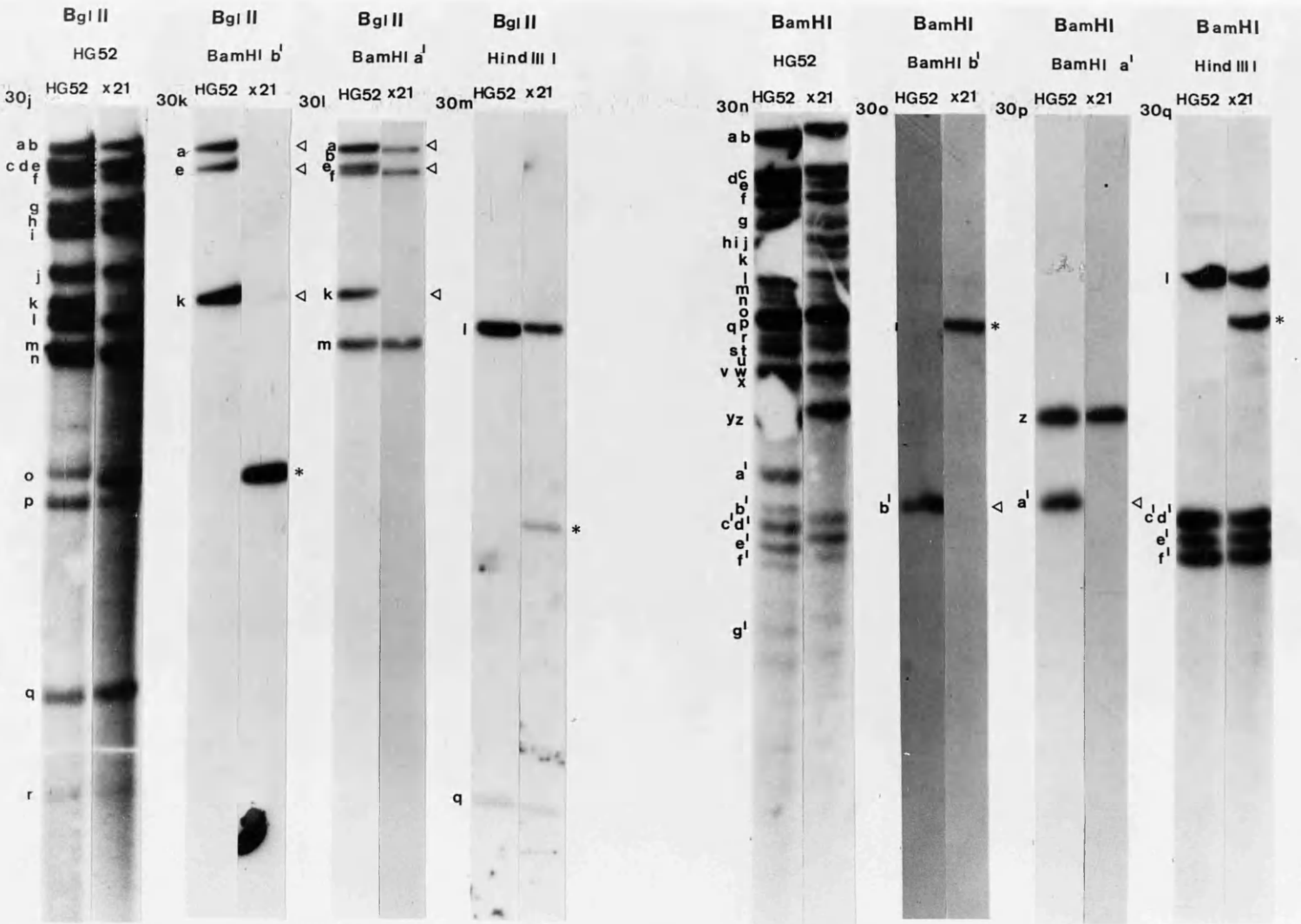
Figure 30(j-m)

Autoradiographs of nitrocellulose strips containing Bgl II restriction fragments of HG52 and HG52X163X21 to which nick-translated HSV-2 DNA probes have been hybridized. The probes were (j) HG52, (k) the recombinant plasmid pGZ67 containing BamH I b', (l) the recombinant plasmid pGZ66 containing BamH I a' and (m) the recombinant plasmid pGZ28 containing Hind III l. The samples shown in (m) are from a different gel than those in (j), (k) and (l) and consequently fragments show slightly different migration. Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.

Figure 30(n-q)

Autoradiographs of nitrocellulose blot strips from a single gel containing BamH I restriction fragments of HG52 and Hg52X163X21 to which nick-translated HSV-2 DNA probes have been hybridized. The probes were (n) HG52, (o) the recombinant plasmid pGZ67 containing BamH I b', (p) the recombinant plasmid pGZ66 containing BamH I a' and (q) the recombinant plasmid pGZ28 containing Hind III l.

Letters refer to specific fragments, ◁ denotes missing fragment, * denotes novel fragment.


Bgl II
HG52
30j
HG52 x21
ab
cdef
ghi
j
kl
mn
o
p
q
r
Bgl II
BamHI b'
30k
HG52 x21
a
e
k
*
Bgl II
BamHI a'
30l
HG52 x21
a
b
e
f
k
m
Bgl II
Hind III 1
30m
HG52 x21
l
q
*
BamHI
HG52
30n
HG52 x21
ab
dcef
g
hij
k
l
mn
o
qpr
st
uvwx
yz
a'
b'c'd'
e'
f'
g'
BamHI
BamHI b'
30o
HG52 x21
l
*
b'
BamHI
BamHI a'
30p
HG52 x21
z
a'
BamHI
Hind III 1
30q
HG52 x21
l
*
c'd'
e'
f'

the Bgl II and BamH I fragments detected by the BamH I b' probe were $0.1x10^6$ daltons larger. The HG52X163X21 EcoR I fragment detected by BamH I b' was approximately $0.2x10^6$ daltons smaller. The HG52X163X21 Hind III fragment of $1x10^6$ daltons detected by BamH I b' was, however, approximately one quarter of the size of the novel HG52X163X14 Hind III fragment.

Using BamH I a' to probe a Bgl II digest of HG52X163X21 (Fig. 30l), the k band and the two k containing joint fragments, a and e, were all missing. The only bands detected were the Bgl II m band and its two joint fragments b and f (due to hybridization to internal inverted repeat sequences). A BamH I digest of HG52X163X21 (Fig. 30p) showed positive hybridization of BamH I a' to BamH I z while a' itself was completely absent. Therefore, like HG52X163X14, HG52X163X21 is missing the DNA contained in the unique portion of BamH I a'.

When Hind III l was used as a probe against a Bgl II digest of HG52X163X21 (Fig. 30m shows a blot from a different gel with slightly different separation), the pattern observed was very similar to HG52X163X14 with the l and q bands appearing as normal plus a novel band of approximately $2.8x10^6$ daltons below o. This was the same band as that detected by the BamH I b' probe. When a Hind III l probe was directed against a BamH I digest of HG52X163X21 (Fig. 30q), there was normal hybridization to the e', f', l, c' and d' bands and to a novel band of approximately $2.6x10^6$ daltons running with BamH I p. This was the same fragment as was detected by a BamH I b' probe.

The most likely explanation is the introduction of a Hind III and an EcoR I site at the novel Xba I j/h site at

0.94m.c.. The fact that the novel HG52X163X21 Bgl II and BamH I bands were slightly larger than the equivalent novel HG52X163X14 bands suggests that there has been a small insertion of DNA containing a Hind III and an EcoR I site. Figure 30r shows a diagram of the proposed structure of HG52X163X21.

It appears that HG52X163X21, like HG52X163X14, is deleted in genes US10, 11, 12 and US9 while gaining a second copy of US1, 2, 3 and 4 and the coding region of US5. Additionally there is loss of the 0.91m.c. Xba I site which lies within the coding region of gG2 and the insertion of sequences around 0.94m.c..

3.4d GROWTH CHARACTERISTICS OF HG52X163X12, HG52X163X14 AND HG52X163X21

The three variants were compared in one step growth experiments at 31^{o}, 37^{o} and 38.5^{o}C with the wild type strain HG52 and the parental strain HG52X163 (Fig. 31).

At 31^{o}C the viruses grew at a rate and to a final yield comparable to that of the parental HG52X163 and the wild type HG52. At 37^{o}C the three variants showed similar rates of growth but grew slightly less well than either the wild type or the parental strain. At 38.5^{o}C the relative patterns of growth of the variants were different to those observed at either of the lower temperatures. As previously noted the wild type strain, HG52, is slightly impaired in its growth at the elevated temperature. The variant HG52X163X14, at 38.5^{o}C, gave a pattern of growth almost identical to that of HG52 with the parental strain HG52X163 growing slightly less well. However, the two variants, HG52X163X12 and HG52X163X21 demonstrated marked impairment of growth at 38.5^{o}C with a

Figure 30(r)

Proposed model for the structure of HG52X163X21 compared to HG52 showing the positions of the various restriction endonuclease sites. The proposed novel fragments created are indicated by vertical arrows and their composition and expected molecular weights are shown on the right.

▽ denotes the position of the proposed insert containing an EcoR I and Hind III site.

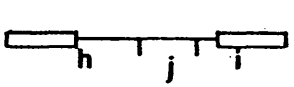
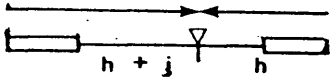
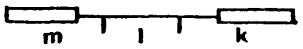
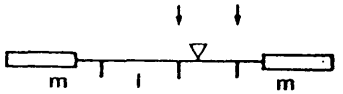
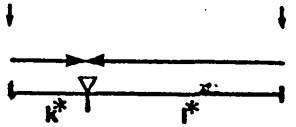
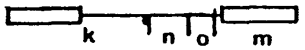
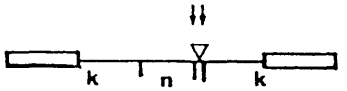
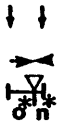
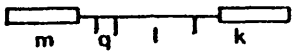
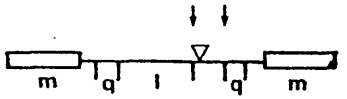
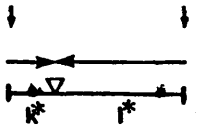
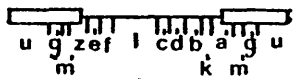
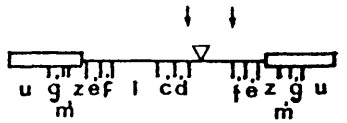
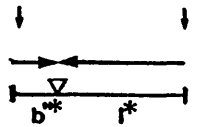
	HG 52	HG52 X163 X21	M.W. x10^6	
Xba I				
Hind III			1·0 +3·0	
EcoR I			0·4 +0·1	
Bgl II			2·8	
BamH I			2·6	

Figure 31

One step growth curves of HSV-2 strain HG52 ● , HG52X163 ■ , HG52X163X12 ○ , HG52X163X14 ▽ and HG52X163X21 □ in BHK21/C13 cells at 31°, 37° and 38.5°C. Cells were infected at a MOI of 5pfu/cell. After absorption for 45min at the appropriate temperature, the monolayers were washed twice with phosphate-buffered saline, overlaid with ETC10% and incubated at the appropriate temperature. Cultures were harvested at the times indicated and virus titres ascertained by plaque assay on BHK21/C13 cells.

To allow comparison zero hour samples were standardized to that of HG52. Actual zero hour values are marked on axis.

38·5°C
37°C
31°C
Virus yield p.f.u. per 10⁷ cells
Time after infection
h
24h
12
8
6
4
2
0
10⁷
10⁶
10⁵
10⁴
10³

24h yield substantially less than the titre of virus immediately post absorption. These growth properties were borne out during the preparation of stocks of the variants. High titre stocks of HG52X163X12, HG52X163X14 and HG52X163X21 were obtained from low multiplicity infections incubated at 31°C. However, when titrated at 31°C and 38.5°C there was evidence of temperature sensitiviy.

	31°C titre (pfu/ml)	38.5°C titre (pfu/ml)
HG52X163	5.1×10^8	1.5×10^8
HG52X163X12	7.7×10^8	$<10^4$
HG52X163X14	1.0×10^8	4.0×10^6
HG52X163X21	1.3×10^8	$<10^3$

HG52X163X12 and HG52X163X21 appeared to be temperature sensitive while HG52X163X14 was impaired at the higher temperature. The particle: pfu ratios of the stocks used in the growth experiments, except for HG53X163, were all rather high:

HG52	1,263: 1
HG52X163	24: 1
HG52X163X12	1,217: 1
HG52X163X14	1,826: 1
HG52X163X21	2,631: 1

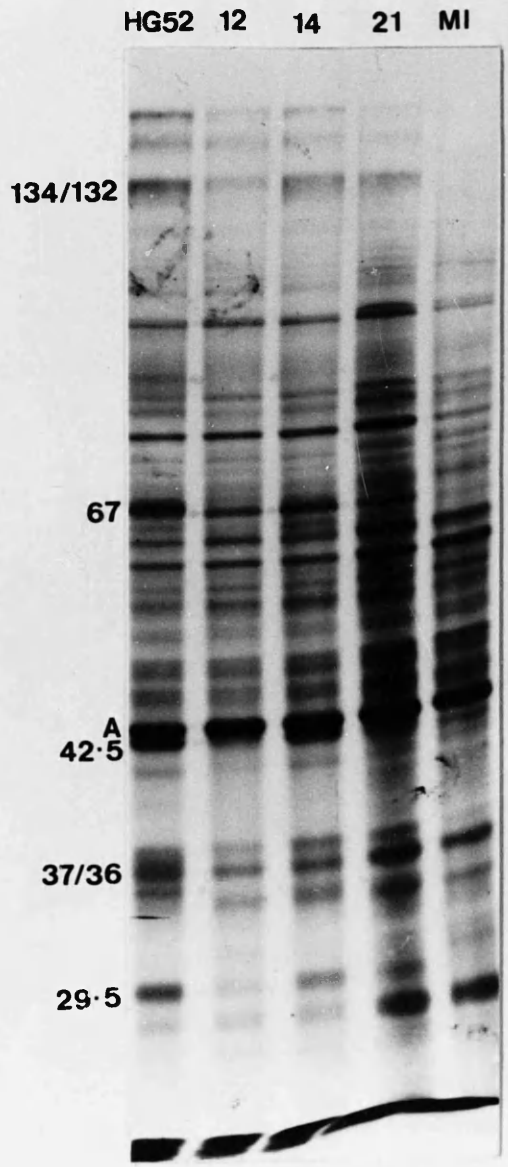
3.4e INFECTED CELL POLYPEPTIDES INDUCED BY VARIANTS ISOLATED AFTER TRANSFECTION OF Xba I TREATED HG52X163 DNA (ie. HG52X163X12, HG52X163X14 and HG52X163X21).

Figure 32 shows general infected cell polypeptide extracts labelled with ^{35}S-methionine run on a denaturing gel. Unfortunately the infections appear poor, however, the only difference apparent between the three variants and the

Figure 32

Autoradiograph of infected cell polypeptides induced in BHK21/C13 cells and separated by SDS-PAGE. Cells were infected at 38.5^{o}C, ^{35}S methionine labelled (3-20h PI) and polypeptides analysed on a 7.5% acrylamide gel.
Lane 1 HG52, lane 2 HG52X163X12, lane 3 HG52X163X14, lane 4 HG52X163X21 and lane 5 mock infected. Numbers show the apparent molecular weights (x10^{3} daltons) of HSV-2 infected cell polypeptides. A represents actin.

32



wild type is the altered mobility in the 37/36kd region of the gel. The altered mobility of bands in this region has already been shown in HG52X163 the parental genome of the three variants (see Fig.18). Further polypeptide analysis of these variants has not been carried out.

CHAPTER 4

DISCUSSION

The original aim of this project was to isolate Xba I site deletion mutants of HSV-2 strain HG52 and to use restriction enzyme sites as unselected markers in recombination studies between otherwise homologous genomes. The intention was to generate HG52 genomes lacking the four Xba I sites (two in U_L and two in U_S) present in the wild type genome and, in conjunction with selected temperature sensitive markers to ascertain (1) the contribution of parental and progeny molecules (2) the possible presence of "hot spots" of recombination and (3) the role (if any) of particular genes in recombination. The same markers could also be used to study intertypic recombination between HSV-1 and HSV-2 where lack of homology between the parental genomes and the variable selective fitness of the generated recombinants would be expected to influence the frequency of generation and the viability of such recombinants.

It has long been known that HSV undergoes intratypic and intertypic recombination in productive infection in cultured cells (Brown et al., 1973; Schaffer et al., 1973, 1974; Brown and Ritchie, 1975; Timbury and Subak-Sharpe, 1973). Two factor recombinational frequencies of 2-40% have been demonstrated in HSV using selected and unselected markers (Honess et al., 1980). Following the suggestion by Brown et al. (1984) that restriction enzyme sites could be used as non-selected markers, recombination studies using two strains of HSV-1 differing in eight restriction enzyme sites were performed and demonstrated an overall recombination frequency of 0.007/kbp with no region of the genome being

shown to be more recombinogenic (Umene, 1985). It is probable, however, that in order to detect possible "hot spots" of recombination it would be necessary to have more frequent markers.

The contributions of viral and host cell factors in the mechanisms of HSV recombination are poorly understood as is the role of parental and progeny genomes. In pseudorabies virus it was deduced on the basis of density labelling experiments that parental molecules are mainly involved in recombination (Ben-Porat et al., 1982). The data for HSV-1 from triparental crosses and time course experiments, however, pointed to a contribution from progeny molecules in recombinational events (Ritchie et al., 1977). This has been substantiated by recombination studies involving Xba I site deletion mutations of HSV-1 as non-selected markers (MacLean, 1988). Using ts markers at 0.41-0.42m.c. and 0.74m.c. to select for recombinant progeny molecules which grew at 38.5^{o}C, the non-permissive temperature for the parental genomes, it was found that at 4h PI >90% of recombinants had only one detectable crossover, whereas, at 24h PI >60% had two or more crossovers. These figures support the theory that progeny molecules must be playing a role in recombination to generate the genomes with multiple crossovers. The fact that at 2h PI there was no detectable recombination, whereas, low levels were apparent by 4h PI indicates that in HSV, DNA replication may be required to allow detectable levels of recombination.

The recombinational frequency between markers approximately 0.2m.c. apart appeared to reach a maximum of approximately 15% after 24h in experiments selecting for genomes crossing over between 0.42-0.74m.c.. The surprising

finding was that, of the 15%, half had recombined between markers approximately 0.2m.c. apart outwith the region of selected recombination. Analysis of the same recombination experiment at 31°C to obtain figures for non-selected recombination (A. MacLean, personal communication) showed that there were recombinational frequencies of approximately 19% between each of the markers which were approximately 0.2m.c. apart. This proves that the high level (ie 50%) of recombination observed outwith the selected region of recombination at 38.5°C is not due to there being a higher tendency towards recombination in these regions. The proposal is, therefore, that some feature of the recombinational process means that genomes which crossover at one point have an increased chance of recombining elsewhere. The simplest explanation is that the alignment of genomes which would allow a single recombination event would predispose the genomes to other crossovers.

The use of restriction enzyme sites as non-selected markers in recombination studies has a number of advantages. Disruption of restriction endonuclease recognition sequences due to single base pair changes should allow the study of recombination between essentially homologous genomes. Large numbers of markers can be created which unlike conditional lethal mutations need not be confined to essential regions. There is also no 'leakiness'; the presence of a restriction site being an all or nothing condition. The nature of alterations causing disruption of restriction enzyme site recognition also means that reversion is liable to be rare and, as the markers are not selected for, amplification of the progeny of such events should not occur.

The main constraint on using enrichment selection as a

method of obtaining site deletion mutants (apart from the probability of non-conservative alterations causing the site loss) is the limit on the number of sites. As the number of sites increases, the chance of any virus remaining viable after digestion of its DNA is reduced. For this reason Xba I, which makes only four staggered cuts in HG52 DNA, was chosen. Preliminary experiments were attempted using the endonuclease Hpa I, which also only cleaves HG52 DNA in four places, but these proved unsuccessful. It was thought that as Hpa I creates blunt ended fragments this might reduce the chance of their ligation to give viable virus and further attempts were abandoned.

The infectivity efficiency of the DNA was crucial to the chances of obtaining successful results from enrichment selection experiments. F. Rixon (personal communication) demonstrated that stocks of HSV-1 DNA prepared in his laboratory contained over 60% fragmented and, therefore, non-infectious molecules. If this level of fragmentation is present in most HSV DNA stocks it would explain the general difficulty in obtaining viable virus after enrichment selection and suggest that purification to obtain unit length genomes prior to treatment should increase the efficiency.

As HG52 DNA has only been partially sequenced the exact positions of the Xba I sites and whether they lie within coding regions or are intergenic is not known in every case. The finding that variants lacking all four restriction sites were temperature sensitive means that further work would be required to isolate non-temperature sensitive mutants if selected temperature sensitivity markers were to be introduced. It is possible, however, that experiments to

study non-selected recombination using the ts Xba I site deleted mutant could be performed. The initial aim of the project to use site deletion mutants in recombination experiments was not pursued, however, because of a change in emphasis of the work due to the unexpected discovery of a large number of variants amongst the individual plaque isolates being screened for site loss.

4.1 LOSS OF Xba I SITES

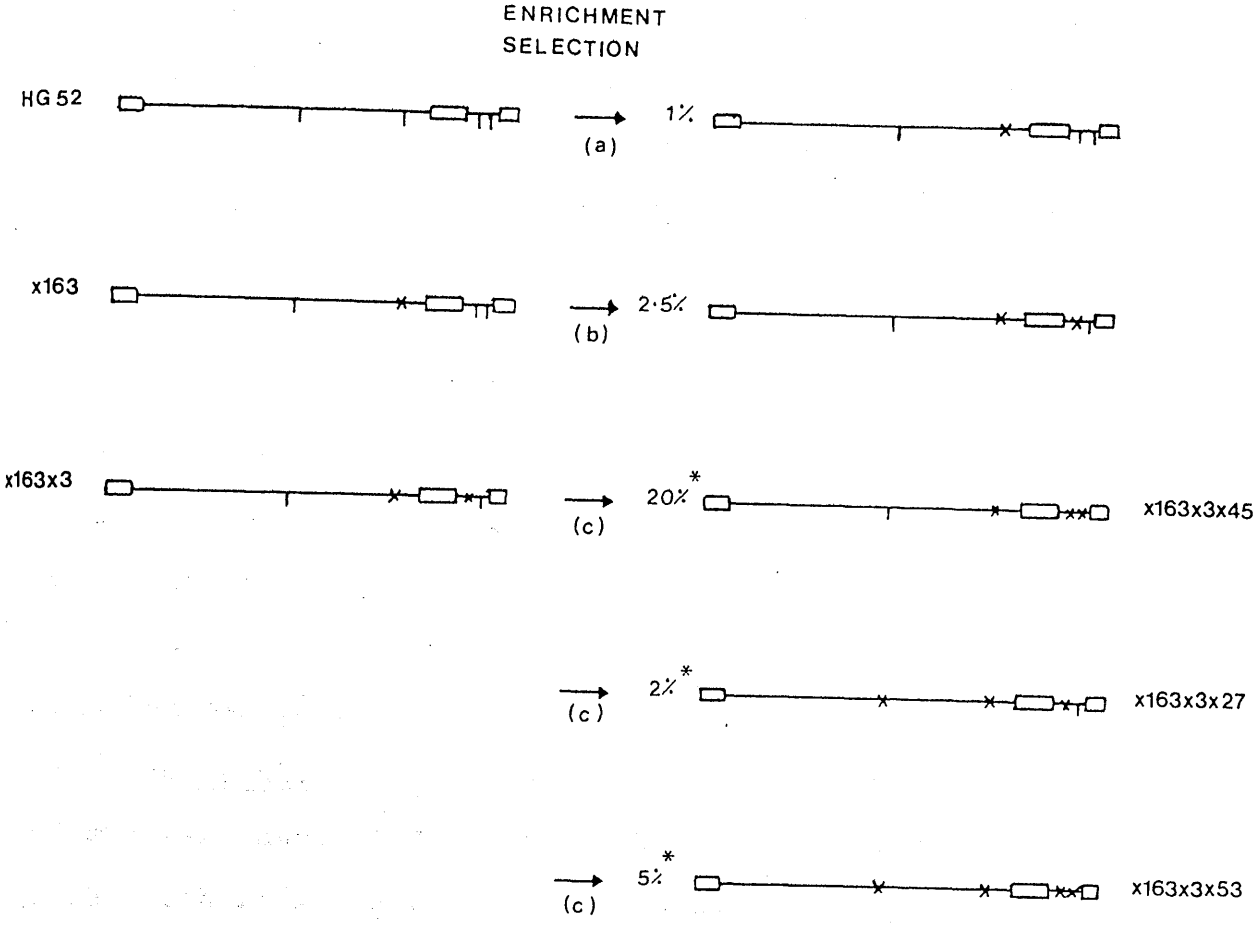
Figure 33 represents diagramatically the frequency of isolation of site deletion mutants following various rounds of enrichment selection. Of two hundred plaques picked after transfection of partially Xba I digested wild type HG52 DNA, two were found to lack the Xba I g/d site at 0.7m.c. (ie a frequency of 1% isolation). When treated in a similar fashion the mutant HG52X163, containing three Xba I sites, yielded one isolate lacking the 0.91m.c. Xba I site from forty plaques picked (a frequency of 2.5% isolation). When the DNA of HG52X163X3, containing two Xba I sites, was treated with Xba I and transfected, one of the sites at 0.94m.c. was lost independently at a frequency of 20%. The 0.45m.c. Xba I c/d site was lost at a frequency of 2%. The frequency of isolation of genomes lacking both of the remaining Xba I sites was 5%.

The above data prompt a number of observations. Firstly, the region of DNA surrounding the 0.7m.c. Xba I g/d site is probably non-essential in lytic growth as variants lacking the 0.7m.c. site were isolated easily and the site losses were associated with a deletion of approximately 150bp and an insertion of approximately the same size. The DNA sequence around the 0.7m.c. Xba I site in HG52 is not known.

Figure 33

This diagram shows the frequency of isolation of Xba I restriction enzyme site deletion mutants during three rounds of enrichment selection. The four Xba I sites are represented as vertical lines; deleted sites are depicted as a cross. Asterisks denote the possibility of certain plaques being clonally related.

The digestion conditions for the three rounds of enrichment selection were (a) 1 unit of Xba I per ug of DNA, digested for 1 or 2h at 37^{o}C, (b) 2 units of Xba I per ug of DNA, digested for 2h at 37^{o}C and (c) 1 unit of Xba I per ug of DNA, digested for 1, 3 or 4.5h at 37^{o}C.


ENRICHMENT
SELECTION
HG 52
1%
(a)
x163
2·5%
(b)
x163x3
20%*
(c)
x163x3x45
2%*
(c)
x163x3x27
5%*
(c)
x163x3x53

In HSV-2, strain 333, the equivalent Xba I site is close to but outwith the coding region of the 65K molecular weight trans-inducing factor (G. Hayward personal communication).

Alterations at the 0.91m.c. and 0.94m.c. Xba I sites causing loss of the recognition sequences without affecting the viability of the viruses were also detected. The 0.91m.c. site is known from sequence data (McGeoch et al., 1985) to lie near the 3' terminus of US4 within the coding region of gG2. Investigation of the gG2 production by the variants lacking the 0.91m.c. Xba I site was felt to be beyond the remit of this study. However, further experiments were performed (Harland and Brown, 1988) and synthesis of gG2 in variants lacking the 0.91m.c. Xba I site was analysed by immunoprecipitation using gG2 specific antibodies. The monoclonal antibodies AP1 and LP5 (Marsden et al., 1984) with unknown epitopes and the anti-peptide serum 14713 (McGeoch et al., 1987) directed against the carboxy terminus of gG2 were used. The mutant HG52X163X3X53 and HG52X163X3 from which it was derived both produce an aberrant form of gG2 precursor, which is either larger than normal or has a different tertiary structure, and which is processed to give a correspondingly aberrant form of gG2. The fact that this aberrant form was recognized by AP1/LP5 but not by 14713 led to the suggestion that the sequence alteration causing loss of the 0.91m.c. site, created a frame shift such that the stop codon for gG2 is no longer read and another stop codon in another frame downstream is used instead. With the variant HG52X163X21 neither gG2 nor its precursor was precipitated by either the monoclonal antibody mixture AP1/LP5 or by the anti-peptide serum 14713. In this case it was proposed that the loss of the Xba I site at 0.91m.c.

might have introduced a stop signal such that a rapidly degraded, truncated form of gG2 is made. The viability of these variants demonstrates that gG2 is non-essential in tissue culture which is supported by the viability of HSV-1 variants lacking US4 (Longnecker and Roizman, 1987).

The apparent ease of isolation of variants lacking the 0.94m.c. Xba I site was not unexpected as it was deduced from sequence data (Whitton, 1984) to be in an intergenic region.

The low frequency of isolation of site deletion mutants at the 0.45m.c. site indicates that changes in this region are liable to be deleterious. This observation is supported by the finding that non-clonally related 0.45m.c. Xba I site deletion mutants all proved to be temperature sensitive. There are no sequence data available for the DNA spanning the 0.45m.c. Xba I site in HG52. However, it is coincident with an Xba I site in HSV-1 strain 17 which has been sequenced and shown to lie within gene UL33 which codes for a polypeptide of predicted molecular weight 14k (McGeoch et al., 1988b). This polypeptide, thought to be involved in DNA packaging, has been shown by mapping of a temperature sensitive mutation to be an essential protein (Al Kobasi, 1989). Mapping of the temperature sensitive lesion in HG52X163X3X53 to confirm that it was associated with the Xba I site loss at 0.45m.c. was attempted by marker rescue with a number of cloned Hind III fragments of HG52 including Hind III e which spans the 0.45m.c. Xba I site. The attempts were unsuccessful and were abandoned for want of time.

A non-temperature sensitive variant of HSV-1 strain 17 lacking the 0.45m.c. Xba I site was isolated by enrichment selection (MacLean and Brown, 1987a). There are various

possible explanations to account for this difference observed between HSV-1 and HSV-2. (1) As the type 2 DNA sequence is not known it is possible that the types 1 and 2 viruses encode differently in that region or that in HG52 the 0.45m.c. Xba I site lies within an adjacent gene. (2) It is possible that the type 1 variant (even if the Xba I site is at the same position within the coding sequence of the same 14k polypeptide) was isolated fortuitously as the result of a conservative change although this would not explain the difference in the frequency of site loss. (3) Possibly the 0.45m.c. Xba I sites in HG52 and strain 17, whilst being contained in the coding sequence of an equivalent 14k polypeptide are not in identical positions within the coding region. The relative ease of isolation of mutants lacking the 0.45m.c. site in HG52 (at least 4% independently generated all of which were temperature sensitive) compared to the difficulty in obtaining the equivalent variant of HSV-1 strain 17 where the isolation frequency was approximately 0.1% (MacLean, 1988) supports the view that the Xba I site in HG52 is probably in a less essential position.

The relatively frequent isolation of mutants lacking both the 0.45m.c. and 0.94m.c. Xba I sites from HG52X163X3 compared to those lacking only the 0.45m.c. Xba I site can be explained by the selective pressure exerted by partially digesting the DNA with Xba I prior to transfection. The fact that molecules lacking all four Xba I sites have an improved chance of surviving the enrichment selection procedure compared to those with only one site supports the view that even partial digestion of the DNA does confer selective pressure for the isolation of site deletion mutants. It is

apparent, however, that the technique would only be useful in isolating site deletion mutants of enzymes which cleave the DNA infrequently as the isolation of viable mutants will decrease with increasing numbers of cleavage sites. There would also be a markedly reduced possibility of isolating site deletion mutants if the sites lie within the coding regions of essential genes.

Another factor which might have a bearing on the frequency of isolation of restriction enzyme site deletion mutants following partial digestion of the DNA is that not all sites are cleaved with equal probability. During the course of this study a number of partial digests was inadvertently produced. The most commonly observed Xba I partial digest band of approximately 10×10^6 daltons ran between Xba I g and h (see Fig. 19a). This band, composed of Xba I j plus i indicates a failure to cleave at the 0.94m.c. Xba I j/i site. When this band was present there was always another of approximately 21×10^6 daltons (running above e) which is a joint fragment composed of j+i+g. This indicates that partial digestion with Xba I might not favour selection of mutants lacking the 0.94m.c. site as much as those lacking the other three Xba I sites. There must be a complex interaction of different forces influencing the selection of site deletion mutants during enrichment selection. Partial digestion may favour selection of mutants with loss of sites which are normally cleaved first. However, this effect is liable to be overshadowed by factors influencing the viability of any mutants produced, for example, whether or not the site lies within an essential region. Therefore, although partial digestion with Xba I during enrichment selection may not favour the selection of mutants lacking

the 0.94m.c. site to the same extent as the other sites, the fact that the site is within a non-coding region would increase their frequency of isolation.

Enrichment selection as a method of isolating restriction enzyme site deletion mutants cannot ensure the isolation of mutants with conservative changes. As was demonstrated, site loss in non-essential regions (eg the 0.7m.c. Xba I site) may be associated with sizable deletions or insertions which would affect homology in recombination studies. Viable genome structures containing alterations producing site loss in essential genes may tend to be very limited. However, even single base changes in essential genes may profoundly affect the growth characteristics of such mutants making them less useful in recombination studies. The only significant change in growth characteristics observed amongst the Xba I site deletion mutants was the temperature sensitivity of HG52X163X27 and HG52X163X3X53 at 38.5^{o}C. Marker rescue experiments failed to prove that the temperature sensitivity was due to the alteration leading to loss of the 0.45m.c. Xba I site. Circumstantial evidence, however, points to the temperature sensitivity being due to alteration at 0.45m.c. in that every isolate lacking the type 2 0.45m.c. Xba I site, including an intertypic recombinant, R12-5X13 (Brown et al., 1984), proved to be temperature sensitive.

The Xba I site deleted mutants isolated following enrichment selection should yield some useful data about intratypic recombination frequencies in HG52. It should even be possible using the temperature sensitive Xba I site deleted mutant, HG52X163X3X53, to perform unselected recombination studies at 31^{o}C although, the lack of any

selection would mean that studies would probably have to be confined to experiments where a reasonable amount of recombination would be expected to occur. Until the HG52 genome is fully sequenced it will not be possible to lose restriction endonuclease sites by site directed mutagenesis where conservative changes could be engineered by recombination with oligonucleotides synthesized with a selected single base pair mismatch. The use of directed mutagenesis would also allow the creation of many more markers along the length of the genome by engineering mutants lacking frequently cutting restriction endonucleases.

4.2 VARIANTS FROM Xba I TREATED HG52 DNA

When HG52 DNA was partially digested with Xba I and transfected, approximately 5% of the progeny exhibited alterations from the wild type structure on screening with Xba I. The largest category of variants consisted of genomes with different sized deletions from IR_L. The observed deletions ranged in size from $1x10^6$ daltons to $6x10^6$ daltons removing the entire internal long repeat. The variant HG52X86 has a deletion of $2.5x10^6$ daltons from IR_L including the BamH I f/p site at 0.775m.c. The variant HG52X85/5 has a deletion of $3.7x10^6$ daltons including the BamH I f/p site. The deletion from the variant HG52X85/4 removes more than $6x10^6$ daltons from IR_L (ie almost the whole of IR_L excluding 'a' sequences). It is not known for any of these variants whether or not the deletion extends beyond the U_L/IR_L junction to remove any unique sequences although the Hind III j/o site at 0.76m.c. and the Hpa I e/f site 28bp to its left were present in each case. The variant HG52X192

which was deleted by $1x10^6$ daltons from IR_L had an equivalent deletion from TR_L. The variant HG52X19 had the most complicated structure with a deletion removing all of IR_L and approximately half of IR_S (ie $9x10^6$ daltons). In place of the deleted sequences a $1x10^6$ dalton sequence was inserted which was reiterated between 1 and 14 times to yield genomes varying in size from approximately $8x10^6$ daltons less than the wild type to approximately $5x10^6$ daltons larger than the wild type genome.

All but one (ie the site deletion mutant HG52X94) of the deletion variants isolated following enrichment selection of HG52 exhibited deletions from IR_L. This observation is biased by the fact that only Xba I was used for initial screening and the large size ($40x10^6$ daltons) of the long terminal fragment Xba I c would have precluded the detection of deletions. The similarity between the deletions in the isolated variants begs the question whether their generation might be connected, for example, by recombination between deleted and wild type genomes. In the case of HG52X85/4 and HG52X85/5 it seems quite probable that there was some common factor in their generation as they were originally isolated together in a single plaque. However, many of the variants were generated independently suggesting that the inverted repeats are either more prone to deletion than most of the unique sequences or that being present in diploid amounts deletion of one copy has less effect on viability. The latter suggestion, however, does not explain why such deletions should be more common than deletions removing any non-essential portion of unique DNA. The majority of HSV deletion variants described are deleted in one of the repeats and the adjacent unique sequences (Brown et al.,

1984; Harland and Brown, 1985; Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987; MacLean and Brown, 1987b, c). It appears probable, therefore, that a functional or structural feature of the repeats makes them more prone to deletion than unique sequences. In HSV-1 strain 17 three groups of tandemly reiterated sequences of high G+C content have been described within the TR_L/IR_L repeat region (Rixon et al., 1984; McGeoch et al., 1988b) which may allow illegitimate recombination giving rise to deletions. As the repeat regions of HG52 have not been fully sequenced it is not yet known if similar reiterations occur in the type 2 genome.

The view that the generation of deletion variants may be related is supported by the observation that the variants isolated after the different rounds of enrichment selection appear to be related to each other yet different to those from other rounds. The first round in which HG52 DNA was partially Xba I digested and transfected yielded genomes with deletions from IR_L and TR_L. The second round yielded a number of variants with alterations in the short region. The third round yielded a large number of variants with altered genomes whose structures have not yet been analysed.

In wild type strains the unique regions are normally bounded by identical repeats indicating that there is pressure towards homology of the repeat regions (Sacks and Schaffer, 1987; Stow and Stow, 1986). The pressure towards homology was first reported by Knipe et al., (1978) who noted that ts mutants in IE3 carry the mutation in both copies of the gene and that sequence modifications in the short repeat appear in both copies if the modifications occur near to the 'a' sequence. The data suggest that

recombination between the inverted repeats occurs at high frequency quickly reinstating homology following small scale alterations in a single copy. For this reason it was thought that the deletions of $1x10^6$ daltons from TR_L and IR_L of HG52X192 were probably identical. This is supported by subsequent marker rescue experiments in which the internal copy of the deletion in HG52X192 was rescued with a wild type joint spanning fragment causing both copies of the long repeat to revert to the wild type structure (Taha et al., 1989a). More recently sequence data have shown that the 1.5kb deletion from each end is identical (Taha et al., 1989b). Wild type genomes or double deletion variants were never detected in the stocks of the single deletion variants HG52X85/4, HG52X85/5 and HG52X86. As a very high level of recombination might be required for such variants to be apparent in the overall population, experiments designed to facilitate self crossing were performed. Each variant was plated out on BHK21/C13 cells at 5pfu/cell and the 24h yield titrated out to pick 50 well separated plaques. Of the 150 plaques analysed, only one (from HG52X86) had reverted to the wild type profile. No double deletion variant was found. The failure to detect recombination between the repeat regions of genomes with larger deletions giving progeny with the same information deleted from each end might be due to the inviability of such molecule. Viruses lacking large portions of sequence from both long repeats could be inviable either because of the loss of both copies of an essential gene or because of the constraints on the size of packaged genomes. However, neither of these explanations would account for the failure to detect the wild type structures which would be the reciprocal progeny of any such

recombination.

The HSV-1 sequence data has conclusively identified only one gene (IE gene 1) within the long repeat region (Perry and McGeoch, 1988). This gene encodes the polypeptide VmwIE110 which has been shown to be non-essential by the viability of a deletion variant lacking both copies of IE1 (Stow and Stow, 1986). It is probable that the type 2 equivalent polypeptide VmwIE118 is also non-essential and that deletion of both copies although probably impairing growth should not prohibit it entirely. During latent infection, transcripts from the long repeat regions of HSV have been identified, which are not detected in lytically infected cells (Stevens et al., 1987). These transcripts termed LATs have been mapped to the opposite strand to the IE1 gene starting downstream but with the 3' terminus overlapping IE1. The significance of these transcripts remains unclear as recent evidence suggests that they are non-essential for the establishment or maintenance of latency although there is some evidence that reactivation from latency of variants lacking the LATs may be reduced in frequency and delayed (Leib et al., 1989b, Steiner et al., 1989).

The presence of a gene within the long repeat region conferring neurovirulence has been reported by Thompson et al. (1983). The gene, located between 0.71 and 0.83m.c. was described in the intertypic recombinant RE6. The variant HG52X192, although showing no significant growth impairment in vitro has subsequently been shown by other workers to be almost completely avirulent in mice (Taha et al., 1989a). Their work clearly demonstrates that deletion of sequences between 0-0.02 and 0.81-0.83m.c. from HG52 has a marked

effect on neurovirulence in mice indicating the presence of such a gene. The viability of HG52X192 demonstrates that the putative gene must be non-essential *in vitro*. It appears unlikely, therefore, that the failure to isolate genomes with large deletions from both copies of R_L is due to the presence of essential genes.

It has been demonstrated that deletions of 15kb can occur from HSV-1 (F) genomes without affecting their viability (Poffenberger *et al.*, 1983). Therefore, the limit on genome size cannot be the only constraint on the generation of genomes with large deletions from both copies of R_L. Duplication of the deletions present in IR_L of HG52X85/5 and HG52X86 would result in overall reductions of only 12kb or less. The fact that recombination apparently occurs in HG52X192 to duplicate the small deletion near the external end of the long repeat but not in HG52X19, HG52X85/4, HG52X85/5 or HG52X86 where the deletions are larger may indicate that the amount of repeat retained on either side of the deleted portion is important in allowing recombination. The small portion of repeat on the terminal side of the deletion from HG52X192 may suffice for recombination as it contains the highly recombinogenic 'a' sequence. The deletions from HG52X19, HG52X85/4, HG52X85/5 and HG52X86 may all extend to include the U_L/IR_L junction. If this were the case then the right hand end of the deletion would efficiently create the novel U_L/IR_L junction. Even if the deletions are contained within the internal repeat there would be only a limited stretch of DNA to the left of the deletion homologous to the terminal repeat. It seems probable that it is the lack of inverted repeat information on each side of the deletion that reduces the

frequency of recombination leading to double deletions or wild type progeny. The appearance of two apparently wild type isolates (Fig. 23d lanes 5 and 6), detected following four rounds of plaque purification of the original isolate of HG52X19 needs to be explained. It is possible that these structures were present as the result of inefficient plaque purification helped by the growth advantage of the wild type virus compared to HG52X19. However, as four rounds of plaque purification should have ensured the removal of such contaminants it is possible that they represent recombinants. As the deletion from HG52X19 spans the L/S junction, any recombination event between unit length molecules could not restore the wild type genome structure. In order to generate wild type genomes, recombination would have to occur between circular or concatameric DNA. However, such recombination would be dependent on the deletion from IR_L not extending as far as the junction with U_L. If sufficient information were retained in IR_L to allow such recombination to occur then it seems probable that inversion of the long segment in this variant would also be observed. The ability of the short region of HG52X19 to invert, albeit inefficiently, despite a deletion removing approximately the terminal half of IR_S including the 'a' sequence also suggests that the internal portion of the short repeat may play a role in recombination. This supports the proposal by Varmuza and Smiley (1985) that segment inversion in HSV may occur as a result of recombination anywhere within the long or short repeats. The presence of short tandem reiterations near to the 'a' sequence has also been implicated with high frequency inversion in this region (Davison and Wilkie, 1981; Varmuza and Smiley, 1984). Longnecker and Roizman

(1986) have also reported that low frequency inversion occurs about a region of the repeat 5kb from the 'a' sequence.

The observation that, under immediate early conditions, the deletion variants HG52X85/5, and HG52X86 produce either no VmwIE64 or reduced levels, whereas the deletion variant HG52X85/4 produces wild type amounts is of interest. The deletions from HG52X85/5 and HG52X86 are both smaller than that from HG52X85/4 and are contained within the portion deleted from HG52X85/4. In each case the deletion ends approximately 1kb from the 3' end of UL54 encoding VmwIE64. The apparent anomaly that a variant with a deletion outwith the IE2 gene produces normal amounts of this polypeptide whilst variants with only part of the same region deleted exhibit reduced or delayed production requires explanation. In HSV-1, VmwIE63 has been demonstrated unequivically to be an essential polypeptide (McCarthy et al., 1989). It is probable, therefore, that functional VmwIE64 must be produced by the HSV-2 variants. MacLean and Brown (1987b) proposed that the HSV-1 deletion variant 1703 which does not synthesise detectable VmwIE63 under immediate early conditions might contain a secondary mutation within a promoter region of IE2 changing its gene category. This could explain why no VmwIE63 was detected at immediate early times despite the fact that some functional polypeptide must be produced. It seems coincidental, however, that variants isolated from both HSV-1 and 2 with deletions from IR_L/U_L stopping well short (at least 500bp) of the coding region of IE2 should all have secondary mutations within that gene.

A possible mechanism was suggested by J. McLauchlan (personal communication) to explain the failure of 1703 to

produce detectable amounts of VmwIE63 under immediate early conditions. The model proposed that as the deletion from IR_L of 1703 removed the 3' end of IE1 and extended into U_L to delete all of UL56 and part of UL55 a large mRNA would be produced under the control of the IE1 promoter. This mRNA would terminate at the end of UL51 which contains the next correctly orientated polyadenylation signal. The suggestion was that at immediate early times this would create antisense mRNA to genes UL52, UL53, UL54 and part of UL55 and that this could result in the observed reduction in the production of VmwIE63 under immediate early conditions. Initially this model also seemed to explain why HG52X85/4 produces wild type quantities of VmwIE64 whilst the variants HG52X85/5 and HG52X86 do not. The large deletion from HG52X85/4, because it removes the entire copy of IE1, would not result in the formation of antisense RNA to UL54 and hence would not affect production of VmwIE64 whereas removal of the 3' end of IE1 in HG52X85/5 and HG52X86 would support the antisense mRNA model. Recent sequence data (D. McGeoch, personal communication), however, do not substantiate the theory. A Hind III digest of HG52X85/5 showed that the o fragment was present proving that the Hind III o/j site is not deleted. The polyadenylation signal for UL56 is 21bp beyond the Hind III o/j site and therefore must be retained. As UL56 is in the same orientation as IE1 (see Fig.4) its polyadenylation signal should effectively stop the creation of antisense message to UL55, UL54, UL53 and UL52 running through from IE1. A Hind III digest of HG52X86 was not performed and although the Hpa I e/f site is present it is 7bp beyond the polyadenylation signal of UL56. Sequence analysis of the different variants both across the deletions

and through UL54 will be necessary before a satisfactory explanation is found for the aberrant VmwIE64 synthesis.

The variant HG52X19 had a deletion of $9x10^6$ daltons removing all of IR_L and approximately half of IR_S replaced by an inserted $1x10^6$ dalton sequence which was reiterated between 1 and 14 times. The ladder of different sized restriction endonuclease cleaved DNA fragments within a population of HG52X19 genomes was similar to that observed in the defective population of the HSV-1 strain Angelotti (Kaerner et al., 1981). The possibility that the ladders in HG52X19 might be due to the presence of defective virus was ruled out by restriction enzyme analysis conclusively proving that the ladders were integrated into the HG52X19 genome.

The deletion of $9x10^6$ daltons (13.5kb) from the L/S junction of HG52X19 is smaller by 1.5kb than that from a type 1 variant, 1358, described by Poffenberger et al.,(1983). The variant 1358 lacking almost the entire IR_L/IR_S region was generated by the insertion of a chimeric thymidine kinase gene containing sequences of Hind III o of strain F (from the left hand end of U_L) which unexpectedly recombined with a portion of IR_S rather than Hind III o as predicted. Roizman and Jenkins (1985) proposed that the large deletion might make such a genome attractive as a possible viral vector allowing the insertion of up to 24kb. The insertion of extraneous DNA into a similar deletion in HG52X19 supports this view although the stability of the recombinant may be in question. It seems probable, however, that the variable copy number of inserted sequences generated in HG52X19 is due to the nature of the inserted sequences rather than to the position of the insert in the

HG52 genome.

It is possible that the host cells may play a role in the instability observed in the HG52X19 insert. Post et al. (1981) described a HSV-1 recombinant, R316, with an insertion of BamH I n into the tk gene. This insert remained stable during passage in Vero cells, however, in other cell lines and in experimental animals deletions of various sizes were created (Sears et al., 1985).

The failure of the insert DNA in HG52X19 to hybridize to HSV DNA was unexpected. Subsequent sequence data from the insert showed that it was composed of bovine satellite DNA. This is not the first report of the introduction of extraneous DNA into HSV genomes as both viral and mammalian genes have previously been specifically introduced at precise locations (Shih et al., 1984; Roizman and Jenkins, 1985). It is different, however, in that the insertion of bovine satellite DNA occurred spontaneously as the result of recombination between HG52 DNA and calf thymus DNA used as a carrier during transfection.

Two recombinants, 1358 and RBMu2 (Poffenberger et al., 1983; Jenkins et al., 1985) both generated following insertional mutagenesis share certain features with HG52X19. 1358 has a 2kb insert containing the tk gene which replaces 15kb of deleted sequences spanning part of U_L and nearly the entire IR_L. RBMu2 which was generated by insertion of an alpha-tk mini-mu chimeric gene into the long component of HSV-1 either near or within the internal copy of the repeat is deleted by approximately 14kb from the internal inverted repeats. The fact that in HG52X19 a similar type of insertion/deletion event has occurred entirely spontaneously supports the suggestion made by Jenkins et al. (1985) that

some property of the repeats might mean that insertional mutagenesis in this region could lead to deletion of the inverted repeats.

It has been shown that 8 satellite DNAs compose 23% of the bovine genome (Gaillard et al., 1981). Bovine satellite 1.715 DNA is made up of reiterations of 1402 bp containing an EcoR I site (Pluciennicak et al., 1982). The EcoR I site in the inserted DNA in the HG52X19 genome delimits a fragment which is almost identical to the published sequence for 1.715 DNA. The likely origin of the bovine sequences incorporated into HG52X19 is the calf thymus DNA used as the carrier in transfection experiments. It is not apparent, however, if some property of the satellite DNA would make it more likely to recombine with viral DNA than other genomic bovine sequences. The relevance of the spontaneous recombination between viral and mammalian DNA sequences in terms of normal infection is hard to guage. Transfection experiments are artificial in that large quantities of purified DNA are introduced simultaneously onto cells which are subsequently treated with DMSO to allow entry of the DNA. It may be that in normal HSV infection there is either physical or temporal separation making recombination between viral and cellular DNA less likely to occur.

The deletion from HG52X19 removes the entire open reading frame of one copy of IE1 and extends to delete at least part of the coding sequence of one copy of IE3. The impairment in growth of HG52X19 compared to the unaltered growth of variants lacking one copy of either IE1 or IE3 is possibly significant as it has been shown in in vitro experiments that the equivalent HSV-1 polypeptides VmwIE 110 and VmwIE 175 act synergistically in mediating viral transcription activity

(Everett, 1984a). The insertion of extraneous DNA in place of the deleted sequences may also affect growth.

As the HG52X19 stock showed a very high particle: pfu ratio, an electron microscopic study was undertaken by J.Aitken to compare monolayers infected with HG52X19 or the parental HG52 for the proportion of empty/total capsids present. The proportion of empty/total capsids in the HG52 infected cells was 36% whereas in a parallel experiment those infected with HG52X19 showed only 23% empty capsids. This infers that in HG52X19 a high proportion of particles containing DNA is non-infectious which may explain its lability at high temperatures. It is possible that genomes containing reiterations of the insert sequence are less stable than those with fewer reiterations. As the mechanism involved in producing the reiterations is unknown it is conceivable that genomes with more than 14 copies of the reiterated sequence are generated but that constraints on the size of genomes which are packaged may prohibit their inclusion in virus particles although this would not explain the larger proportion of full but non-infectious particles.

Due to the inherent variability within the HG52 stock care is required when correlating changes in growth characteristics with observed changes in genome structure. Unless the growth pattern is outwith the normal range observed within the wild type population any difference from the wild type is liable to be due as much to the effect of sampling as to the genome alteration. The only deletion variant isolated following enrichment selection of HG52 which showed marked alteration in growth was HG52X19 which was impaired in single cycle growth at 31^{o}C, 37^{o}C and 38.5^{o}C.

4.3 WILD TYPE STRAIN VARIANTS

As it has been shown that transfection of DNA (Calos et al., 1983) or possibly pre-treatment with an endonuclease might cause mutation, single plaque isolates of untreated HG52 were analysed. A high proportion (24%) of the individual plaques, eight passes after the initial isolation demonstrated altered genome structures. Only two novel structures were present, both of which had been detected amongst the 200 plaques isolated following enrichment selection of HG52. The difference in the comparable detection rate after enrichment (only 2%) could be due to sampling variation or to some negative selective pressure during the additional rounds of growth involved in producing the DNA stock and its transfection. It seems unlikely that the enrichment selection procedure would bias the result in this way.

It is probable that the 10% of genomes represented by HG52/5 which were identical to HG52X86 were all derived from the same genome. This view is supported by the finding that the other variant, HG52/10, detected at 14%, contained a second unrelated mutation in all seven of the isolates. This secondary mutation was present in HG52X192 which points to it being clonally related to HG52/10. The 24% of altered genomes present by pass eight appear, therefore, to be derived from only two variants. This implies that either these variants were generated very shortly after plaque purification or else that if generated in later rounds they have been amplified during virus passage. In either case it is apparent that they have little selective disadvantage in tissue culture.

To establish if HG52 is intrinsically variable or if the variation observed within the elite stock was due to chance a plaque isolate which during screening exhibited a normal wild type genome structure was passaged and the DNA of single plaques analysed for alterations. Of fifty plaques isolated after four passes none exhibited DNA variation from the wild type. A temperature sensitive mutant, tsl, derived from and, therefore, essentially homologous to HG52 was also analysed. Again no variant was observed in a stock of tsl which had been treated identically to the HG52 stock. Both of these results support the view that the high proportion of variants in a stock of HG52 at pass eight is due to chance rather than a predisposition to mutation.

Variation within a stock of virus might be affected by factors other than the strain of virus in question. As it is known, for example, that recombination frequencies vary with different batches of BHK21/Cl3 cells (Taylor, 1976) it seems possible that external factors might also affect the chances of events causing the observed structural alterations. The significance of the relatively low incidence of variation detected in the majority of other wild type strains examined compared to HG52 is, therefore, hard to determine. It is clear, however, that HG52 is not unique as the type 2 strain 186 exhibited a low level of genome variation and the type 1 strain McKrae also contained a high proportion of variants. No conclusions can be drawn from the high variability observed in the McKrae stock as its history is virtually unknown.

The conclusion from these analyses is that at least a proportion of the isolated variants pre-existed in the HG52 stock and were, therefore, not generated as the result of

either the digestion of HG52 DNA with Xba I or by the subsequent mutagenic effect of transfection. The data demonstrate that there is a high degree of heterogeneity within the HG52 wild type stock after a small number of passages following plaque purification. Care must be taken when attributing alterations observed within subcloned populations to experimental procedures. It is possible to over estimate the mutagenic potential of procedures such as transfection if an assumption is made about the homogeneity of the stock and if the proper controls of picking equal numbers of plaques from untreated stock are not performed. However it is apparent that the alteration in HG52X19 in which bovine satellite DNA has been introduced into an HSV-2 genome must have occurred during the transfection procedure.

4.4 VARIANTS ISOLATED AFTER Xba I TREATMENT OF HG52X163 DNA

When DNA from the mutant HG52X163 lacking the 0.7m.c. Xba I site was partially digested with Xba I and transfected, apart from isolating a mutant, HG52X163X3, lacking the 0.94m.c. Xba I site, three variants HG52X163X12, HG52X163X14 and HG52X163X21 were also isolated.

The variant HG52X163X12 has a deletion removing sequences from approximately 0.94m.c. to 0.99m.c.. As the short region inverted normally it was assumed that the 'a' sequence portion of TR_S was still present. The DNA sequence is not known for the part of the genome deleted from HG52X163X12, however, the equivalent sequence is known for HSV-1. In HSV-1 the right hand end of U_S contains three 3' co-terminal genes, US10, 11 and 12 (McGeoch et al., 1985) which have been shown to have equivalent transcripts in HSV-2 strain HG52 (Whitton, 1984). US10 encodes a polypeptide of 33k

molecular weight (Lee et al.,1982) whose function is unknown. From sequence data US11 was calculated to code for a polypeptide of 18k molecular weight thought to be equivalent to two observed polypeptides of 21k and 22k molecular weight. These proteins were shown to have a preferential affinity for 'a' sequences and it was postulated that they might play a role in inversion or packaging (Dalziel and Marsden, 1984). As the equivalent HSV-2 gene can be deleted the US11 gene product cannot be essential for either function. This is supported by the isolation of similar deletion variants in HSV-1 (Longnecker and Roizman, 1986; Umene, 1986) and work by MacLean et al. (1987) showing that the binding of the 21/22k polypeptide to 'a' sequences was not apparent in vivo where there were different patterns of localization of the virus DNA and the 21k protein. US12 codes for an immediate early polypeptide, VmwIE12, of unknown function (Watson et al., 1979) which has been shown to have a type 2 equivalent VmwIE 12.5 (Marsden et al., 1982).

The deletion from HG52X163X12 removes almost the entire TR_S region excluding the 'a' sequence. This causes loss of one copy of ori_S (Stow and McMonagle, 1983) and one copy of IE3. Variants lacking a single copy of ori_S have been described in type 1 (Longneckner and Roizman, 1986) and a type 1 variant lacking ori_L has also been isolated (Polvino-Bodnar et al., 1987). It has been reported, however, that one copy of ori_S is essential (Smith et al., 1989). VmwIE182 is equivalent to VmwIE175 in HSV-1 (Morse et al., 1978). In HSV-1 it is known that the IE gene 3 product, VmwIE175, is an essential protein (Preston, 1979b; Dixon and Schaffer, 1980; Watson and Clements, 1980) and in vitro

experiments have demonstrated that in conjunction with VmwIE110 it has an enhancing effect on early polypeptide transcription (Everett, 1984a; O'Hare and Hayward, 1985a). The viability of both HG52X163X12 and HG52X19 demonstrate that one copy of IE3 suffices for viability in tissue culture.

The structures of HG52X163X14 and HG52X163X21 are similar involving deletion of sequences analogous to those missing from HG52X163X12. However, in the case of these two variants the deleted sequences have been replaced by sequences from 0.83-0.91m.c. (see Figs. 29r and 30r for models). These genomes, therefore, have two copies of the sequences between the IR_L/IR_S junction and approximately 0.91m.c. making this the novel short inverted repeat. These genomes, therefore, contain two copies of the genes US1, 2, 3 and 4 and the coding region of US5 whilst lacking US10, 11 and 12. Although similar, there are differences in the structures of HG52X163X14 and HG52X163X21. Duplication of sequences from the left hand end of the short region replacing sequences from the right hand end results in the unexplained generation of a novel Hind III and EcoR I site in HG52X163X21 which are not present in HG52X163X14. There is also loss of the 0.91m.c. Xba I site from HG52X163X21 which remains unaffected in HG52X163X14.

It is possible that the novel structures observed in HG52X163X14 and HG52X163X21 have arisen as the result of the enrichment selection procedure. As recombination occurred in each case at, or very close to, the 0.91m.c. and 0.94m.c. Xba I sites in Xba I treated DNA it seems possible that Xba I treatment of the DNA may have contributed to the generation of the variants. However, Umene (1986) has

described the isolation of a spontaneously generated variant, N38, of HSV-1 in which the short unique sequence 0.865-0.880m.c. has been converted to inverted repeat whilst unique sequence 0.937-0.955m.c. has been deleted. Sequence analysis proved that the variant, N38, had been generated by a recombination event between two 5bp stretches of homology (5'-CCCCG-3') arranged in inverted orientation.

The extended short repeats in HG52X163X21 and HG52X163X14 lend support to a mechanism proposed by Whitton and Clements (1984b) for the expansion of repeats by non-homologous recombination. In HSV-1 the 5' non-coding regions of US1 and US12 encoding VmwIE68 and VmwIE12 respectively are identical being within R_S while the coding regions within U_S are different. They found that the first translational initiation codon of US12 has the A of ATG as the first base in U_S and they speculated that it defines the R_S/U_S junction by acting as a buffer preventing expansion of R_S. They also predicted that crossover beyond the 3' termini of the genes would lead to the generation of genomes in which only one of the genes US1 or US12 was retained.

The isolation of HG52X163X12, HG52X163X14 and HG52X163X21 demonstrates that the type 2 equivalents of US10, 11 and 12 can be deleted from HG52 without loss of viability although there is some impairment in growth at elevated temperatures. In addition HG52X163X12 retains only a single copy each of IE3 and ori_S. Although not proven it seems probable that the impairment in growth may be a consequence of the deleted genes. Further work needs to be done to ascertain the possible consequences of such deletions on growth both *in vitro* and *in vivo*.

FUTURE PROSPECTS

Before studying recombination in HG52 it will be necessary to investigate the mutation in HG52X163X3X53 which renders it temperature sensitive and by marker rescue, if possible, to isolate a non-temperature sensitive Xba I site lacking mutant. Identified temperature sensitive markers could then be introduced by marker rescue at known locations. The use of distant temperature sensitive markers would allow the selection of recombinant progeny from crosses between Xba I site deletion mutants and wild type virus. Alternatively unselected recombination between HG52X163X3X53 and HG52 could be analysed. The distribution of Xba I sites in HG52 (two in U_L and two in U_S) allows the contribution of the four genomic isomers in recombination to be assessed. At present the nucleotide sequence for much of HG52 remains unknown. However, as sequence data become available it would be possible to introduce selected single base substitutions disrupting the restriction enzyme recognition sequences. The use of synthetic oligonucleotides would allow the construction of isolates containing conservative single base changes. This should mean that the sites of frequently cutting enzymes could be employed as markers allowing finer analysis of recombination frequencies in particular regions of the genome.

It will be of interest to sequence the end points of the deletions from HG52X85/5 and HG52X86. If the sequence data confirm the restriction enzyme data that the deletions from HG52X85/5 and HG52X86 do not extend into UL56 it will be necessary to sequence across UL54 to find out if a secondary mutation accounts for the reduction in VmwIE63 synthesis. These analyses, together with information on the equivalent

type 1 virus, 1703, would hopefully explain the effects on VmwIE64 production. Comparison of the accumulated data on the end points of deletions from both type 1 and type 2 viruses should determine if certain sequences or portions of the repeat region of HSV are more prone to recombination than others.

Sequencing of the end points of the deletion from HG52X19 and the inserted information has already begun. Analysis shows that the reiterated material is composed of bovine satellite DNA. It appears, however, that at least one end of this reiterated material is flanked by unidentified DNA sequences, probably bovine in origin. It has been recently postulated that a large latency associated transcript (approximately 8.3kb) is produced which crosses the L/S junction (Rock et al., 1987; Mitchell et al., 1990). In HG52X19 this large LAT could not be produced as the deletion from the internal repeats and failure of the long segment to invert would mean that in unit length molecules there is never a normal L/S junction formed. It would, therefore, be of interest to look at the latency and pathogenesis characteristics of HG52X19 in vivo and possibly, if results warrant it, to construct a genome deleted in a similar fashion to HG52X19 but without the complication of inserted DNA.

The finding that HG52X192 was completely avirulent in mice is already under detailed study. It will be necessary to narrow down the sequences within the 1.5kb deletion responsible for neurovirulence and to determine if there is an as yet unidentified polypeptide coded for by this region which is responsible for neurovirulence. This polypeptide could be identified using antisera raised against peptides

from candidate open reading frames, especially those conserved between HSV-1 and 2. Attempts should also be made to determine if deletion of this region, and hence a potentially homologous polypeptide, in HSV-1 will also cause attenuation. It would also be important to investigate if the lack of neurovirulence is apparent in species other than mice. It is possible that in the future avirulent variants could form the basis of live HSV vaccines.

Sequencing of the region around the 0.91m.c. Xba I site in the variant HG52X163X21 would reveal the alteration leading to the failure to produce any detectable gG2. The site deletion mutant HG52X163X3 and its progeny virus HG52X163X3X53 also fail to produce normal sized gG2. The theory that the aberrant form of gG2 produced by these mutants might be the result of the alteration at 0.91m.c. causing failure of the normal stop codon usage could also be investigated by sequence analysis. It has been suggested that the alteration in these mutants would affect the transmembrane portion of the gG2 polypeptide. It would, therefore, be of interest to discover if the aberrant gG2 is incorporated into virions. The lack of gG2 has not been shown to have any effect on the <u>in vitro</u> viability of the viruses but these gG2 impaired variants have markedly reduced neurovirulence in mice. This aspect of these mutants should also be studied.

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A Herpes Simplex Virus Type 2 Variant in which a Deletion across the L–S Junction Is Replaced by Single or Multiple Reiterations of Extraneous DNA

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SUMMARY

We have isolated and characterized a novel variant of herpes simplex virus type 2 (HSV-2) strain HG52 which has a deletion of 13·5 kb across the L–S junction of the genome, resulting in all of the IR_L region, half of the IR_S region and the intervening L–S junction 'a' sequence(s) being removed. The deleted DNA has been replaced by single or multiple (up to 14) reiterations of a DNA sequence approximately 1 kb in length. Individual genomes within the population range in size from approximately 12 kb smaller than unit length to unit length or marginally larger. The L component of the genome is fixed in the prototype orientation while the S component inverts inefficiently. The variant is viable in tissue culture, is not temperature-sensitive, demonstrates impaired single-cycle growth characteristics and, apart from altered mobility of a single species (29·5K), its polypeptide profile in infected cells appears normal. Southern blot analysis has failed to identify the inserted sequences as being derived from HSV-2.

INTRODUCTION

The genomes of herpes simplex virus (HSV) type 1 and type 2 share a common structure consisting of two covalently linked components, long (L) and short (S). Each component comprises a unique sequence (U_L and U_S) bounded by inverted repeats (TR_L and IR_L; IR_S and TR_S). The inverted repeat sequences of the long component have been designated ab and b′a′ and those of the short component as c′a′ and ca. The HSV-2 'a' sequence is constant in size at 251 bp and contains no reiterated sequences. The majority of DNA molecules in a population have single 'a' sequences at the L–S joint and at each terminus, however, some have two or more as tandem direct repeats at the L–S joint and the L terminus; there is always a single copy at the S terminus. Viral DNA extracted from virions or infected cells contains four genomic arrangements in equal proportions. These have been termed P (prototype), I_L (L inverted), I_S (S inverted) and I_{SL} (L and S inverted) (Roizman, 1979). It has been shown that the 'a' sequence located at the termini and at the L–S junction is the site-specific, cis-acting sequence mediating the inversion of HSV DNA (Mocarski *et al.*, 1980; Chou & Roizman, 1985). Cleavage and packaging signals for HSV DNA also reside within a 250 bp subfragment of the terminal repeat (Varmuza & Smiley, 1985; Deiss *et al.*, 1986).

We have previously reported the isolation of variants with a deletion in one copy of the long repeat of HSV-2 (parental strain HG52). These deletions had various lengths with the maximum being 9 kb, i.e. the entire long repeat with the exception of the 'a' sequence. The genomes of the deletion variants isomerized normally (Harland & Brown, 1985). Similarly, we have isolated HG52 deletion variants in which all of the TR_S and part of U_S have been removed, i.e. the U_S genes 10, 11 and 12, one copy of IE3 and one copy of ORI_S (Brown & Harland, 1987). These were similar to the deletion variants of HSV-1 reported by Longnecker & Roizman (1986) and Umene (1986). Variants of HSV-1 strain 17 with deletions in U_L/IR_L removing genes UL55 and UL56 and one copy of IE1 have also been isolated in our laboratory (MacLean & Brown, 1987*a*, *b*). In

three variants the deleted sequences have been replaced by insertion of HSV sequences from another part of the genome. These are two HG52 variants in which deletions of 7·5 kb extending from 0·94 to 0·99 map coordinates (m.c.) have been replaced by inserted duplication of sequences between 0·83 and 0·91 m.c. such that the molecules have short region inverted repeats extended by 6 kb on either side (Brown & Harland, 1987). In the third case a variant of HSV-1 strain 17 has a 1·5 kb deletion in U_L adjacent to IR_L which has been replaced by sequences from the left end of U_L such that the long repeats are extended by 4·5 kb and the overall genome size by 3 kb (MacLean & Brown, 1987*b*). All the HG52 and strain 17 deletion variants are competent *in vitro*.

Poffenberger *et al.* (1983) reported the isolation of a recombinant virus (1358) of HSV-1 (F) generated by insertion of a chimeric thymidine kinase gene within the reiterated sequence of the S component eliminating most of the internal inverted repeats at the junction between the L and S components. The deletion was 15 kb in size. A characteristic of the mutant was that L and S were frozen in the prototype orientation and as the mutant was viable, it was again concluded that inversion of L and S was not required for replication of viral DNA. Further studies on 1358 (Poffenberger & Roizman, 1985) revealed the presence of two submolar populations among packaged DNA. One consisted of defective genomes which had arisen by recombination through a directly repeated sequence inserted in place of the deleted L–S junction. The second population consisted of 1358 DNA linked head to tail (Poffenberger & Roizman, 1985).

This paper reports the isolation of a spontaneous variant (JH2614) of HSV-2 strain HG52 with a deletion of approx. 13·5 kb across IR_L/IR_S such that all of IR_L, half of IR_S and the intervening L–S 'a' sequences have been removed. The molecule has its L component fixed in the prototype orientation but S still inverts, albeit inefficiently. The deletion has been replaced by one to 14 copies of a DNA sequence approx. 1 kb in size such that a population of genomes contains individuals of different sizes. The genomes range in size from approx. 12 kb smaller than unit length to unit length or marginally longer. Southern blot analysis with intact HG52 DNA or cloned HG52 DNA fragments suggests that the inserted sequences are not derived from HSV-2. When a fragment comprising solely inserted sequences was isolated, nick-translated and used as a probe in Southern blot experiments, it hybridized to itself and to discrete bands containing insert plus HSV sequences. There was no hybridization to any other HSV fragment, confirming that the insert sequences were not derived from HSV-2. Virus having the variant genome was viable in tissue culture.

METHODS

Growth of virus. Virus stocks were grown and titrated as described previously (Brown *et al.*, 1973). The HSV-2 strain HG52 was the parental virus (Timbury, 1971).

Preparation of virion DNA. Virion DNA was prepared according to the method of Wilkie (1973) and Stow & Wilkie (1976) as described by Brown *et al.* (1984).

Transfection of virus DNA. Intact and *Xba*I-digested DNA (1 unit *Xba*I/μg DNA for 4 h) was transfected at 1 to 2 μg/plate on to BHK-21/C13 monolayers (4×10^6 cells) using the calcium phosphate infectivity assay technique (Stow & Wilkie, 1976). Single plaques obtained after transfection of *Xba*I-treated DNA were isolated.

Plaque purification. Individual plaques from *Xba*I-digested DNA were picked into 0·5 ml phosphate-buffered saline (PBS) containing 5% calf serum (CS) and sonicated. A sample (100 to 250 μl) was re-plated on BHK-21/C13 cells and after incubation at 37 °C, 10 well separated plaques were isolated (first round). Each of the 10 plaques was re-plated on separate plates and after incubation one plaque was picked from each plate. The procedure was repeated for the third round of 10 plaques. Fifty mm plate stocks of individual plaques were grown at each stage of plaque purification. Roller bottle stocks were grown from plate stocks.

Preparation of total cellular and encapsidated (DNase-resistant) DNA. The method used was a modification of that of Stow *et al.* (1983). Four $\times 10^6$ BHK-21/C13 cells preincubated for 24 h in phosphate-free Eagle's medium containing 1% CS were infected at an m.o.i. of 5 p.f.u./cell and incubated at 37 °C for 1 h. After washing and overlaying with 2 ml phosphate-free Eagle's medium containing 1% CS the infected cells were incubated at 31 °C for 2 h when 25 MBq/plate of $^{32}P_i$ was added and incubation was continued at 31 °C for 48 h. To prepare total cellular DNA, the medium was removed from the plates, 2 ml lysis buffer (0·6% SDS, 10 mM-EDTA, 10 mM-Tris–HCl pH 7·5, 500 μg/ml Pronase) was added and incubation was continued at 37 °C for 4 h. NaCl was added to a final concentration of 200 mM after which the DNA was extracted sequentially with phenol and chloroform and precipitated overnight at −20 °C with ethanol. After centrifuging and redissolving in water, 10 μg/ml of RNase

A/Tl was added and digestion carried out at 37 °C for 2 h. To prepare encapsidated DNA, the growth medium was removed and the cells scraped into 1 ml RSB. Fifty μl of 10% NP40 and 20 μl of 10 mg/ml DNase I were added and incubation was continued at 37 °C for 2 h. After the addition of 1 ml of 2 × lysis buffer (1·2% SDS, 20 mM-Tris-HCl pH 7·5, 20 mM-EDTA, 500 μg/ml Pronase) incubation was continued for a further 2 h at 37 °C. DNA was prepared and extracted as for total cellular DNA.

Restriction enzyme analysis of virus genomes. Restriction enzyme analysis was carried out using the Linbro well technique (Lonsdale, 1979). Cells were infected in the presence of $^{32}P_i$ at an m.o.i. of 10 p.f.u./cell of titrated virus stocks obtained from single plaques; the cells were incubated at 31 °C for 24 to 48 h and the DNA was extracted with SDS–phenol and ethanol-precipitated. ^{32}P-labelled viral DNA was treated with a range of restriction endonucleases at concentrations sufficient to give complete digestion in 4 h at 37 °C. Digests were analysed by electrophoresis overnight in agarose gels of appropriate concentration (0·5 to 1·2%). Gels were air-dried and exposed to Kodak XS1 film for 24 to 48 h.

DNA–DNA hybridization. DNA fragments from restriction endonuclease digests were transferred from agarose gels to nitrocellulose filters (BA85, Schleicher & Schuell) by the method of Southern (1975) and hybridized with nick-translated DNA (Rigby *et al.*, 1977). Nick-translated DNA was prepared from total HSV-2 (HG52) DNA in the recombinant plasmids pGZ11, -12, -13, -14, -15, -26 and -28 containing HSV-2 *Hin*dIII *b*, *k*, *o*, *a*, *e*, *h* and *l* fragments respectively and the recombinant plasmid pGZ1 containing HSV-2 *Bam*HI *g*. The hybridization conditions were as described by Brown *et al.* (1984).

Single-cycle growth experiments. BHK-21/C13 (10^6) cells were infected at an m.o.i. of 5 p.f.u./cell. Absorption was carried out for 45 min at either 31, 37 or 38·5 °C and after two washes with phosphate-buffered saline containing 5% CS and the addition of an overlay of Eagle's medium containing 10% CS incubation was continued at either 31, 37 or 38·5 °C. Cells were harvested into the medium (2 ml) and virus released by sonication was titrated at 37 °C. Samples were harvested at 0, 2, 4, 6, 8, 12 and 24 h. The viruses used were HG52 and JH2614.

Infected cell polypeptide analysis. Twenty-four-well tissue culture dishes were seeded with 2 × 10^5 BHK-21/C13 cells per well in Eagle's medium containing 10% CS and incubated at 37 °C. After 24 h the cells were infected with either HG52 or JH2614 at an m.o.i. of 20 p.f.u./cell. After absorption for 1 h at 37 °C the cells were washed with methionine-reduced medium (E Met/5 CS 2%) before the addition of 450 μl of the same medium. After a further 3 h at 37 °C, 20 μCi of [^{35}S]methionine was added to each well in 50 μl of E Met/5 CS 2% and the samples were returned to 37 °C. After 24 h absorption the medium was removed, the cells were washed once with PBS and harvested into 15 μl of 1:3 sample buffer (150 mM-Tris–HCl pH 6·7, 6·28% w/v SDS, 0·15% v/v 2-mercaptoethanol, 0·31% glycerol, 0·1% bromophenol blue). After boiling at 95 °C for 2 min the samples were run on polyacrylamide gels of 7·5% and 5 to 12% (Marsden *et al.*, 1976, 1978).

Isolation and purification of 'insert' sequences and their use in Southern blot experiments. The presence of an *Eco*RI site within the reiterated insert sequence in JH2614 meant that it was possible to isolate an *Eco*RI fragment composed entirely of insert material. The small size of the fragment (M_r 0·6 × 10^6 to 1·0 × 10^6) made its isolation without contamination from fragmented HSV DNA difficult. It was necessary therefore to purify JH2614 DNA on a sucrose gradient to isolate unit length molecules. Approximately 0·5 ml containing 25 μg of JH2614 DNA was run on a 10 ml isokinetic gradient of 5 to 29·8% sucrose in NTE (0·01 M-Tris–HCl, 0·1 M-NaCl, 0·001 M-EDTA, pH 7·4) with a 0·5 ml 60% sucrose cushion. The gradient was run at 40000 r.p.m. for 5 h at 18 °C in a TST41 rotor. Fractions containing apparently unit length DNA were pooled and the DNA was ethanol-precipitated. The DNA was digested with *Eco*RI and run on a 0·7% agarose gel containing ethidium bromide. The insert band was visualized under long-wave u.v. illumination, cut from the gel and the DNA eluted. The DNA was purified on a Sephacel column (Maniatis *et al.*, 1982) and subsequently nick-translated to give a ^{32}P-labelled probe.

RESULTS

Isolation and restriction endonuclease characterization of the variant JH2614

During our studies on *Xba*I site-negative mutants we have isolated a number of variants, the analysis of which has been useful in studying the structure of the HSV genome (Brown *et al.*, 1984; Harland & Brown, 1985; Brown & Harland, 1987; MacLean & Brown, 1987*a*, *b*). After transfection of *Xba*I-digested HG52 DNA, a plaque was isolated whose DNA had a novel *Xba*I digestion profile. The profile was determined from DNA of a stock that had not been plaque-purified. Restriction endonuclease maps of HG52 are shown in Fig. 1. The *Xba*I profile of JH2614 (Fig. 2*a*) shows that the genome has a slightly aberrant *g* band (this is more apparent in Fig. 2*c*) and *g*-containing joints *e* (*g* + *h*) and *f* (*g* + *i*) in addition to a ladder of bands between *g* and *e*. Two rounds of plaque purification were carried out on the original plaque and the *Xba*I profiles of 11 progeny plaques were compared to that of the parental HG52 (Fig. 2*b*). Extreme

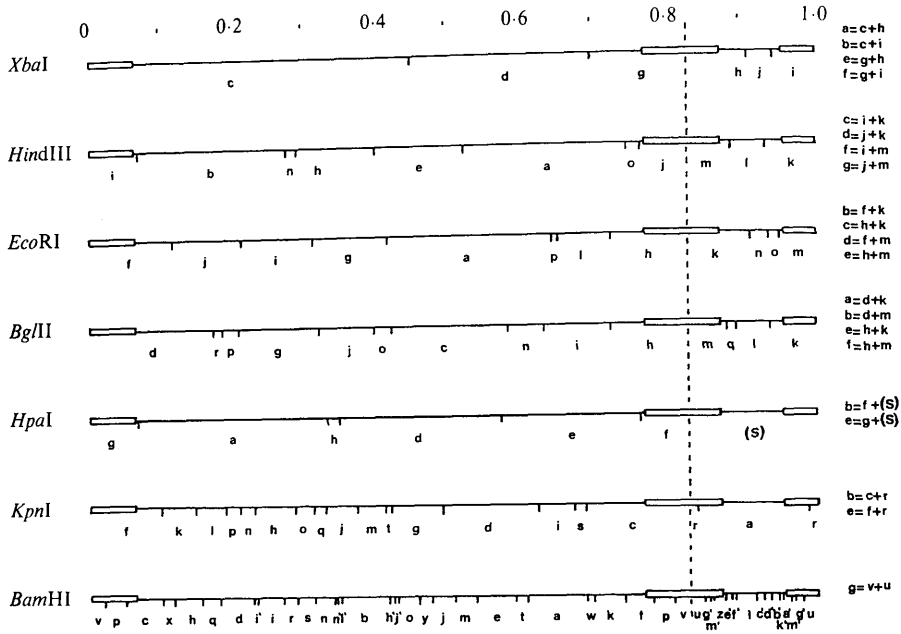

Fig. 1. Restriction endonuclease maps for the DNA of HSV-2 strain HG52 from Cortini & Wilkie (1978).

structure variation is seen among the individual plaques, from the absence of some bands and presence of other discrete bands, e.g. plaque no. 4, to what we have termed a 'laddering effect' as exemplified by plaque no. 1. The alteration in the genome of HG52 that produced JH2614 therefore introduced instability. Plaque no. 4 (Fig. 2*b*, lane 4) was subjected to a further round of plaque purification and Fig. 2(*c*) shows the *Xba*I profile of six progeny plaques. As well as exhibiting apparent fragment deletions, the molarities of individual fragments are not consistent e.g. *Xba*I *i*. The isolate shown in Fig. 2(*c*) (lane 2) was then subjected to a further round of plaque purification from which the DNA profiles of 10 plaques digested with *Xba*I are shown in Fig. 2(*d*). Again it is apparent that there is exceptional variability in the *Xba*I profiles of the progeny genomes arising from one individual plaque including what appears to be reversion to the wild-type profile (lane 6).

Some indication of the nature of the genomic alteration was provided by comparing the profiles in Fig. 2(*c*), lanes 2 and 3. The relative abundance of the *h* and *i* fragments in the two lanes and the relative intensities of the novel bands running above and below the normal position of *g* indicated that the novel bands are probably abnormal L–S joint fragments *e** and *f**. The fragments *h* and *i* being short terminal fragments are normally 0·5 M. In lane 2 the molarity of *h* is increased and that of *i* decreased. Conversely in lane 3 the molarity of *i* is increased and that of *h* decreased. The isolate represented in lane 2 appears therefore to have the S region in predominantly the inverted orientation with the *h* fragment forming an end. The isolate represented in lane 3 has the *h* fragment predominantly forming a joint and the *i* fragment an end.

The *Xba*I analysis therefore indicated that the genome is deleted by 6×10^6 in *Xba*I *g* such that the resulting fragment makes novel joints *e** and *f**. The new *g* band is not apparent on any of the *Xba*I gels although its predicted size should mean that it migrates just above *Xba*I *i*. Its absence suggested that the L segment of the genome was present in only one orientation where *g** was always forming a joint. Unfortunately the other L terminal *Xba*I fragment *c* comigrates with *a* and *b* and therefore is not distinguishable. However, the deletion also prevents the

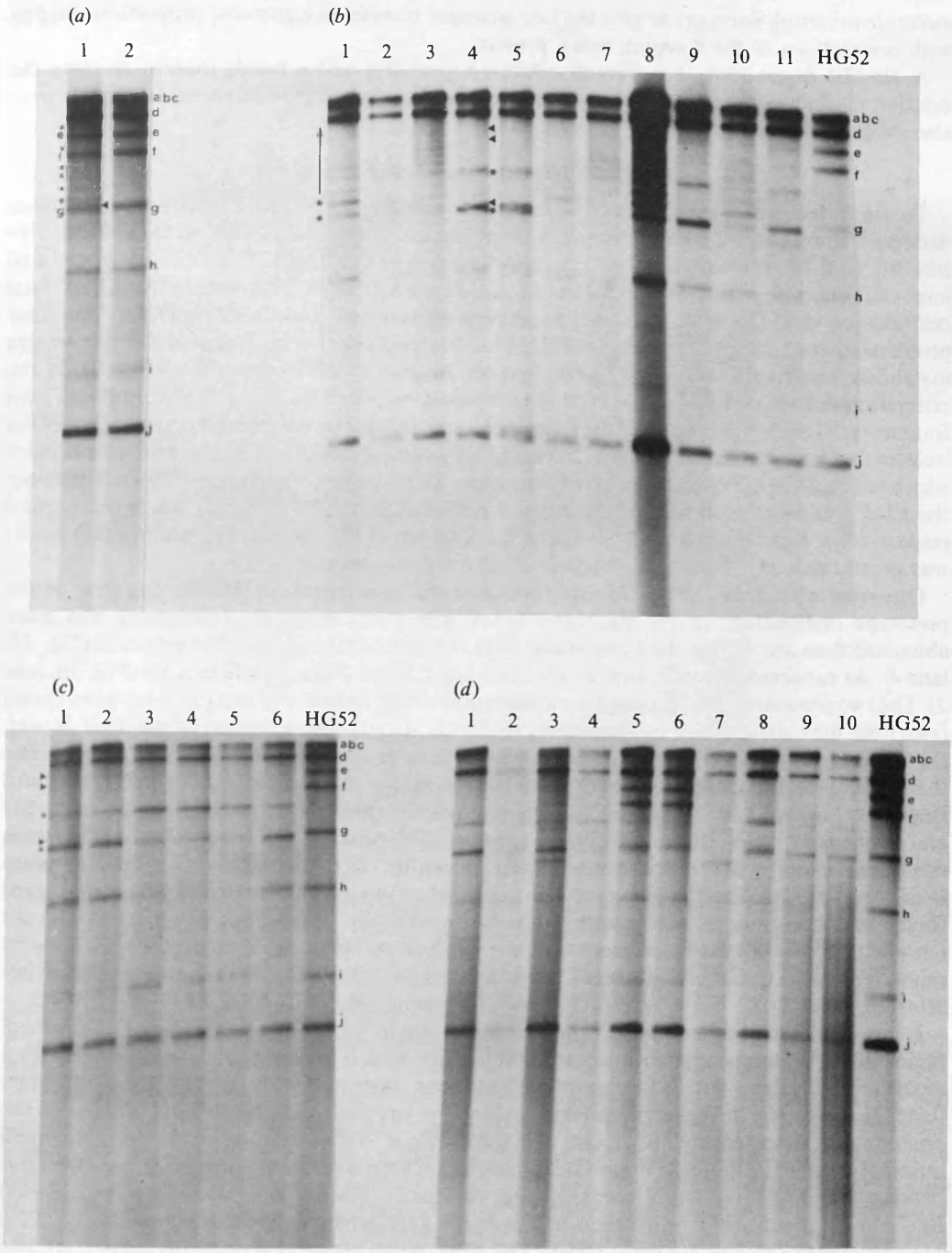

Fig. 2. Autoradiographs of *Xba*I digests of viral DNA ^{32}P-labelled *in vivo*. (*a*) Lane 1, JH2614; lane 2, HG52. Letters refer to specific fragments, asterisks indicate novel bands. Arrowhead indicates missing fragments. (*b*) Lanes 1 to 11, single plaque isolates of JH2614 derived from the plaque shown in (*a*), lane 1. Lane 12, HG52. Letters refer to specific fragments; arrowheads indicate missing fragments; asterisks indicate novel fragments and arrow indicates 'ladder' of fragments. (*c*) Lanes 1 to 6, single plaque isolates of JH2614 derived from the plaque shown in (*b*), lane 4. Lane 7, HG52. Letters refer to specific fragments; arrowheads indicate missing fragments and asterisks indicate novel fragments. (*d*) Lanes 1 to 10, single plaque isolates of JH2614 derived from the plaque shown in (*c*), lane 2. Lane 11, HG52. Letters refer to specific fragments.

molecule inverting normally to give the four genomic isomers in equimolar proportions despite both orientations of the S region being present.

A *Hin*dIII digest (data not shown) exhibited normal *a* and *o* bands thereby limiting the deletion in *Xba*I *g* to the IR portion of the fragment. Ladders of different sized fragments were also evident.

Structure of encapsidated and total viral DNA

To study the relative abundance of end and joint fragments and to determine whether 'laddered' molecules were packaged, analysis of encapsidated viral DNA was carried out. ^{32}P-labelled viral DNA from DNase-resistant virions was extracted and the intensity of end and joint restriction fragments in the mutant and wild-type virus was compared to those from total cell-released viral DNA which would be expected to contain concatemeric DNA. The *Xba*I profile of some of the plaque isolates of JH2614 indicated a deletion in *Xba*I *g* such that there was instability resulting in reiteration of sequences to give a 'laddering' effect (Fig. 2). If the reiteration did not contain an *Xba*I site then it would be predicted that the *g**-containing joint fragments, *e** and *f**, would be of different sizes with regular incremental increases. One of the isolates that had shown pronounced 'laddering' of joint fragments was grown into a virus stock which was used in the encapsidation experiments. There was no significant difference between the *Xba*I profiles of total and encapsidated viral DNA of this isolate (Fig. 3*a*, lanes 2 and 4 respectively). Again abnormal inversion of the S region of the genome was seen in that *Xba*I *i* was greatly reduced in molarity whereas *Xba*I *h* was increased.

Digestion with *Eco*RI (Fig. 3*b*) confirmed that the long region of JH2614 was fixed in the prototype orientation. In the packaged DNA, the terminal *Eco*RI *f* fragment was more abundant than normal but the *f*-containing joint fragments *b* and *d* were not apparent (Fig. 3*b*, lane 4). As expected, the total viral DNA exhibited *f*, *b* and *d* in normal amounts (Fig. 3*b*, lane 2). The two remaining joint fragments containing *Eco*RI *h*, i.e. *c* (*h*+*k*) and *e* (*h*+*m*), were absent from both the encapsidated and concatemeric DNA. As was demonstrated by the *Xba*I digests, the S region of the genome is predominantly in the inverted orientation with *Eco*RI *m* being less than, and *Eco*RI *k* more than, 0·5 M. The *Eco*RI profile did not exhibit 'laddering' of joint fragments but three novel bands were apparent, one of approx. M_r 6 × 10^6 running between *l* and *m*, one of M_r 1·8 × 10^6 running below *n* and one of approx. M_r 0·6 × 10^6 below *o*. The bands can be explained by the existence of an *Eco*RI site within the reiterated DNA. If the reiteration is of constant size then the site would be repeated at regular intervals giving constant sized fragments on digestion. This model would be expected to create four novel bands: (i) the remainder of the *Eco*RI *h* fragment plus the reiteration up to the first *Eco*RI site, (ii) only reiterated material between the *Eco*RI sites, (iii) and (iv) the portion of the reiterated sequence after the final *Eco*RI site plus *Eco*RI *m* and, less commonly, plus *Eco*RI *k*.

It would therefore follow that a different *Eco*RI profile would be seen in isolates with the S region in the prototype orientation compared to those with it inverted. Fig. 4(*a*) shows an *Xba*I digest of two isolates with S in opposite orientations compared with HG52 (lane 1). The first (lane 2) shows the S region in predominantly the inverted orientation, i.e. *Xba*I *h* is the predominant short terminal fragment. The second (lane 3) shows *Xba*I *i* as the predominant S terminal fragment. On *Eco*RI digestion, the isolate with the S region predominantly inverted has bands *c* and *e* and presumably *h* missing (Fig. 4*b*, lane 2). The inverted orientation is confirmed by the predominance of *k* rather than *m*. The *b* (*f* + *k*) fragment molarity is increased due to the high proportion of concatemeric DNA. The three novel bands described previously are also apparent. When the S region is in the prototype orientation *b* and *k* are reduced and *m* is predominant (Fig. 4*b*, lane 3). The three novel bands are again present. The M_r 0·6 × 10^6 and 6 × 10^6 bands appear to be present in the same quantities in both isolates. However the M_r 1·8 × 10^6 band is clearly reduced in intensity in the second isolate (lane 3). The predominance of this band when the S region is inverted suggests that it contains information from the *m* fragment. If this band is composed of insert sequences plus *Eco*RI *m* then the fourth proposed novel fragment should consist of insert sequences plus *k*, should have an approx. M_r of 5 × 10^6 and should run above *m*. The proposed novel band composed of insert plus the remainder of *h* is also about

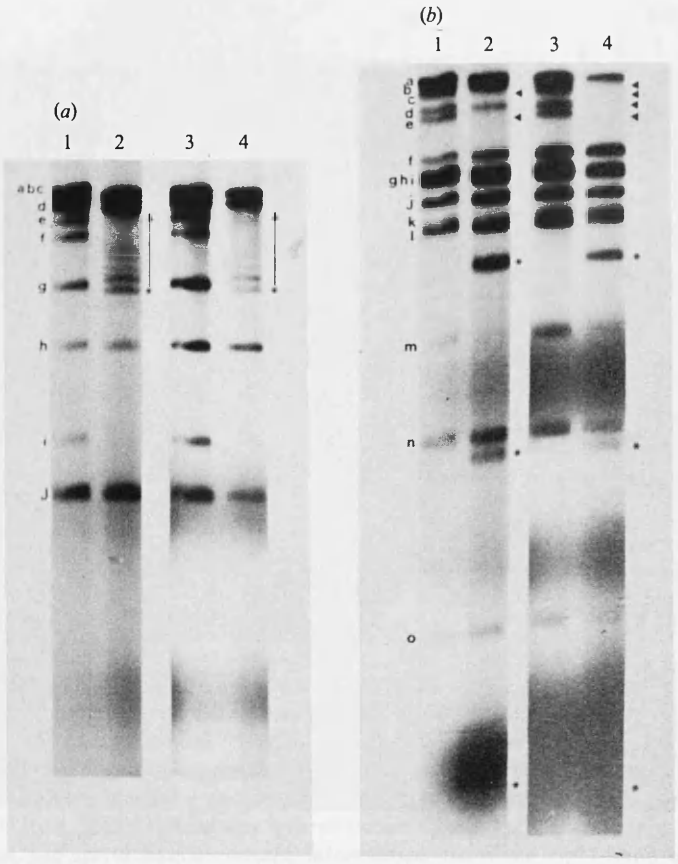

Fig. 3. (*a*) Autoradiographs of *Xba*I digests of viral DNA ^{32}P-labelled *in vivo*. Lanes 1 and 3, HG52; lanes 2 and 4, JH2614. Lanes 1 and 2, total viral DNA from infected cells. Lanes 3 and 4, DNase-resistant encapsidated DNA. Letters refer to specific fragments; asterisks indicate novel bands; arrows indicate 'ladder' of novel bands. (*b*) Autoradiographs of *Eco*RI digests of viral DNA ^{32}P-labelled *in vivo*. Lanes 1 and 3, HG52; lanes 2 and 4, JH2614. Lanes 1 and 2, total viral DNA from infected cells; lanes 3 and 4, DNase-resistant encapsidated DNA. Letters refer to specific fragments; arrowheads indicate missing fragments, asterisks indicate novel fragments.

5×10^6 in size. The one band seen in this region calculated to be of approx. M_r 6×10^6 and apparently greater than 1 M is therefore thought to be (k + insert) plus (h + insert). The band composed of m + insert sequences is less than half the size of m indicating that the deletion extends across the IR_L/IR_S junction and removes sequences from the internal portion of m. Both *Bgl*II and *Kpn*I digestion confirmed the proposed structure (data not shown). The presence of 'ladders' indicated the absence of a *Bgl*II site in the reiterated sequences. The absence of ladders indicated the presence of a *Kpn*I site in the reiterations.

The restriction endonuclease analysis led to the conclusion that JH2614 is a variant in which there is deletion of sequences from the internal repeat regions of the genome, spanning the IR_L/IR_S junction. The instability is due to the insertion of sequences which are reiterated a variable number of times resulting in genomes of differing sizes. The insert contains *Eco*RI and *Kpn*I sites but not *Xba*I, *Hin*dIII or *Bgl*II sites. *Bam*HI digestion provided additional information about the extent of the deletion. It can be seen in Fig. 5 that the *f* fragment is not in the expected position, one copy of both p and g' are missing and therefore presumably one copy each of v and u. As m' has run off the gel it is not possible to see whether one copy is absent.

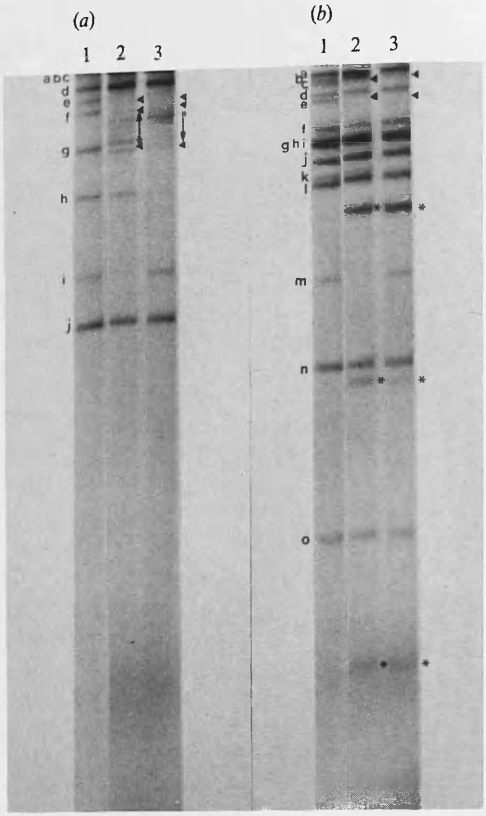

Fig. 4

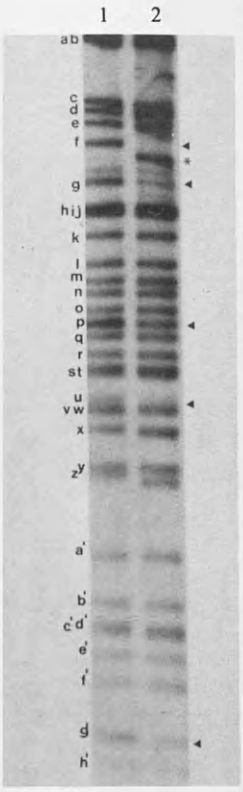

Fig. 5

Fig. 4. (*a*) Autoradiographs of *Xba*I digests of viral DNA ^{32}P-labelled *in vivo*. Lane 1, HG52; lane 2, JH2614 with S in predominantly the inverted orientation; lane 3, JH2614 with S in predominantly the prototype orientation. Letters refer to specific fragments; arrowheads indicate missing fragments; asterisks indicate novel fragments and arrows indicate 'ladder' of novel fragments. (*b*) Autoradiographs of *Eco*RI digests of viral DNA ^{32}P-labelled *in vivo*. Lane 1, HG52; lane 2, JH2614 with S predominantly inverted; lane 3, JH2614 with S predominantly in the prototype orientation. Letters refer to specific fragments; arrowheads indicate missing fragments and asterisks indicate novel fragments.

Fig. 5. Autoradiograph of *Bam*HI digests of viral DNA ^{32}P-labelled *in vivo*. Lane 1, HG52; lane 2, JH2614. Letters refer to specific fragments; arrowheads indicate missing fragments and asterisk indicates novel fragments.

However, *z*, a known variable fragment adjacent to *m'*, is present and of normal size. The deletion therefore removes part of *f*, all of *p*, *v*, *u*, and at least part of *g'*. The deletion is therefore between 7×10^6 and 9×10^6 M_r and the more stable structures seen in Fig. 2(*b*) contain an insert with an M_r of at least 1×10^6, i.e. one copy of the insert. Subsequent Southern blot data from a *Bam*HI digest of JH2614 probed with insert sequences indicated the absence of a *Bam*HI site within the insert (data not shown).

DNA–DNA hybridization

On the assumption that the insert contains HSV sequences, only three regions of the HG52 genome contain, within a sequence of M_r 1×10^6, *Eco*RI and *Kpn*I, but not *Hin*dIII, *Bgl*II, *Bam*HI or *Xba*I sites. These are located around 0·21 m.c., 0·31 m.c. and 0·42 m.c. and are located within *Hin*dIII *b*, *h* and *e*, respectively. When Southern blot hybridization was carried out with

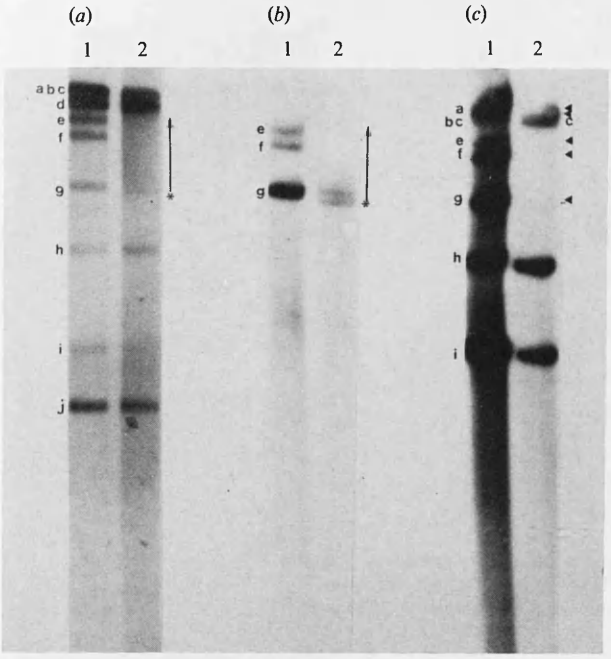

Fig. 6. Autoradiographs of nitrocellulose blot strips containing *Xba*I restriction fragments of HSV-2 HG52 (lane 1) and JH2614 (lane 2) DNA to which DNA probes have been hybridized. The probes were (*a*) HG52 DNA, (*b*) the *Hin*dIII *o* fragment of HG52 (pGZ43) and (*c*) the *Bam*HI *g* fragment of HG52 (pGZ1). Letters refer to specific fragments; arrowheads indicate missing fragments; asterisks indicate novel fragments and arrows indicate a 'ladder' of novel fragments.

*Hin*dIII *b*, *h* and *e* there was no detectable hybridization to the novel *Xba*I fragments seen on restriction endonuclease digestion (data not shown).

When *Hin*dIII *o* was used as a probe with an *Xba*I digest of HG52 and JH2614, there was as expected hybridization to *Xba*I *g* and *g*-containing joint fragments *e* and *f* (Fig. 6*b*, lane 1). In addition, in JH2614 (lane 2) there was hybridization to the 'ladder' fragments supporting the conclusion that the novel bands contain information from the right-hand end of U_L plus reiterated sequences.

When *Bam*HI *g* (*v* + *u*) was used as a probe with an *Xba*I digest of HG52 and JH2614, there was positive hybridization to fragments *c*, *g*, *h*, *i*, *a*, *b*, *e* and *f* (Fig. 6*c*, lane 1) of HG52 as expected. With JH2614 there was hybridization to *Xba*I *c* but not to the *c*-containing joint fragments as they are not present in packaged viral DNA (due to the fixed orientation of L). The lack of hybridization to the novel 'ladder' confirms that information from *v* and *u* is not present in the novel joint fragments and must have been deleted. However, *h* and *i* in the terminal position are intact as they are of normal size.

In order to span most of the genome, each *Hin*dIII fragment (except *n*, *j*, *i* and *m*) of HSV-2 HG52 was used as a probe to detect the origin of the inserted sequences. As stated *Hin*dIII *b*, *e* and *h* failed to hybridize to novel fragments. When *Hin*dIII *o* was used as a probe with an *Eco*RI digest of JH2614, there was no hybridization to *h*, *e* and *c* as seen with wild-type virus (Fig. 7*a*, lane 3), but there was positive hybridization to the band of M_r 6 × 10^6 running below *l* (Fig. 7*a*, lane 4). Similarly *Hin*dIII *a* hybridized to this band (Fig. 7*b*, lane 4). As predicted, the M_r 6 × 10^6 band contains sequences from the right-hand end of U_L. When *Hin*dIII *l* (0·89 to 0·93 m.c.) was used there was also hybridization to the novel *Eco*RI band running below *l* (Fig. 7*c*, lane 4). Using *Hin*dIII *k* (0·93 to 1·0 m.c.) there was positive hybridization to the novel 6 × 10^6 *Eco*RI band and also to the band of 1·8 × 10^6 running below *n* (Fig. 7*c*, lane 6).

These hybridization results support the interpretation of the restriction digest of JH2614 that the band seen below *Eco*RI *l* is composed of two fragments, *Eco*RI *h* plus insert and *Eco*RI *k* plus insert. *Hin*dIII *a* and *o* hybridize to *Eco*RI *h* and *Hin*dIII *l* and *k* hybridize to *Eco*RI *k*. Hybridization of *Hin*dIII *k* to the M_r 1·8 × 10^6 *Eco*RI fragment substantiates the conclusion that this band is composed of part of *Eco*RI *m* plus insert.

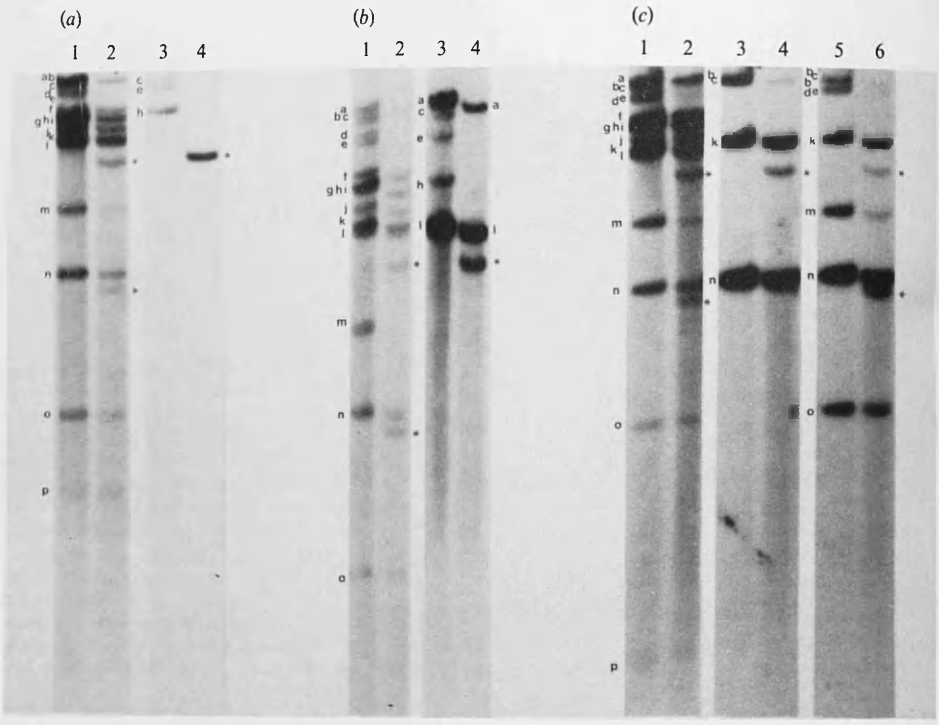

Fig. 7. Autoradiographs of nitrocellulose blot strips containing *Eco*RI restriction fragments of HSV-2 HG52 (lanes 1, 3 and 5) and JH2614 (lanes 2, 4 and 6) DNA to which DNA probes have been hybridized. The probes used were as follows: (*a*, *b* and *c*) lanes 1 and 2, HG52; (*a*) lanes 3 and 4, the *Hin*dIII *o* fragment of HG52 (pGZ43); (*b*) lanes 3 and 4, the *Hin*dIII *a* fragment of HG52 (pGZ26); (*c*) lanes 3 and 4, the *Hin*dIII *l* fragment of HG52 (pGZ28); (*c*) lanes 5 and 6, the *Hin*dIII *k* fragment of HG52 (pGZ12). Letters refer to specific fragments; asterisks indicate novel fragments.

The lack of any positive hybridization to insert sequences (i.e. $0{\cdot}6 \times 10^6$ M_r band seen in *Eco*RI digests of labelled DNA seen in Fig. 4*b*) led to the conclusion that either the insert was not derived from HSV-2 DNA or that very short non-contiguous HSV-2 DNA had been inserted in such a way as not to be picked up by the probes. The similarity of the observed ladders to those seen in defective genomes (Kaerner *et al.*, 1981) led to the speculation that the insert may contain an origin of replication, either Ori_L or Ori_S. The sequence around 0·42 m.c. contains not only the correct combination of restriction enzyme sites but also Ori_L. It was thought that as Ori_L is known to become deleted in a cloned fragment, the lack of hybridization seen with *Hin*dIII *e* may be accounted for in this way. To overcome this problem a fragment containing Ori_L i.e. *Hin*dIII/*Xba*I *e'* (0·4 to 0·45 m.c.) was extracted from a gel and purified. Its purity was checked by hybridization and it was used in Southern blot hybridization against JH2614. However, it also failed to hybridize to either the $0{\cdot}6 \times 10^6$ *Eco*RI band thought to be entirely insert sequences or to the other novel bands (data not shown).

The restriction endonuclease and Southern blot analyses showed that JH2614 has a deletion of M_r between 7×10^6 to 9×10^6. It removes most of IR_L and at least half of IR_S. The internal copies of the *a* sequences are thus removed which fixes the L region in the prototype orientation. Inefficient inversion of the S region is presumably occurring through the remaining sequences of the short repeat. Removal of the entire long repeat does not allow inversion to occur in this manner in the L region. Part of the deletion is replaced by insertion of DNA approximately $0{\cdot}6 \times 10^6$ M_r which can be reiterated from one to 14 times. The insert contains *Eco*RI and *Kpn*I sites. The origin of the insert was not deduced using a range of probes covering the entire HSV-2

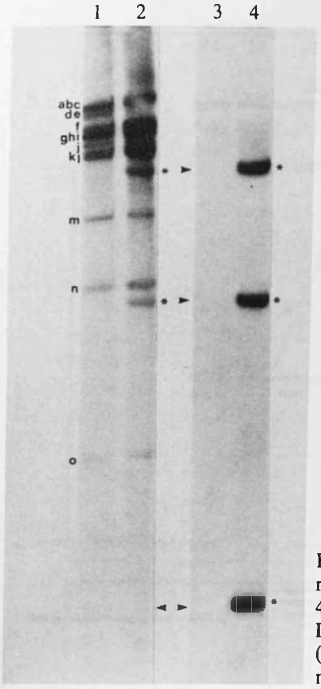

Fig. 8. Autoradiographs of nitrocellulose blot strips containing *Eco*RI restriction fragments of HSV-2 HG52 (lanes 1 and 3) and JH2614 (lanes 2 and 4) DNA to which DNA probes have been hybridized. The probes were HG52 DNA (lanes 1 and 2) and the insert ($0{\cdot}6 \times 10^6$ M_r) *Eco*RI fragment of JH2614 (lanes 3 and 4). Letters refer to specific fragments; arrowheads indicate negative hybridization and asterisks indicate novel fragments.

genome with the exception of the sequences between 0·02 and 0·07, 0·28 and 0·29, 0·75 and 0·81, and 0·86 and 0·87 m.c. This suggests that the insert may not contain normally occurring HSV-2 sequences. Substantiation of this conclusion is provided by the fact that when the whole HG52 genome is used as a probe in Southern blot hybridization the $0{\cdot}6 \times 10^6$ M_r band seen on an *Eco*RI digest is not detectable although the two other novel *Eco*RI fragments of $1{\cdot}8 \times 10^6$ and 6×10^6 are readily apparent (Fig. 7*a*, lane 2 and Fig. 7*c*, lane 2).

From the restriction endonuclease analysis it was determined that the novel low M_r band ($0{\cdot}6 \times 10^6$ M_r) seen on *Eco*RI digestion of JH2614 (Fig. 4*b*, lanes 2 and 3) was entirely composed of insert sequences. Using both intact HG52 DNA and cloned HG52 fragments, Southern blot analysis failed to identify this fragment as being composed of HSV sequences. To substantiate this conclusion, the small fragment was excised from *Eco*RI-digested DNA previously subjected to stringent purification (see Methods). When the fragment was nick-translated and used as a probe in Southern blot experiments with *Eco*RI-digested JH2614 DNA, it predictably hybridized to the $0{\cdot}6 \times 10^6$ M_r fragment and also to the two bands previously identified as being composed of (insert + k* and insert + *h**) (6×10^6 M_r) and insert + *m** ($1{\cdot}8 \times 10^6$ M_r) (Fig. 8, lane 4). There was no hybridization to *Eco*RI-digested HG52 DNA (Fig. 8, lane 3). Southern blot hybridization using nick-translated insert sequences ($0{\cdot}6 \times 10^6$ M_r) failed to show any hybridization to *Eco*RI-cleaved HSV-1 strain 17 DNA under conditions in which HSV-1 sequences (*Bam*HI *z*; $1{\cdot}2 \times 10^6$ M_r) hybridized. The insert sequences under the same conditions hybridized only to three novel bands of *Eco*RI-cleaved JH2614 DNA (data not shown). These results confirm that the reiterated insert of DNA in JH2614 is not composed of HSV sequences. A diagram showing the proposed structure of JH2614 is shown in Fig. 9.

Growth properties of JH2614

High titre stocks of JH2614 were obtained by low multiplicity infection of BHK-21/C13 cells at 31 °C over 3 days. A typical stock gave a titre of $5{\cdot}3 \times 10^7$ p.f.u./ml at 31 °C, 7×10^7 p.f.u./ml at 37 °C and $5{\cdot}4 \times 10^7$ p.f.u./ml at 38·5 °C. A typical HG52 wild-type stock gives a titre of $2{\cdot}2 \times 10^8$ p.f.u./ml at 31 °C, 6×10^8 p.f.u./ml at 37 °C and $3{\cdot}2 \times 10^8$ p.f.u./ml at 38·5 °C. Single-cycle

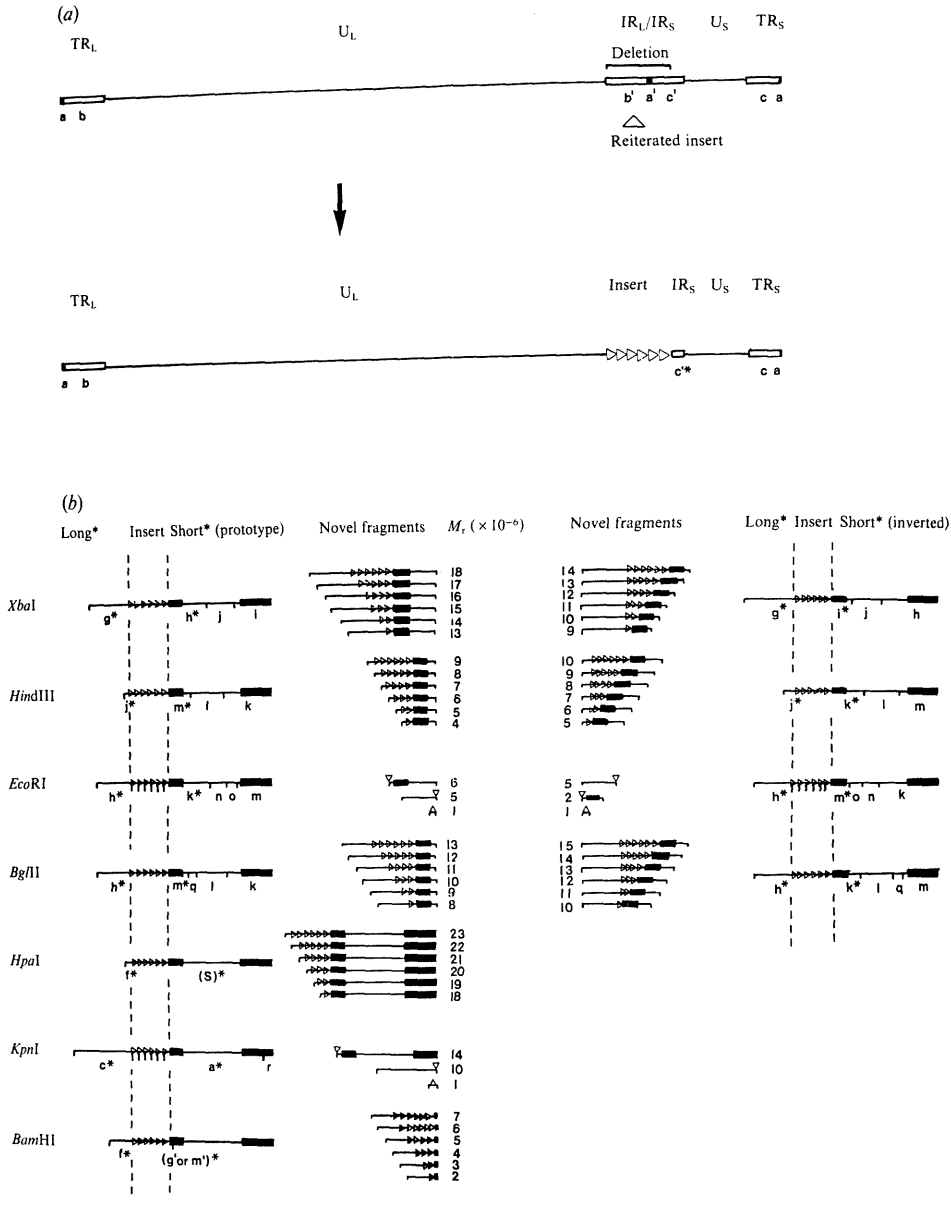

Fig. 9. Proposed model for the structure of JH2614. (*a*) Deletion of IR_L and part of IR_S and their replacement by inserted sequences reiterated a variable number of times resulting in genomes of different lengths. Asterisk denotes a deleted fragment. (*b*) Derivation and approximate M_r values of the novel fragments containing a range of one to six reiterations of the insert when digested with different restriction enzymes. Asterisk denotes a deleted fragment; ▷ indicates one insert; ▷ indicates an insert containing a restriction enzyme site; ▽ denotes the presence of part of an insert before or after a restriction enzyme site; △ denotes a fragment which is the same length as the insert but which is composed of part of one insert after a restriction enzyme site plus part of the adjacent insert up to the next site. The maximum number of reiterations of inserts apparent on gels was 14. The diagram shows only the fragments produced in the absence, not conclusively demonstrated, of a *Hpa*I site within the insert.

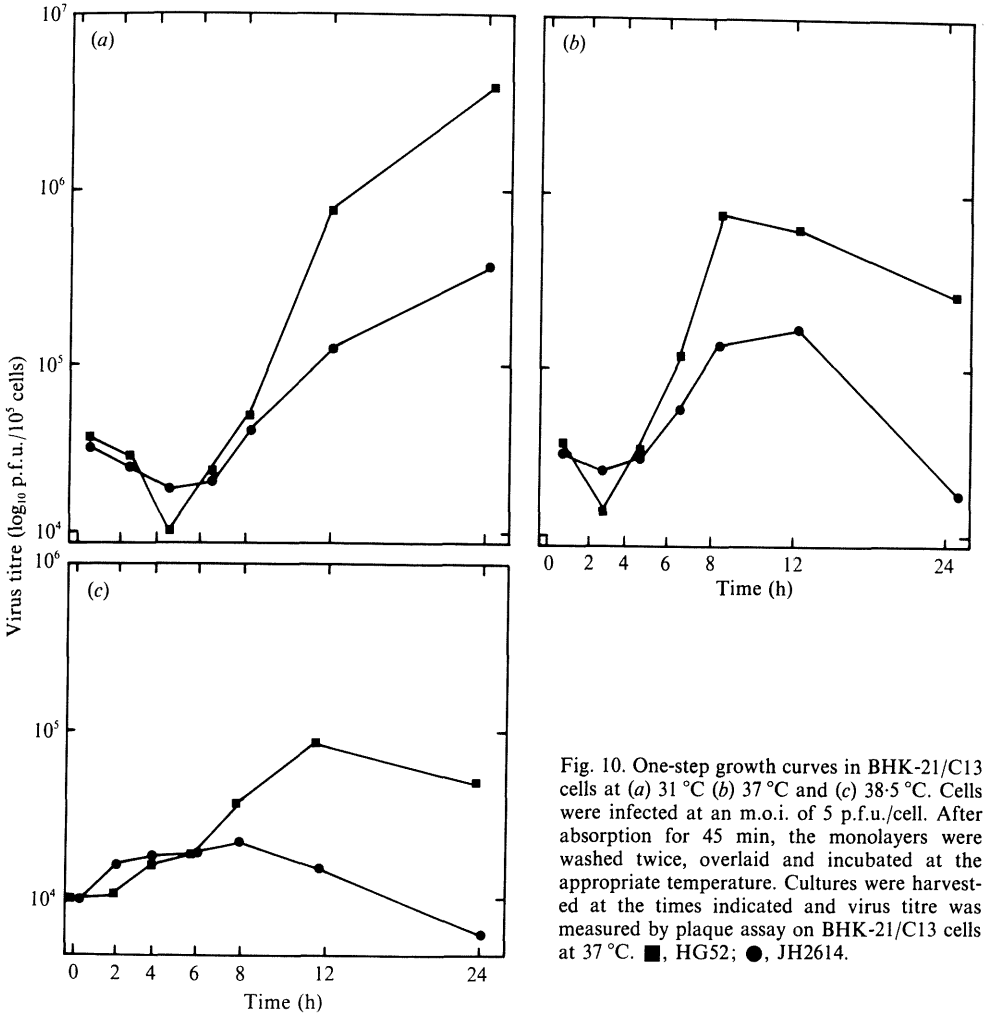

Fig. 10. One-step growth curves in BHK-21/C13 cells at (*a*) 31 °C (*b*) 37 °C and (*c*) 38·5 °C. Cells were infected at an m.o.i. of 5 p.f.u./cell. After absorption for 45 min, the monolayers were washed twice, overlaid and incubated at the appropriate temperature. Cultures were harvested at the times indicated and virus titre was measured by plaque assay on BHK-21/C13 cells at 37 °C. ■, HG52; ●, JH2614.

growth experiments were carried out at three temperatures in BHK-21/C13 cells as described and the results are shown in Fig. 10. At 31 °C, JH2614 displayed the same rate of growth as wild-type virus but the final titre at 24 h was 10-fold lower. A similar pattern was apparent when growth occurred at 37 °C, and at 38·5 °C the temperature restriction demonstrated by wild-type HG52 (Harland & Brown, 1985) was also apparent in JH2614. The particle : p.f.u. ratio of the wild-type stock of HG52 used in these experiments was 1263 : 1 and that of JH2614 was 7220 : 1 which is very high even for an HSV-2 stock. The particle : p.f.u. ratios of HG52 obtained in this laboratory range from 40 : 1 to 1500 : 1.

Infected cell polypeptide analysis

Infected cell polypeptides synthesized by HG52 and the variant JH2614 were prepared as described. [^{35}S]Methionine was incorporated 3 to 24 h post-infection and the polypeptides were analysed by either 7·5% single concentration or 5 to 12·5% gradient SDS–PAGE. Infections were carried out at 31 and 38·5 °C. Fig. 11 shows the polypeptides synthesized by HG52 and JH2614 at 38·5 °C analysed on a 7·5% gel (*a*) and at 31 °C analysed on a 5 to 12·5% gel (*b*). It can be seen in Fig. 11 (*a*) that JH2614 shows a wild-type profile with the exception of the

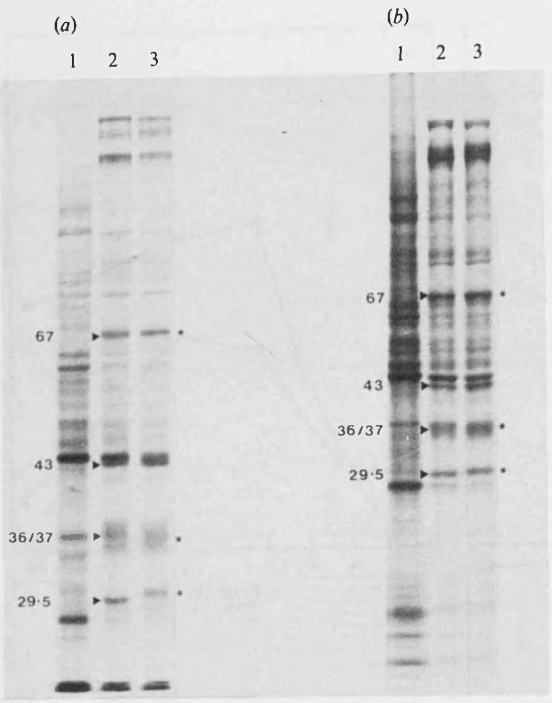

Fig. 11. Autoradiographs of infected cell polypeptides induced in BHK-21/C13 cells and separated by SDS–PAGE. (*a*) Cells infected at 38·5 °C and polypeptides analysed on a 7·5% gel. (*b*) Cells infected at 31 °C and polypeptides analysed on a 5 to 12·5% gel. Lanes 1, mock-infected; lanes 2, HG52; lanes 3, JH2614. Numbers show the apparent M_r ($\times 10^{-3}$) of HSV-2-infected cell polypeptides. Asterisks denote altered migration or intensity.

29·5 $\times$ 10^3 M_r band which is migrating slower than the equivalent wild-type band in JH2614 and is of higher M_r. There is also some alteration in the mobility of the bands in the 36/37 $\times$ 10^3 M_r region in the JH2614 profile. Fig. 11(*b*) also shows the slower migration of the 29·5 $\times$ 10^3 M_r polypeptide in JH2614 in addition to a change in mobility and intensity of a band in the 67 $\times$ 10^3 M_r region.

DISCUSSION

Most of the deletions reported in the HSV genome involve one of the repeats and adjacent unique sequences (Davison & Wilkie, 1983; Brown *et al.*, 1984; Harland & Brown, 1985; Longnecker & Roizman, 1986; Umene, 1986; Brown & Harland, 1987; MacLean & Brown, 1987*a*,*b*). It is probable that deletions originate from distinct regions of instability in the DNA, e.g. banks of tandemly reiterated short sequences like those found in the long repeat near the U_L/IR_L junction, in the middle of IR_L, near the IR_L/IR_S joint and in U_S of HSV-1 (Rixon *et al.*, 1984; McGeoch *et al.*, 1988). These reiterations have been proposed to favour unequal recombination. Now that the whole of HSV-1 and part of HSV-2 have been sequenced, it will be possible to sequence the relevant regions of the numerous deletion variants isolated in this laboratory and determine the endpoints of the deletions.

In this paper we have described a variant of HSV-2 strain HG52 in which again the deletion removes sequences from the repeat regions of the genome. The deletion removes all of IR_L, the L–S junction and half of IR_S. The variant was isolated from a plaque derived from transfected DNA which had been digested with the restriction endonuclease *Xba*I. We had previously speculated that deletions may result from restriction enzyme treatment or from the transfection procedure. This proposal was discounted on finding deletion variants within untreated populations of HG52 genomes. That such treatment may increase the frequency of genomic deletions can not however be ruled out.

Genomic deletions across the IR_L/IR_S junction have been reported previously in HSV-1 by Poffenberger *et al.* (1983). They described the recombinant virus (1358) generated by insertion

of a chimeric thymidine kinase gene within the reiterated sequences of the S component and deletions that eliminated most of the internal inverted repeats at the junction between the L and S components. The insertion contained the thymidine kinase gene and sequences of *Hin*dIII *o* which are normally located in U_L. It had been generated by unexpected recombination of the thymidine kinase/*Hin*dIII *o* chimeric fragment at the L–S junction and not within *Hin*dIII *o*. Further characterization of 1358 (Poffenberger & Roizman, 1985) revealed the presence of two submolar populations in packaged DNA. The first consisted of defective genomes with a subunit size of 36 kbp and the second consisted of 1358 DNA linked head to tail.

This was not the first report of insertion of HSV sequences into a novel position in the genome. Davison & Wilkie (1983) reported the analysis of an HSV-1/HSV-2 recombinant (RE4) in which a 10·5 kbp deletion including IR_L and a short adjacent region of U_L had been partially replaced by sequences from U_L. Both 1358 and RE4 were fixed in both the L and S regions of the genome. The variant described in this paper is fixed in the prototype orientation in the L region of the genome as neither the junction 'a' sequence nor any sequences within IR_L have been retained. However, the identification of both S region internal and terminal fragments, albeit in unequal proportions, indicates that the 'a' sequence at the L–S junction is not a prerequisite for isomerization. As only part of IR_S is retained in JH2614, it is thought that inefficient inversion is taking place through homologous sequences in TR_S and the remainder of IR_S. Varmuza & Smiley (1985) have already proposed that HSV genome segment inversion could result from the summed recombinational activity of sequences dispersed throughout the 15 kb L–S junction region. The existence of JH2614 shows that sequences in R_S outside the 'a' sequence can act as recombinational hotspots.

From our experiments we have concluded that JH2614 is deleted by 13·5 kb across the L–S joint and this is partially compensated by insertion of single or multiple copies of a 1 kb DNA fragment. JH2614 was first detected by its unusual DNA pattern on *Xba*I digestion. The profile indicated a ladder of bands which decreased in intensity with what appeared to be incremental increases in M_r (Fig. 2*a* and *b*). Single plaque isolation from the original plaque revealed a number of genomes with disparate *Xba*I profiles. Some retained the ladder appearance; some showed discrete bands but not in their normal position and a proportion had returned to the wild-type profile (Fig. 2*d*, lane 6). Further restriction enzyme analysis determined that these alterations involved repeat and joint fragments. Step-wise patterns have been previously recognized in the DNA of defective virus of the Angelotti strain (Kaerner *et al.*, 1981). Again a population of defective DNA was shown to consist of different homopolymers each containing a constant number of inserts in all of its repeats. The possibility of defective genomes being the cause of the aberrant features in JH2614 DNA was ruled out by certain observations. First, the step-wise pattern of DNA fragments was not identifiable as a separate entity. It was always part of an altered HSV genome profile. Secondly, apart from the initial unpurified isolate (Fig. 2*a*), the ladders were not present in a profile in addition to the normal complement of wild-type sized fragments and, thirdly, novel bands were shown to consist of identifiable HSV fragments fused to inserted sequences, e.g. the M_r 6×10^6 and $1{\cdot}8 \times 10^6$ *Eco*RI bands.

The step-wise increments were present only with certain restriction enzymes; others showed discrete aberrant bands. This was due to the absence or presence of particular restriction sites in the DNA insert.

One of the causes of a reiteration may be the presence of an origin of replication. However, Southern blot analysis either with a cloned sequence in which Ori_L normally resides or with an Ori_L-containing sequence extracted from a gel failed to indicate the insert as having originated from this region of the genome.

Using either cloned individual HG52 fragments covering 87% of the genome, or the complete HG52 genome in Southern blot analysis, the origin of the DNA insert was not identified as HG52. Of fundamental importance to this assertion is the finding that when the entire HG52 genome was used as a probe, the bands composed of insert plus HG52 sequences were readily identified (Fig. 7*a*, *b*, *c* bands of M_r 6×10^6 and $1{\cdot}8 \times 10^6$) but the low M_r $0{\cdot}6 \times 10^6$ fragment which is present in the insert only did not appear. In contrast, its presence is obvious on an *Eco*RI digest of JH2614 DNA ^{32}P-labelled *in vivo* (Fig. 4*b*). Failure to detect this fragment by

Southern blotting was repeatable and was not due to poor hybridization of a low M_r fragment; the *Eco*RI *o* and *p* bands of similar M_r ($0{\cdot}8 \times 10^6$ and $0{\cdot}4 \times 10^6$ respectively) are clearly apparent in Fig. 7(*a*) and (*c*). Confirmation that the inserted sequences are not from HSV-2 was provided by using the novel low M_r *Eco*RI fragment as a probe in Southern blot experiments. Positive hybridization in JH2614 was demonstrated with the putative insert fragment and with those concluded to be insert + HSV sequences. There was no hybridization to any other HSV fragments in JH2614 and more importantly there was no hybridization with the wild-type HG52 DNA (Fig. 8). The insert was also shown not to be HSV-1 in origin.

JH2614 grows to high titre on low multiplicity passage, is impaired in single-cycle high multiplicity growth, and titrates equally well at 31, 37 and 38·5 °C; i.e. the virus is viable though impaired *in vitro*. Its high particle : p.f.u. ratio, however, suggests that either a lower than normal proportion of genomes is packaged or that the packaged DNA is defective and not infectious. It could be envisaged that genomes with multiple reiterations of insert DNA which result in sizes appreciably larger than unit length may not be packaged. Also the absence of the 'a' sequence at every second L–S junction in concatemeric DNA no doubt affects packaging. On electron micrograph analysis there was a higher proportion of full to empty particles in the JH2614 virus preparation (77%) than in HG52 (64%) which may suggest the more likely explanation that a proportion of packaged DNA is non-infectious.

The infected cell polypeptide profiles of JH2614 were normal at 31 and 38·5 °C with the exception of a change in mobility of the 29·5K species. This is equivalent to the HSV-1 28K polypeptide encoded by UL32 located between m.c. 0·44 and 0·45. The 29·5K polypeptide of JH2614 migrated slower than that of the wild-type indicating either increased M_r or a conformational change. Restriction enzyme analysis failed to detect any alteration in fragments in which UL32 is located, indicating that the size change in the polypeptide is due to a point mutation or small undetectable deletion/insertion. There is no reason to postulate a connection between the IR_L/IR_S deletion/insertion in JH2614 and the altered mobility of this polypeptide, although the possibility can not be entirely ruled out.

This is the first reported isolation of a HSV genome with a large genomic deletion into which extraneous DNA has been inserted. Nucleotide sequencing of the insert is in progress and comparisons are being made with sequenced DNA from sources other than HSV. The role of deleted genomes as vectors is shown by the isolation of a variant with the genomic structure of JH2614, but it is yet to be proven that such constructs are stable.

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Generation of a Herpes Simplex Virus Type 2 Variant Devoid of *Xba*I Sites: Removal of the 0·91 Map Coordinate Site Results in Impaired Synthesis of Glycoprotein G-2

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SUMMARY

Herpes simplex virus (HSV) type 2 (strain HG52) has four *Xba*I sites at map coordinates (m.c.) 0·45, 0·7, 0·91 and 0·94, i.e. two in the unique long and two in the unique short regions of the genome. Previously, we had isolated a genome containing only the 0·45 and 0·94 *Xba*I sites. Here we report the isolation of a mutant (JH2611) in which all four *Xba*I sites have been removed using an enrichment selection procedure, without any loss of viability. Removal of each site has been shown to be due to a base change or small undetectable deletion/insertion of less than 100 bp. In HSV-1, the *Xba*I site at 0·45 m.c. is in an open reading frame coding for a polypeptide of 14K. Both the 0·7 and 0·94 m.c. HSV-2 *Xba*I sites are in intergenic positions. The 0·91 m.c. *Xba*I site has been shown to be within the coding sequence of the glycoprotein gG-2. Synthesis of gG-2 by JH2611 and two other mutants, JH2610 (formerly HG52X163X3) and JH2609 (formerly HG52X163X21), in which the 0·91 m.c. site has been deleted was analysed by immunoprecipitation using the gG-2-specific monoclonal antibodies AP1 and LP5 and the anti-peptide serum 14713. In the mutants JH2610 and JH2611 neither gG-2 nor its precursor were detected but the monoclonal antibodies detected two polypeptides migrating above the normal positions of gG-2 and the gG-2 precursor; these were not precipitated by the anti-peptide serum. With the mutant JH2609 neither gG-2 nor its precursors could be detected by either the monoclonal antibodies or the anti-peptide serum. The results strongly suggest that gG-2 is non-essential for the growth of HSV-2 *in vitro*.

INTRODUCTION

Recombination in herpes simplex virus (HSV) is poorly understood and has for the most part been studied by using temperature-sensitive (*ts*) mutants (Brown *et al.*, 1973; Brown & Ritchie, 1975; Schaffer *et al.*, 1974). However, following our suggestion of using restriction endonuclease sites as unselected markers for the study of recombination in HSV (Brown *et al.*, 1984), Umene (1985) used two HSV type 1 (HSV-1) strains with eight restriction enzyme site differences to evaluate recombination frequency. In order to study recombination within a single strain and to use genomes which are totally homologous in genetic content apart from restriction endonuclease site differences, we have removed *Xba*I sites from HSV-1 strain 17 and HSV type 2 (HSV-2) strain HG52 (Brown *et al.*, 1984; Harland & Brown, 1985; Brown & Harland, 1987; MacLean & Brown, 1987*a*). In conjunction with *ts* markers these special genomes will enable us to study the role of sequence homology in recombination by determining the distribution of restriction endonuclease sites in intra- and intertypic crosses. In addition, by studying the time course of recombination the contribution of parental and progeny molecules in multiple rounds of mating will become apparent. The introduction of *ts* mutations of different genes into restriction endonuclease site-negative genomes should allow determination of the role of specific genes in recombination. The present paper reports the isolation of an HSV-2 (strain HG52)

genome in which the four *Xba*I sites have been deleted by enrichment selection (Brown *et al.*, 1984).

The glycoprotein gG-2 (formerly g92K) has been shown to map in the unique short (U_S) region (Marsden *et al.*, 1978, 1984; Roizman *et al.*, 1984; Olofsson *et al.*, 1986). Recent work by McGeoch *et al.* (1987) has determined the sequence of the gG-2 gene and shown that the *Xba*I site at map coordinate (m.c.) 0·91 lies within the coding region for the gG-2 polypeptide at residue 4785 near the carboxy terminus. Balachandran & Hutt-Fletcher (1985) have demonstrated the processing steps involved in the synthesis of gG-2 through a 120K precursor to the final processed form of 108K. The 108K polypeptide was shown to be equivalent to gG-2. The construction of mutants with changes in the *Xba*I site in the gene coding for gG-2 has made it possible to study the effect of these specific mutations on the production of the gG-2 glycoprotein.

METHODS

Growth of virus. Virus stocks were grown and titrated as described previously (Brown *et al.*, 1973). Strains of virus used were HSV-2 strain HG52 (Timbury, 1971), JH2602 (formerly HG52X163) (Harland & Brown, 1985), JH2610 (formerly HG52X163X3), JH2608 (formerly HG52X163X14) and JH2609 (formerly HG52X163X21) (Brown & Harland, 1987).

Preparation of virion DNA. Virion DNA was prepared according to the method of Wilkie (1973) and Stow & Wilkie (1976) as described by Brown *et al.* (1984).

Restriction endonuclease digestion of viral DNA. Digestion of DNA at 50 to 100 μg/ml was carried out at 37·5 °C in 0·006 M-Tris–HCl pH 7·5, 0·006 M-$MgCl_2$, 0·006 M-2-mercaptoethanol, 0·02 M-KCl, 1 mg/ml bovine serum albumin using 1 unit of *Xba*I per μg of viral DNA. Incubation was for 1, 3 or 4·5 h.

Transfection of virus DNA. Intact and *Xba*I-treated DNA was transfected at 1 to 2 μg/plate onto BHK21/C13 monolayers (4×10^6 cells) using the calcium phosphate infectivity assay technique (Stow & Wilkie, 1976). Single plaques obtained from transfection of *Xba*I-treated DNA were isolated, plaque-purified three times, grown into individual stocks and titrated.

Restriction enzyme analysis of virus genomes. Restriction enzyme analysis was carried out using the Linbro well technique (Lonsdale, 1979). Cells were infected in the presence of ^{32}P at an m.o.i. of 10 p.f.u./cell of titrated virus stocks obtained from single plaques; the cells were incubated at 31 °C for 24 to 48 h. ^{32}P-labelled viral DNA was treated with a range of restriction endonucleases at concentrations sufficient to give complete digestion in 4 h at 37 °C. Digests were analysed by electrophoresis on agarose gels of appropriate concentrations (0·5% to 1·2%). Gels were air-dried and exposed to Kodak XSI film for 24 to 48 h.

Immunoprecipitation. The monoclonal antibodies used were a mixture of AP1 and LP5 (Marsden *et al.*, 1984) which have been shown to precipitate specifically the M_r 92K glycoprotein gG-2; for the experiments, AP1 and LP5 were used in a 1:1 ratio. Cells (4×10^6) were infected with mutant or wild-type virus at an m.o.i. of 20 p.f.u./cell and maintained in Eagle's MEM containing one-fifth of the normal methionine concentration plus 2% calf serum. Labelling was carried out from 3 to 24 h post-infection with [^{35}S]methionine (50 μCi/ml), 100 μCi having been added to each plate. The cells were then washed twice with phosphate-buffered saline. Two ml of extraction buffer (0·05 M-Tris–HCl pH 7·2, 0·15 M-NaCl, 1% sodium deoxycholate, 0·1% SDS, 1% Triton X-100, 0·5 mM-PMSF) was added to each plate and the cells were scraped off. The suspension was sonicated, and ultracentrifuged in a Ti50 rotor at 35000 r.p.m. at 4 °C for 1 h. The supernatant was the antigen extract. Ten μl of the monoclonal antibody mixture was mixed with 500 μl antigen and incubated at room temperature for 25 min. Sixty μl of Protein A–Sepharose diluted 1:1 in extraction buffer was added and the mixture incubated for 2 h at 4 °C. The pellet was washed four times in extraction buffer and proteins were eluted by boiling in 100 μl sample buffer. Fifty μl of the sample was run on either a 7·5% or a 5% to 12·5% polyacrylamide gel (Marsden *et al.*, 1978).

In pulse–chase experiments, cells were labelled for 10 min with [^{35}S]methionine (200 μCi/ml) at 5 h post-infection and chased in medium containing 100 times the normal concentration of unlabelled methionine and cycloheximide (50 μg/ml) for 5 h. Cells were harvested either immediately after the pulse or after the 5 h chase.

In addition, immunoprecipitation was carried out using antiserum 14713 raised against amino acids 687 to 698 of U_S gene 4 (US4); these form a near C-terminal sequence but exclude the C-terminal residue itself (McGeoch *et al.*, 1987). Confluent monolayers of BHK cells were infected with 20 p.f.u./cell of virus and labelled with 100 μCi/ml [^{3}H]mannose from 5 to 12 h post-infection. Immunoprecipitation was carried out as described by Frame *et al.* (1986) and precipitated proteins were analysed by electrophoresis in 5% to 12·5% polyacrylamide gels.

Reciprocal labelling experiments were carried out using [^{3}H]mannose-labelled extracts with a mixture of AP1 and LP5 and [^{35}S]methionine-labelled extracts with 14713.

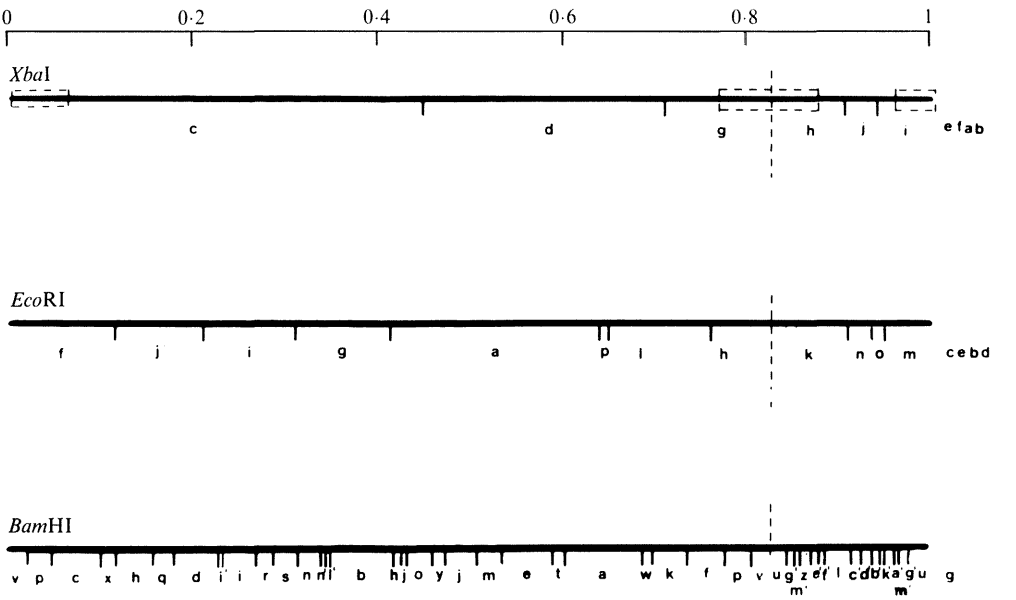

Fig. 1. Restriction endonuclease maps for the DNA of HSV-2 strain HG52 from Cortini & Wilkie (1978). The origin of the joint fragments is as follows: *Xba*I, $a = c + h$, $b = c + i$, $e = g + h$, $f = g + i$; *Eco*RI, $b = f + k$, $c = h + k$, $d = f + m$, $e = h + m$; *Bam*HI, $g = u + v$.

Nomenclature. As the number of HG52 *Xba*I site deletion mutants and mutants with large genomic deletions has increased to a point where the current nomenclature is too cumbersome, the mutants will from now on be designated as follows.

HG52XD94 = JH2601	HG52X163 = JH2602	HG52XD86 = JH2603
HG52XD192 = JH2604	HG52XD85/5 = JH2605	HG52XD85/4 = JH2606
HG52X163X12 = JH2607	HG52X163X14 = JH2608	HG52X163X21 = JH2609
HG52X163X3 = JH2610	HG52X163X3X53 = JH2611	HG52X163X3X45 = JH2612
	HG52X163X3X27 = JH2613	

RESULTS

Removal of the 0·45 and 0·94 m.c. Xba*I sites*

Wild-type HG52 contains four *Xba*I sites at 0·45, 0·7, 0·91 and 0·94 m.c. (Fig. 1; Cortini & Wilkie, 1978) i.e. two in the long unique and two in the short unique region of the genome. Using the enrichment selection procedure described previously (Brown *et al.*, 1984), we isolated a mutant, JH2602, lacking the *Xba*I site at 0·7 m.c.; the site loss was due to an approximately 150 base pair (bp) insertion as demonstrated by the altered mobility of the *Bam*HI *k* fragment in which the *Xba*I site is situated (Harland & Brown, 1985). JH2602 was used as the parental virus from which a variant lacking the 0·91 m.c. site in addition to the 0·7 m.c. site was isolated (Brown & Harland, 1987). The variant with the two deleted sites, JH2610, had lost the 0·91 m.c. site by a base change or small undetectable deletion or insertion. The *Bam*HI *l* fragment containing the 0·91 m.c. site had an unaltered mobility. A JH2610 DNA preparation was digested with *Xba*I (1 unit/μg DNA) for 1, 3 or 4·5 h at 37 °C. The digested DNA was transfected onto BHK21/C13 monolayers and 32 plaques were picked from two plates for each time point. The DNA from each plaque was subjected to restriction endonuclease digestion. In order to identify mutants lacking the 0·45 m.c. site it was necessary to carry out *Xba*I/*Eco*RI double digestions. *Xba*I alone would not clearly identify such mutants as the fragments generated would comigrate at the top of the gel. The *Xba*I site at 0·45 m.c. is within the *Eco*RI *a* fragment and the *Xba*I site at 0·94 m.c. is within the *Eco*RI *o* fragment. Thus *Eco*RI *a*, a

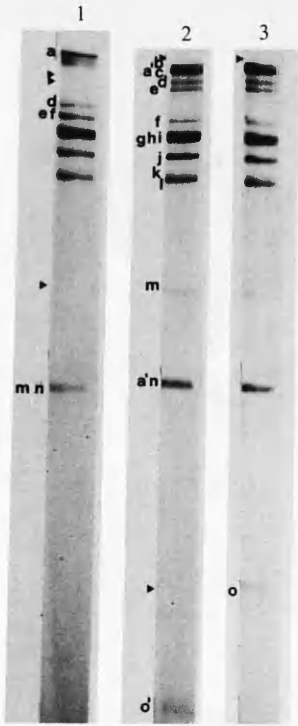

Fig. 2

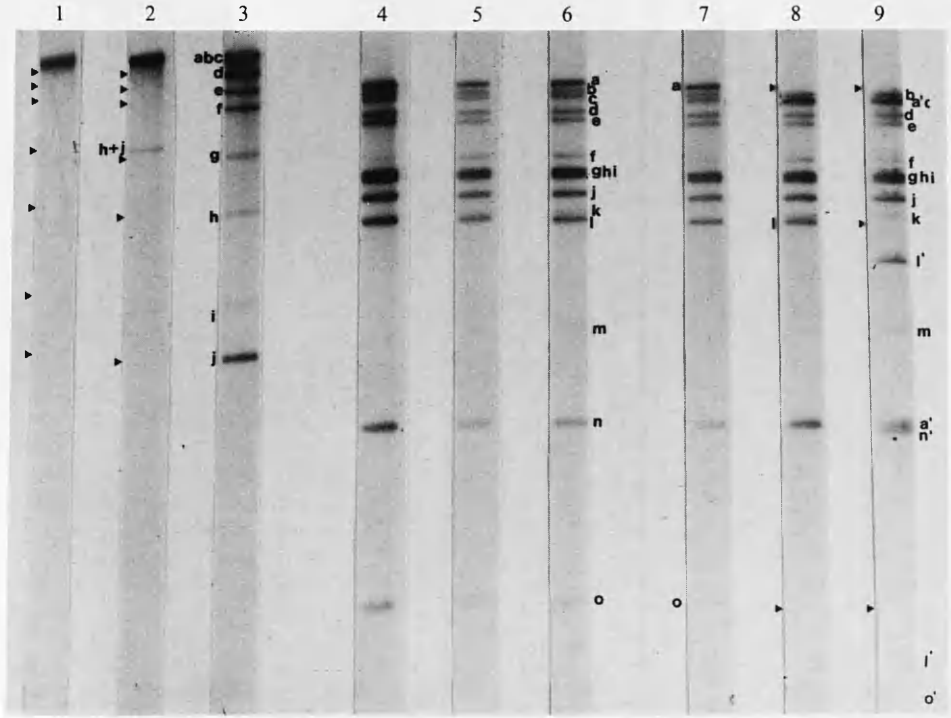

Fig. 3

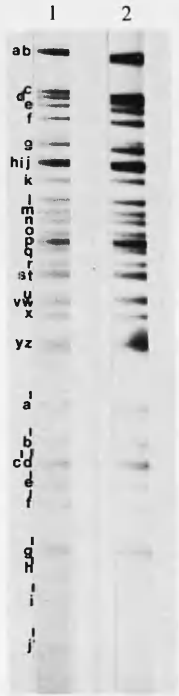

Fig. 4

Fig. 2. Autoradiographs of *Xba*I/*Eco*RI digests of viral DNA ^{32}P-labelled *in vivo*. Lane 1, JH2613; lane 2, JH2610; lane 3, JH2612. Letters refer to specific fragments and ▶ indicates missing fragments.

Fig. 3. Autoradiographs of restriction digests of viral DNA ^{32}P-labelled *in vivo*. Lanes 1, 2 and 3, *Xba*I; lanes 4, 5 and 6, *Eco*RI; lanes 7, 8 and 9, *Xba*I/*Eco*RI. Lanes 1, 4 and 7, JH2611; lanes 2, 5 and 8, JH2610; lanes 3, 6 and 9, HG52. Letters refer to specific fragments and ▶ indicates missing fragments.

Fig. 4. Autoradiograph of a *Bam*HI digest of viral DNA ^{32}P-labelled *in vivo*: lane 1, JH2610; lane 2, JH2611.

20 × 10^6 M_r fragment, is cleaved by *Xba*I to two *a'* fragments of M_r approximately 18 × 10^6 and 2 × 10^6. The *Eco*RI *o* fragment (0·8 × 10^6 M_r) is cleaved by *Xba*I to two *o'* fragments of M_r approximately 0·6 × 10^6 and 0·2 × 10^6.

Of the 96 plaques analysed, two appeared to have lost the *Xba*I site at 0·45 m.c. An *Xba*I/*Eco*RI digest of one of these, JH2613, is shown in Fig. 2. When the parental JH2610 was cleaved with *Xba*I/*Eco*RI it could be seen that the *Eco*RI *a* band disappeared and the two resulting *a'* bands comigrated with *c* and *n* (lane 2). When JH2613 DNA was cut with *Xba*I/*Eco*RI, the *Eco*RI *a* band ran in its normal position at the top of the gel (lane 1). Hence this mutant had lost the 0·45 m.c. *Xba*I site in addition to the 0·7 and 0·91 m.c. sites. This mutant also had a deletion in the short region of the genome such that *Eco*RI *m* comigrated with *n* and the two joint fragments containing *m*, i.e. *d* and *e*, were not in their normal position; *e* comigrated with *f* and *d* ran above *f*. The deletion was similar to that described in JH2607 (Brown & Harland, 1987). The *Xba*I/*Eco*RI analysis also revealed that 20 of the 96 plaques appeared to have lost the *Xba*I site at 0·94 m.c. An *Xba*I/*Eco*RI digest of one of these, JH2612, is shown in Fig. 2 (lane 3). When the parental JH2610 DNA was cut with *Xba*I/*Eco*RI, it could be seen that the *Eco*RI *o* band was missing and one *o'* band ran at the bottom of the gel. The other *o'* band of M_r 0·2 × 10^6 had migrated off the gel (lane 2). In the mutant JH2612 the *Eco*RI *o* band migrated in its normal position (lane 3). The *Xba*I site at 0·94 m.c. had therefore been deleted.

Restriction endonuclease analysis revealed that five of the 96 plaques had lost both the 0·45 and 0·94 m.c. sites. *Xba*I, *Eco*RI and *Xba*I/*Eco*RI digests of one of these mutants (JH2611) are shown in Fig. 3. *Xba*I digestion of JH2610 (lane 2) showed missing *g*, *h*, *j*, *d*, *e* and *f* bands; the *i* band was present and the new band running above the *g* position was made up of *h* and *j*. The mutant had lost the 0·7 m.c. *d*/*g* site and the 0·91 m.c. *h*/*j* site. *Xba*I analysis of JH2611 showed only a large band at the top of the gel (lane 1). *Eco*RI digestion of both JH2610 (lane 5) and JH2611 (lane 4) gave an identical pattern to that of the wild-type HG52 (lane 6). All the fragments were present and their mobilities were normal. When HG52 was cut with *Xba*I and *Eco*RI (lane 9) the *Eco*RI *a* band was cut to give two *a'* fragments as described for Fig. 2, the *Eco*RI *l* band was cut to give two *l'* fragments, the *Eco*RI *n* fragment was cut to give a slightly smaller *n'* fragment and an *n'* fragment which was too small to be seen on the gel, and the *Eco*RI *o* fragment was cut to give two *o'* fragments as also described for Fig. 2. In JH2610 (lane 8) the 0·7 m.c. *Xba*I site was absent, therefore the *Eco*RI *l* band was not cut and migrated normally; the 0·91 m.c. *Xba*I site was also absent, therefore *Eco*RI *n* migrated in its normal position. The 0·45 and 0·94 m.c. sites were present giving rise to the two *Eco*RI *a'* and two *o'* bands (lane 8). *Xba*I/*Eco*RI digestion of JH2611 showed the *Eco*RI *a*, *l*, *n* and *o* bands in their normal positions (lane 7). In other words the *Xba*I/*Eco*RI double digest was indistinguishable from the *Eco*RI digest, demonstrating that no *Xba*I site was present in the mutant. The 0·94 m.c. site loss must have been due to a base change or small undetectable deletion or insertion. Any deletion or insertion > 100 b.p. would have been detected in the mobility of the *Eco*RI *o* fragment (lane 4). The 0·45 m.c. *Xba*I site lies within *Bam*HI *o*. It can be seen in Fig. 4 (lane 2) that the mobility of *Bam*HI *o* of JH2611 was unaltered compared to the parental JH2610 (lane 1). The site loss at 0·45 m.c. was therefore due to a base change or small undetectable deletion or insertion. The mutants JH2611, JH2612 and JH2613 were all viable in tissue culture and grew normally to give titres of at least 10^8 p.f.u./ml.

Effect of removal of the 0·91 m.c. XbaI *site on the US4 gene product, gG-2*

The monoclonal antibodies AP1 and LP5 have been shown to immunoprecipitate specifically the polypeptide gG-2 (g92K; Marsden *et al.*, 1984) which has been shown to be encoded by US4 of HSV-2 (McGeoch *et al.*, 1987). The *Xba*I site at 0·91 m.c. lies within the coding region for gG-2 starting at residue 4785. Our analysis has led to the identification of three mutants in which the *Xba*I site at 0·91 m.c. has been lost. These are JH2610, JH2609 (Brown & Harland, 1987) and JH2611. The 0·91 m.c. site loss in the mutant JH2610 was shown to be due to either a base change or a small undetectable deletion or insertion in that the *Bam*HI *l* fragment in which the site resides was shown to have an unchanged mobility compared to wild-type virus on restriction endonuclease analysis (JH2610 retains the *Xba*I sites at 0·45 and 0·94). The mutant JH2609 on

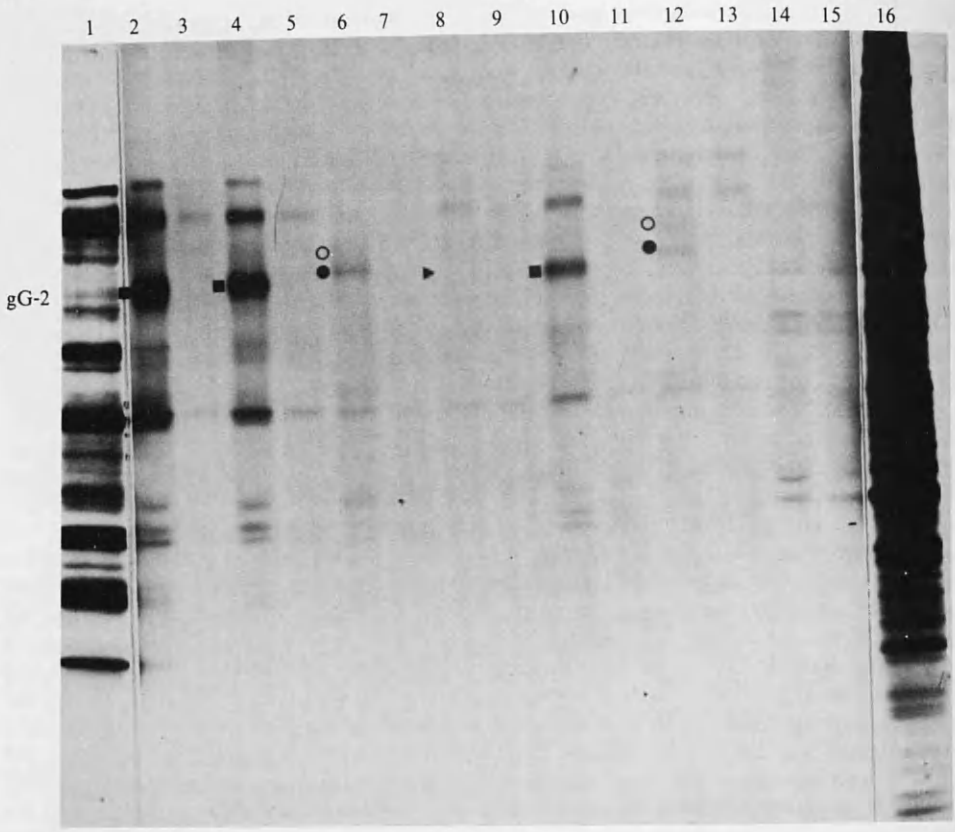

Fig. 5. Immunoprecipitation of HSV-2 glycoprotein G by the monoclonal antibodies AP1 and LP5 (Marsden *et al.*, 1984). Immunoprecipitations were carried out on extracts of [^{35}S]methionine-labelled, HSV-2-infected BHK cells and precipitated species were resolved by gel electrophoresis and detected by autoradiography. Lane 1, an extract from HSV-2-infected cells; lanes 14, 15 and 16, extracts from mock-infected cells; lanes 3, 5, 7, 9, 11 and 13, controls using normal ascitic fluid; lane 2, gG-2 from HG52-infected cells as precipitated by AP1 and LP5; lane 4, gG-2 from JH2602; lane 10, gG-2 from JH2608; lane 8, no specific precipitation from JH2609; lane 6, precipitation of two species from JH2610; lane 12, precipitation of two species from JH2611. The location of gG-2 is marked ■. The absence of gG-2 is marked ▶. The two aberrant species precipitated are marked ○ and ●.

*Xba*I digestion showed a fused *Xba*I *h* and *j* fragment indicating loss of the 0·91 m.c. *Xba*I site; the mobility of the *Bam*HI *l* fragment was unchanged. In addition, the information in *Xba*I *i* was replaced by the information within *Xba*I *h*. A detailed structure of this mutant genome which again retains the *Xba*I sites at 0·45 and 0·94 is shown by Brown & Harland (1987). The third mutant JH2611 was derived from JH2610 and had lost all four *Xba*I sites.

We have previously shown the one-step growth kinetics of JH2610 and JH2609 (Brown & Harland, 1987). JH2610 showed normal growth curve characteristics but JH2609 demonstrated poor single cycle growth kinetics. However, as this mutant in addition to the deleted 0·91 *Xba*I site has lost the genes US10, -11, -12, one copy of immediate early (IE) gene 3 and one copy of an origin of replication (ORI_S), the growth restriction cannot be attributed to a specific gene. The mutant JH2611 derived from JH2610 grew to high titre ($> 10^8$ p.f.u./ml) under normal growth conditions.

To determine whether the removal of the 0·91 m.c. *Xba*I site from these mutants had any effect on the synthesis of gG-2, immunoprecipitation experiments were carried out using a 1 : 1 mixture of the monoclonal antibodies AP1 and LP5, both of which specifically precipitate gG-2.

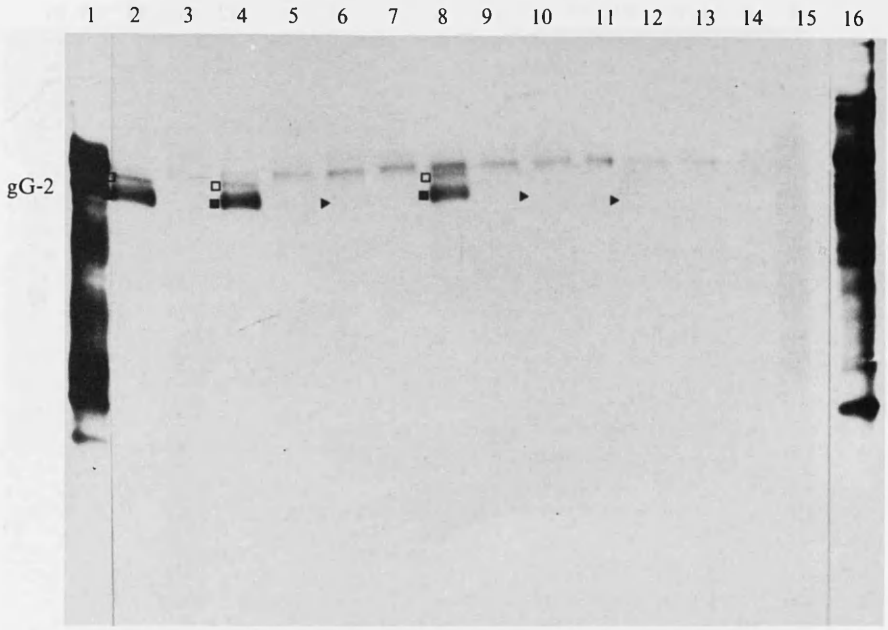

Fig. 6. Immunoprecipitation of HSV-2 glycoprotein G by the anti-peptide serum 14713 (McGeoch *et al.*, 1987). Immunoprecipitations were carried out on extracts of [^{3}H]mannose-labelled, HSV-2-infected BHK cells, and precipitated species were resolved by gel electrophoresis and detected by autoradiography. Lane 1, an extract from HG52-infected cells; lanes 14, 15 and 16, extracts from mock-infected cells; lane 2, gG-2 and it precursor from HG52-infected cells as precipitated by 14713. Similarly, lane 4, gG-2 and its precursor from JH2602 and lane 8, gG-2 and its precursor from JH2608. No specific precipitation was detected in extracts from the mutants JH2610 (lane 6), JH2609 (lane 10) or JH2611 (lane 12). Lanes 3, 5, 7, 9, 11 and 13 are controls using 14713 with the addition of 50 μg of the C-terminal peptide. The position of gG-2 is marked ■ and its precursor □. The absence of gG-2 is marked ▶.

Viruses used as controls were HG52, JH2602 which has the *Xba*I site at 0·7 m.c. deleted, and JH2608 which has a structure similar to JH2609 except that the 0·91 m.c. *Xba*I site has been retained. Ascites fluid was used as a further control with each mutant. The results are shown in Fig. 5. It can be seen that as expected AP1 and LP5 specifically precipitated gG-2 of HG52 (Fig. 5, lane 2) and of mutants JH2602 (lane 4) and JH2608 (lane 10). In contrast, with the mutants JH2610 (lane 6) and JH2611 (lane 12) the monoclonal antibodies did not precipitate a band in the normal gG-2 position but precipitated two novel bands of apparent higher M_r running just above gG-2. In the mutant JH2609 (lane 8) there was no detectable specific precipitation.

To confirm these findings, immunoprecipitation experiments were carried out using the antiserum 14713 directed against the dodecapeptide of the near C-terminal portion of US4 (McGeoch *et al.*, 1987). The label used was [^{3}H]mannose whereas in the AP1 and LP5 experiments that used was [^{35}S]methionine. Competition controls were set up by addition of the specific peptide. Fig. 6 shows that the oligopeptide antiserum specifically precipitated gG-2 of HG52 (lane 2) and of the mutants JH2602 (lane 4) and JH2608 (lane 8). No specific precipitation was seen with the mutants JH2609 (lane 10), JH2610 (lane 6) and JH2611 (lane 12). Reciprocal labelling experiments using [^{35}S]methionine with 14713 and [^{3}H]mannose with AP1 and LP5 gave confirmatory results. Fig. 7 shows an immunoprecipitation of [^{3}H]mannose-labelled extracts with AP1 and LP5. With HG52 (lane 2), JH2602 (lane 4) and JH2608 (lane 8) the monoclonal antibodies precipitated both the precursor and the processed forms of gG-2. With the mutant JH2609 (lane 10) no precipitate was detectable. With the mutants JH2610 (lane 6)

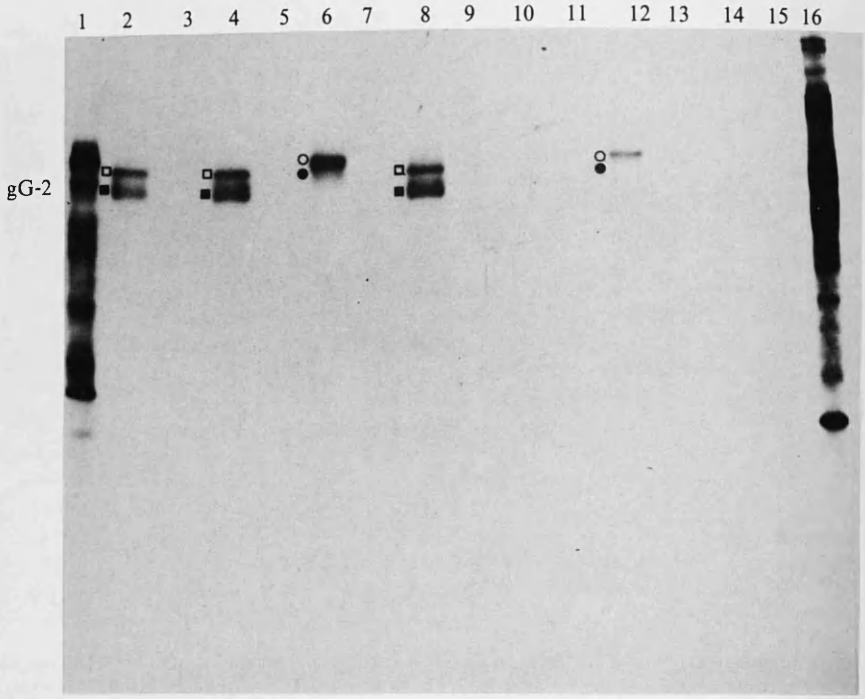

Fig. 7. Immunoprecipitation of HSV-2 glycoprotein G by the monoclonal antibodies AP1 and LP5 (Marsden *et al.*, 1984). Immunoprecipitations were carried out on extracts of [^{3}H]mannose-labelled, HSV-2-infected BHK cells, and the precipitated species were resolved by gel electrophoresis and detected by autoradiography. Lane 1, an extract from HG52-infected cells; lanes 14, 15 and 16, extracts from mock-infected cells; lane 2, gG-2 and its precursor from HG52-infected cells as precipitated by AP1 and LP5. Similarly, lanes 4 and 8 show gG-2 and its precursor from JH2602- and JH2608-infected cells, respectively. Lanes 6 and 12, the aberrant gG-2 precursor and a small amount of the aberrant processed form of the precursor from JH2610- and JH2611-infected cells, respectively. Lane 10, no specific precipitation from JH2609-infected cells. Lanes 3, 5, 7, 9, 11 and 13 are controls using normal ascitic fluid. Location of gG-2 is marked ■ and its precursor □. The location of the aberrant precursor is marked ○ and the aberrant processed form of gG-2 is marked ●.

and JH2611 (lane 12) there was precipitation of aberrantly migrating precursor polypeptides with little detectable precipitation of the processed form.

To determine whether the apparent higher M_r species precipitated from extracts of the mutants JH2610 and JH2611 were precursors of gG-2, pulse–chase experiments were carried out. The wild-type HG52 was labelled with [^{35}S]methionine for 10 min at 5 h post-infection and samples were harvested immediately. In addition, after labelling for 10 min at 5 h post-infection, a further 5 h chase was allowed before harvesting of the sample. As controls, wild-type and mutant were labelled from 3 to 20 h with [^{35}S]methionine. The results with HG52 using the monoclonal antibodies AP1 and LP5 are shown in Fig. 8. When a pulse was given for 10 min at 5 h post-infection with HG52, a single labelled species was specifically precipitated (lane 6). This would be equivalent to the 120K polypeptide described by Balachandran & Hutt-Fletcher (1985) under similar conditions. When the 10 min pulse was followed by a 5 h chase (lane 8), the only species specifically precipitated was the processed form of gG-2 equivalent to the 108K polypeptide seen by Balachandran & Hutt-Fletcher (1985). This migrated to the same position as gG-2 seen in a 3 to 20 h labelled infection (lane 2). As already seen in Fig. 5 when JH2611 was labelled from 3 to 20 h, an aberrant precursor polypeptide was specifically precipitated and a small amount of an aberrant processed form was also precipitated (Fig. 5, lane 4). The aberrant precursor migrated above the precursor seen in (Fig. 8, lane 6) and therefore was not the 120K polypeptide described as the normal precursor of gG-2.

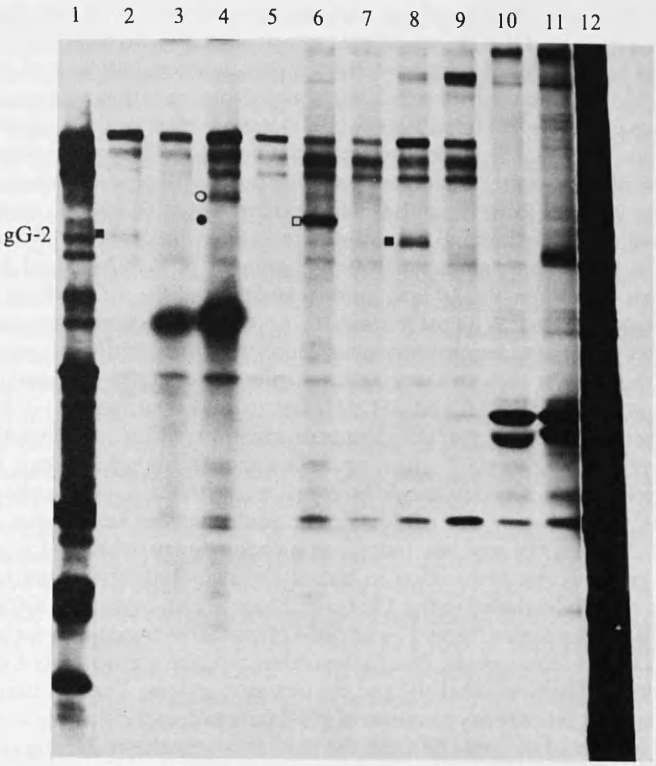

Fig. 8. Immunoprecipitation of HSV-2 glycoprotein G by the monoclonal antibodies AP1 and LP5 (Marsden *et al.*, 1984) in a pulse–chase experiment. Immunoprecipitations were carried out on extracts of [^{35}S]methionine-labelled, HSV-2-infected BHK cells, and the precipitated species were resolved by gel electrophoresis and detected by autoradiography. Lane 1, extract from HG52-infected cells; lanes 10, 11 and 12, extracts from mock-infected cells. Lane 2, gG-2 from HG52-infected cells labelled from 3 to 20 h post-infection; lane 4, the aberrant precursor and aberrant gG-2 from JH2611-infected cells labelled from 3 to 20 h post-infection; lane 6, the gG-2 precursor from HG52-infected cells labelled for 10 min at 3 h post-infection; lane 8, gG-2 from HG52-infected cells labelled for 10 min at 5 h post-infection and chased for 5 h in medium containing 100 times the normal concentration of unlabelled methionine plus cycloheximide (50 μg/ml). Lanes 3, 5, 7 and 9 are controls using normal ascitic fluid. Location of gG-2 is marked ■ and its precursor □. The location of the aberrant precursor is marked ○ and the aberrant processed form of gG-2 is marked ●.

DISCUSSION

The 0·45 m.c. *Xba*I site in HSV-1 has been shown to be within an open reading frame coding for a polypeptide of 14K (D. J. McGeoch, personal communication). As the 0·45 m.c. site is coincident in HSV-1 and HSV-2 and has been lost without affecting the viability of the virus it must be assumed that either the DNA alteration causing the site loss is conservative or the gene function is non-essential. The 0·7 m.c. site lies very close to the coding region for 65K but in HSV-2 strain 333 it has been shown that the *Xba*I site is not within *Bgl*II *i* which contains the coding sequence for 65K. No sequence data are available for the 0·7 m.c. site of HSV-2 strain HG52 but as for the 0·45 m.c. site, it must be assumed that the DNA insertion causing the site loss at 0·7 m.c. is conservative, intergenic or, less likely, in the coding sequence for a non-essential polypeptide. The 0·94 m.c. *Xba*I site is in an intergenic position (Whitton, 1984) and therefore any DNA alteration should be of no consequence.

The *Xba*I site at 0·91 m.c. lies near the 3′ end of US4 starting from residue 4785 of HSV-2 (McGeoch *et al.*, 1987). Immunoprecipitation experiments using a mixture of the monoclonal antibodies AP1 and LP5 showed that in HG52 infections, gG-2 was specifically precipitated.

The mutants JH2602 and JH2608 which retained the 0·91 m.c. *Xba*I site also synthesized a normal gG-2. The mutants JH2610 and JH2611 which were derived from JH2602 and in which the *Xba*I site has been removed by a base change or a deletion/insertion of less than 100 bp, showed no specific precipitation of gG-2 but specific precipitation of two bands of higher apparent M_r. Balachandran & Hutt-Fletcher (1985) have shown that the processing of gG-2 is complex. They proposed that an unglycosylated precursor of 110K is glycosylated to give a high mannose peptide of 120K which undergoes peptide cleavage to give a product of 108K; this is equivalent to the 92K gG-2. In the pulse–chase experiment with HG52, when the virus was pulse-labelled with [^{35}S]methionine for 10 min at 5 h post-infection only a single species equivalent to the main 120K precursor was precipitated by the AP1 and LP5 monoclonal antibody mixture, but when the 10 min pulse was followed by a 5 h chase the single 92K polypeptide was precipitated. With the mutant JH2611 the pulse–chase experiment showed that the higher M_r species was an aberrant precursor which was inefficiently processed to the smaller polypeptide of the doublet seen in the long label infection (data not shown).

The data suggest that JH2610 and JH2611 fail to make normal gG-2 but synthesize a polypeptide either of higher M_r or of different conformation, which is inefficiently processed to give a form corresponding to gG-2 which again is either of higher M_r than normal or has a different tertiary structure. The data would be consistent with the sequence alteration removing the *Xba*I site at 4785, altering the reading frame such that the stop codon at residue 4939 (McGeoch *et al.*, 1987) is not read but another stop codon downstream and in another frame is used. The only out-of-frame stop codon in one of the two alternative reading frames in the coding region is actually included in the *Xba*I site. There are no other out-of-frame stop codons until residues 5007 (alternative frame 1) and 5024 (alternative frame 2) which would result in either an extra 23 or 29 amino acids. Due to the frameshift, the amino acids encoded will all be changed between the destroyed *Xba*I site and the new stop codons. The fact that the antipeptide serum directed against the carboxy terminus of gG-2 fails to detect either the aberrant precursor or its processed form is what would be expected from this hypothesis. Upstream of the carboxy terminus of gG-2 are the sequences thought to confer the transmembrane properties of gG-2. If the normal reading frame is not used the transmembrane ability of the polypeptide may be impaired. Experiments are currently under way to determine whether the aberrant gG-2 is incorporated into virions. Until we have sequenced the US4 gene of the mutants, the exact nature of the deletion and hence its specific consequences cannot be determined.

With the mutant JH2609 neither gG-2 nor its precursors were precipitated by either the monoclonal antibody mixture or the anti-peptide serum 14713. The results obtained by the use of both sets of reagent make it unlikely that the explanation is non-recognition by all three reagents. 14713 is known to recognize the carboxy terminus of gG-2, although the precise epitopes recognized by AP1 and LP5 are unknown. The mutants JH2608 and JH2609 have essentially the same structure except for the missing *Xba*I site in JH2609, and thus absence of any gG-2-related polypeptide must be a direct consequence of the genomic alteration removing the *Xba*I site of JH2609. The unidentified sequence rearrangement which removes the *Xba*I site could have introduced a stop signal such that a rapidly degraded truncated form of gG-2 is made. The *Xba*I recognition signal is TCTAGA. Removal of one nucleotide from between T-CT or insertion there of two nucleotides bring the TAG stop codon into the reading frame. Similarly insertion of a T between A and G would produce an in-frame stop codon in the *Xba*I site. Other deletions or insertions which take out the site and introduce an in-frame stop codon would have the same effect. Until the sequencing data are available for JH2609, the reason for lack of detectable polypeptide can only be conjecture.

The data demonstrate that (i) a fully processed form of gG-2 is not necessary for viable growth in tissue culture and (ii) gG-2 is either dispensable or needed only in undetectable amounts in the lytic cycle of the virus. It has recently been shown that US4 of HSV-1 is non-essential *in vitro* (Longnecker & Roizman, 1987). Varicella-zoster virus has been shown to lack the glycoprotein gene equivalent to HSV US4 (McGeoch, 1984; Davison & McGeoch, 1986). Perhaps the lack of gG-2 in the HSV mutants could be compensated by the evolutionarily related gD (McGeoch *et al.*, 1987).

The HG52 mutant with all four *Xba*I sites deleted arose at a frequency of five plaques out of 96 isolated after transfection of *Xba*I-digested JH2610 DNA. As the five could have been clonally related, this isolation frequency is approximately 1%. This high frequency of isolation has been maintained throughout the study on the removal of *Xba*I sites from HG52 (Harland & Brown, 1985; Brown & Harland, 1987). In addition, deletions in the long repeat regions of HG52 occur at a frequency of 24% and in the short repeat/unique region at a frequency of 7% (Harland & Brown, 1985; Brown & Harland, 1987).

In HSV-1 strain 17, genomes with deleted *Xba*I sites at 0·07 and 0·63 m.c. were isolated at a frequency of 3·6% (Brown *et al.*, 1984). However, genomes lacking the 0·29 m.c. *Xba*I site (which lies within the gH gene) were not detected despite over 2500 plaques being screened after enrichment selection. The 0·29 m.c. *Xba*I site was eventually disrupted by insertion of a synthetic oligonucleotide (MacLean & Brown, 1987*a*). The *Xba*I site at 0·45 m.c. in strain 17 was shown to be deleted in 14 out of 196 plaques screened after a single transfection of *Xba*I-treated DNA. Again as these plaques could have been clonally related this represents an isolation frequency of approximately 0·5%. However, this mutant was detected only after screening over 2500 plaques for the loss of the 0·45 m.c. site. Similarly, in screening HSV-1 strain 17 plaques for genomic deletions, only four genomes with deletions have been isolated from over 5000 plaques tested. These mutations occurred around the U_S/IR_S junction (MacLean & Brown, 1987*b*,*c*). It is concluded that genomic alterations, i.e. base changes, deletions and insertions, occur at a much higher frequency in HSV-2 strain HG52 than in HSV-1 strain 17.

The isolation of JH2611 will allow its use in intratypic and intertypic recombination studies and as a eukaryotic vector or recipient for viral plasmids cloned into desired positions, similar to adenovirus (Jones & Shenk, 1978; Stow, 1981). The role of restriction endonuclease site-negative mutants in superinfection experiments has already been exploited (Cook & Brown, 1987) and they are currently being used to study recombination *in vivo*.

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Three Mutants of Herpes Simplex Virus Type 2: One Lacking the Genes US10, US11 and US12 and Two in which R_S Has Been Extended by 6 kb to 0·91 Map Units with Loss of U_S Sequences between 0·94 and the U_S/TR_S Junction

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SUMMARY

In the process of generating restriction endonuclease site deletion mutants, we have isolated and characterized three mutants of herpes simplex virus type 2 (HSV-2), strain HG52, with large genomic deletions in U_S and TR_S. The deleted sequences (7·5 kb) extend from 0·94 map coordinates (m.c.) to 0·99 m.c. and are presumed to contain the HSV-2 gene equivalents of US10, 11 and 12, one copy of immediate early (IE) gene 3 and one copy of an origin of replication (ORI_S). One of the mutants (HG52X163X12) has a simple deletion whereas in the two others (HG52X163X14 and HG52X163X21) the deleted sequences have been replaced by inverted duplications of U_S/IR_S sequences between 0·83 and 0·91 m.c. such that the molecules have short region inverted repeats extended by 6 kb on either side. All three are viable, stable and grow in tissue culture indicating that the polypeptides coded by the HSV-2 genes equivalent to US10, 11 and 12 are non-essential for lytic growth in BHK21/C13 cells. In addition the lack of one copy of the HSV-2 equivalent of IE gene 3 and ORI_S in HG52X163X12 shows that only one copy of each suffices for viability. However the temperature restriction of the mutants at 38·5 °C suggests that one or more of the polypeptides coded by the deleted sequences may be required in conjunction with another polypeptide(s) for viral growth or stability at the higher temperature.

INTRODUCTION

Deletions in one copy of the long repeat sequences of herpes simplex virus type 2 (HSV-2) strain HG52 occur at a frequency of 24% by passage eight from the original field isolation (Harland & Brown, 1985). The described deletions range in size with the maximum being 9 kb and encompass the entire long repeat with the exception of the '*a*' sequences. Other strains of HSV-2 exhibit similar deletions showing that the HG52 strain is not exceptional. Deletions in the long repeat had been observed previously in intertypic recombinants when Davison *et al.* (1981) suggested that they probably arose as a consequence of heterotypic repetitive regions. Our observations show that this could not be the explanation but that deletions within one copy of the long repeats of the HG52 genome are relatively common. These deletion variants do not have a selective disadvantage (Harland & Brown, 1985); thus only one copy of immediate early (IE) gene 1 and the other coding information within the long repeats is sufficient for viable growth in tissue culture. The product of IE gene 1, i.e. V_{mw}IE110, in conjunction with V_{mw}IE175 has an enhancing effect on early polypeptide transcription (Everett, 1984) but it has not been shown whether V_{mw}IE110 is essential for lytic growth.

The high frequency of spontaneous deletions within the long repetitive sequences raises the question of whether similar deletions could occur in the short repetitive sequences of the genome. The repeat (TR_S) and unique short (U_S) regions of the HSV-1 genome have been fully sequenced (Murchie & McGeoch, 1982; McGeoch *et al.*, 1985). The IE3 gene coding for V_{mw}IE175 is contained entirely within the R_S component (Murchie & McGeoch, 1982) and is

diploid in the virus genome (Clements *et al.*, 1979; Watson *et al.*, 1979; Mackem & Roizman, 1980; Davison & Wilkie, 1981); V_{mw}IE175 is required continuously during infection to maintain early and late mRNA and protein synthesis (Preston, 1979; Dixon & Schaffer, 1980; Watson & Clements, 1980). Part of the US12 gene (IE5) coding for V_{mw}IE12 is also contained within TR_S i.e. the promoter and upstream sequences, most of the 5′ non-coding region and the intron, whereas the coding region is entirely within U_S. The function of V_{mw}IE12 is unknown but Marsden *et al.* (1982) suggested that its cytoplasmic location and the observation that it is not phosphorylated may point to a different role from other IE polypeptides.

Two other HSV-1 genes have been identified at the right end of U_S (McGeoch *et al.*, 1985). These are US11 and US10 and their reading frames which differ partially overlap. US11 codes for a polypeptide of 21 000 mol. wt. (21K), a late basic species previously identified as binding strongly to DNA (Bayliss *et al.*, 1975) and subsequently shown to bind preferentially to '*a*' sequences (Dalziel & Marsden, 1984). US10 which is 3′ coterminal with US12 and US11 codes for a late polypeptide (mol. wt. 33K) of unknown function (Lee *et al.*, 1982).

The short repetitive regions of HSV-1 also contain an origin of replication, ORI_S, which has been shown to reside in a 535 bp segment within the untranscribed region between the IE5 (US12) and IE3 genes (Stow & McMonagle, 1983).

The complete sequence of the HSV-2 short region is still unknown, but it is known that there are transcripts equivalent to genes US10, 11 and 12 of HSV-1 (D. J. McGeoch, personal communication). A polypeptide V_{mw}IE182 equivalent to V_{mw}IE175 has been identified (Morse *et al.*, 1978) and similarly a polypeptide of mol. wt. 12·5K in HSV-2 has been identified as equivalent to V_{mw}IE12 of HSV-1 (Marsden *et al.*, 1982). In the process of deleting *Xba*I sites we have now identified and isolated three separate viable mutants with deletions spanning the short terminal repeat and the right hand end of the short unique region of strain HG52 of HSV-2. Two of the mutants additionally exhibit duplications of sequences within U_S. This paper reports the characterization of the three mutants with major rearrangements in the short region of the genome and also of the DNA structure of one *Xba*I site deletion mutant.

METHODS

Growth of virus. Virus stocks were grown and titrated as described previously (Brown *et al.*, 1973). Strains of virus used were HSV-2 strain HG52 (Timbury, 1971) and HG52X163 (Harland & Brown, 1985).

Preparation of virion DNA. Virion DNA was prepared according to the method of Wilkie (1973) and Stow & Wilkie (1976) as described by Brown *et al.* (1984).

Restriction endonuclease digestion of viral DNA. Digestion of DNA at 50 to 100 μg/ml was carried out at 37 °C in 0·006 M-Tris–HCl pH 7·5, 0·006 M-$MgCl_2$, 0·006 M-2-mercaptoethanol, 0·02 M-KCl, 1 mg/ml bovine serum albumin using 1 unit of *Xba*I per μg viral DNA. Incubation was for 1, 2 or 4 h. DNA restriction enzyme profiles were obtained by electrophoresis through 0·5% agarose gels stained with ethidium bromide.

Transfection of virus DNA. Intact and *Xba*I-digested DNA was transfected at 1 to 2 μg/plate onto BHK21/C13 monolayers (4×10^6 cells) using the calcium phosphate infectivity assay technique (Stow & Wilkie, 1976). Single plaques obtained from transfection of *Xba*I-treated DNA were isolated, plaque-purified three times, grown into individual stocks and titrated. The mutants described in this paper were all isolated from a single transfection of *Xba*I-digested DNA of the *Xba*I site mutant HG52X163 (Harland & Brown, 1985). In the nomenclature of mutants the numbers after X indicate plaque number.

Restriction enzyme analysis of virus genomes. Restriction enzyme analysis was carried out using the Linbro well technique (Lonsdale, 1979). Cells were infected in the presence of $^{32}P_i$ at a m.o.i. of 10 p.f.u./cell of titrated virus stocks obtained from single plaques; the cells were incubated at 31 °C for 24 to 48 h. ^{32}P-labelled viral DNA was treated with a range of restriction endonucleases at concentrations sufficient to give complete digestion in 4 h at 37 °C. Digests were analysed by electrophoresis overnight on agarose gels of appropriate concentrations (0·5 to 1·2%). Gels were air-dried and exposed to Kodak XS1 film for 24 to 48 h.

DNA–DNA hybridization. DNA fragments from restriction endonuclease digests were transferred from agarose gels to nitrocellulose filters (BA85, Schleicher & Schüll) and hybridized with nick-translated DNA by the method of Southern (1975). Nick-translated DNA was prepared from total HSV-2 DNA (HG52), a recombinant plasmid (pGZ1) containing HSV-2 *Bam*HI fragment *g* (Davison & Wilkie, 1981), a recombinant plasmid (pGZ66) containing HSV-2 *Bam*HI *a′*, a recombinant plasmid (pGZ67) containing HSV-2 *Bam*HI *b′* (Whitton, 1984), a recombinant plasmid (pGZ12) containing HSV-2 *Hin*dIII *k* and a recombinant plasmid (pGZ28) containing HSV-2 *Hin*dIII *l*. The hybridization conditions were as described by Brown *et al.* (1984).

One-step growth experiments. 10^6 BHK21/C13 cells were infected at a m.o.i. of 5 p.f.u./cell. Adsorption was carried out for 45 min at either 37 °C or 38·5 °C and after washing twice with phosphate-buffered saline containing 5% calf serum and overlaying with Eagle's medium containing 10% calf serum, incubation was continued at either 37 °C or 38·5 °C. Cells were harvested into the medium (2 ml) and virus released by sonication was titrated at 37 °C. Samples were harvested at 0, 2, 4, 6, 8, 12 and 24 h post-adsorption. The viruses used were HG52, HG52X163, HG52X163X3, HG52X163X12, HG52X163X14 and HG52X163X21.

RESULTS

Removal of the Xba*I site at 0·91 map units*

The genome of HG52X163 has lost the *Xba*I site at map coordinate (m.c.) 0·7, due to an insertion of about 150 base pairs (bp) as demonstrated by the alteration in the mobility of the *Bam*HI *k* fragment which contains the *Xba*I 0·7 site (Harland & Brown, 1985). The relevant restriction endonuclease maps for HSV-2 strain HG52 from Cortini & Wilkie (1978) are shown in Fig. 1. HG52X163 was the parental genome from which new mutants have been isolated. After treating viral DNA with 2 units of *Xba*I per 1 μg of DNA for 2 h at 37 °C, the digested DNA was transfected and any resulting plaques were picked, grown into plate stocks and their DNA was prepared and digested by the method of Lonsdale (1979). Virus from one of the resulting plaques (HG52X163X3) lacked the *Xba*I site at m.c. 0·91. On *Xba*I digestion of HG52X163X3 DNA it was found that the molar *j* band and the 0·5 M *h* band were both absent, but the 0·5 M *i* band was present. As the parental virus was HG52X163, the *g*, *d*, *e* and *f* bands were already missing due to the loss of the 0·7 *d*/*g* *Xba*I site. In HG52X163X3 a novel 0·5 M band of mol. wt. $12·5 \times 10^6$ could be seen running above the position of the *g* band (Fig. 2*a*). This was considered to have been generated by fusion of the *h* and *j* bands on removal of the 0·91 *Xba*I site. Confirmation came from a *Hin*dIII/*Xba*I double digestion (Fig. 2*b*); with HG52X163 (lane 1) the *Hin*dIII *e* band gave two new *e'* bands, the *k* band gave two new *k'* bands and the *l* band gave two new *l'* bands which were of similar molecular weight. The joints containing the new *k'* fragment i.e. *c'* and *d'* ran with *f*, *g* and *h*. The *Xba*I site at 0·91 m.c. lies within the *Hin*dIII *l* fragment and it can be seen that in HG52X163X3 (lane 2) the two *l'* fragments had gone and the *l*

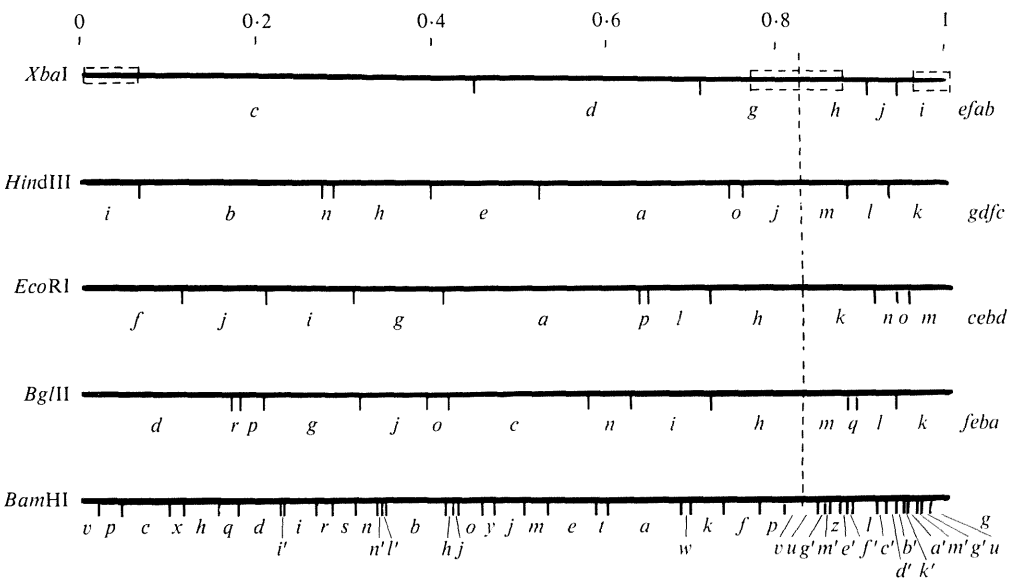

Fig. 1. Restriction endonuclease maps for the DNA of HSV-2 strain HG52 from Cortini & Wilkie (1978). The origin of the joint fragments is as follows. *Xba*I: $a = c + h$, $b = c + i$, $e = g + h$, $f = g + i$. *Hin*dIII: $c = i + k$, $d = j + k$, $f = i + m$, $g = j + m$. *Eco*RI: $b = f + k$, $c = h + k$, $d = f + m$, $e = h + m$. *Bgl*II: $a = d + k$, $b = d + m$, $e = h + k$, $f = h + m$. *Bam*HI: $g = v + u$.

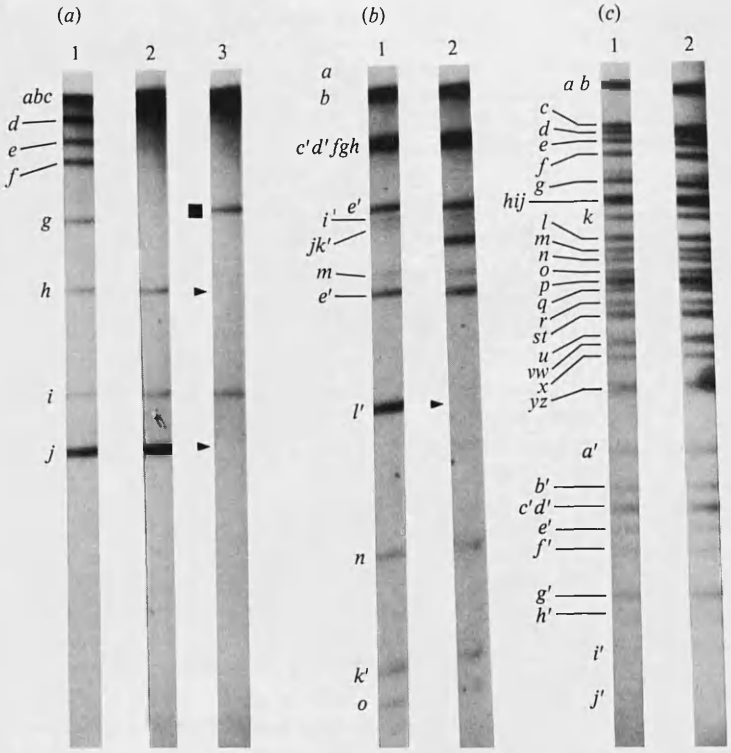

Fig. 2. Autoradiographs of restriction digests of viral DNA ^{32}P-labelled *in vivo*. (*a*) *Xba*I digests of HG52 (lane 1), HG52X163 (lane 2) and HG52X163X3 (lane 3). (*b*) *Hin*dIII/*Xba*I digests of HG52X163 (lane 1) and HG52X163X3 (lane 2). (*c*) *Bam*HI digests of HG52 (lane 1) and HG52X163X3 (lane 2). Letters refer to specific fragments; ■, new fragments; ►, missing fragments.

band had returned to its normal position below *j*. A *Bam*HI digest of the mutant showed that the site loss was probably due to a base change or a small undetectable deletion or insertion, or conceivably a small inversion. The *Xba*I site lies within the *Bam*HI *l* fragment which had unchanged mobility (Fig. 2*c*).

Isolation of a mutant with a 7·5 kb deletion in U_S/TR_S of HSV-2

*Xba*I analysis of one of the plaques (HG52X163X12) isolated after *Xba*I digestion of HG52X163 showed that the 0·5 M *i* band and the 1 M *j* band were missing and a new 0·5 M band was observed above the position of the *j* band (Fig. 3*a*). The *Xba*I site at 0·94 m.c. divides the genome into the *i* and *j* fragments and mere loss of this site would result in a band migrating above the position of the *h* band. The new 0·5 M band (mol. wt. approx. 5×10^6) actually observed suggested a deletion of 5×10^6 containing the 0·94 *Xba*I site and spanning the short unique and short terminal repeat regions of the genome. Confirmation came from an *Eco*RI digest in which the *o* and *m* fragments were missing, the *n* fragment was present whereas the joints containing *m* showed increased mobility: *d* ran just above *f*, and *e* comigrated with *f* (Fig. 3*b*, lane 2). This showed that the *Eco*RI *n*/*o* site was present, but the *m*/*o* site had been lost; the changes in position of the *d* and *e* joints indicated that this loss resulted from a 4×10^6 to 5×10^6 mol. wt. deletion. A *Hin*dIII digest showed that in HG52X163X12 as expected the *c* and *d* joints containing the terminal *k* fragment now migrated above *i* (Fig. 3*c*). Similarly the *Bgl*II digest also showed loss of the terminal *k* fragment and displacement of the *k*-containing joints, i.e. *a* to above *c*/*d* and *e* to below *g* (Fig. 3*d*). Final confirmation of the large deletion came from a *Bam*HI digest (Fig. 4) where the *a'*, *b'* and *k'* fragments were missing; *g'* appeared to be reduced

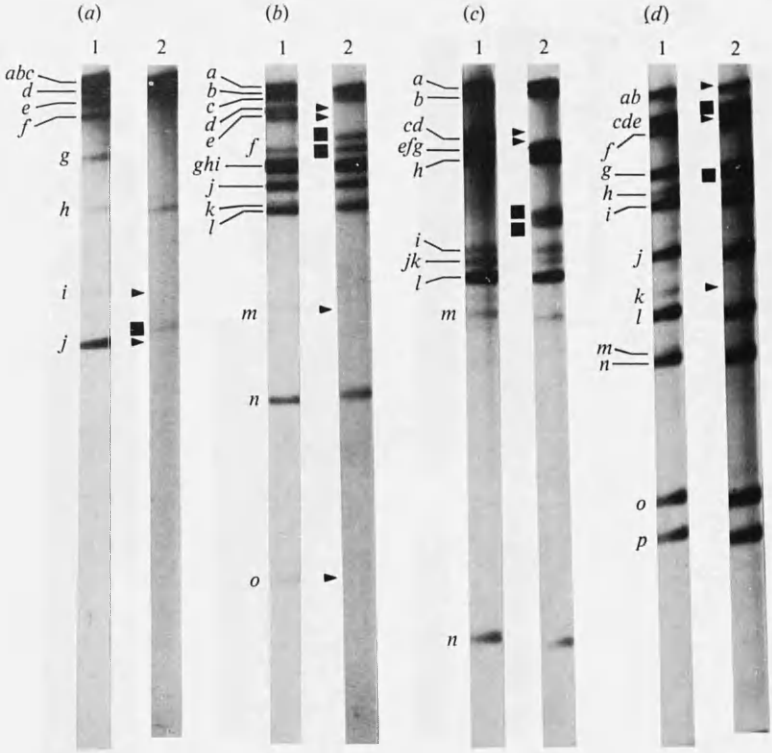

Fig. 3. Autoradiographs of restriction digests of viral DNA ^{32}P-labelled *in vivo*. (*a*) *Xba*I, (*b*) *Eco*RI, (*c*) *Hin*dIII and (*d*) *Bgl*II digests of HG52 (lanes 1) and HG52X163X12 (lane 2). Letters refer to specific fragments; ■, new fragments; ►, missing fragments.

in molarity suggesting that one *g′* fragment had been removed; the joint *g* fragment (*u* + *v*) also seemed to be reduced suggesting that the deletion involved at least part of the terminal *u* fragment. The deletion therefore starts in *Bam*HI *b′* and extends into the terminal *u* fragment (approximately 0·94 to 0·99 m.c.). There was no indication of the deleted DNA being inserted into any other region of the genome. The genome structure of HG52X163X12 was deduced from a stock (P1) from a single plaque isolate obtained after DNA transfection. After three rounds of stringent plaque purification the mutant was grown into a large-scale virus stock and the DNA profile of the stock was again determined by restriction endonuclease digestion. The structure stably retained all features initially observed in the single plaque stock (P1). The mutant was therefore viable and did not require helper virus for lytic growth in tissue culture.

DNA–DNA hybridization

To ensure that the sequences deduced to have been deleted from the mutant HG52X163X12 were not migrating to novel undetected positions, Southern blot hybridization experiments were carried out. DNA from HG52 as well as from HG52X163X12 was digested with *Eco*RI and *Bam*HI and hybridized with nick-translated total HSV-2 DNA, pGZ1 (*Bam*HI *g* clone of HSV-2 containing *Bam*HI *u* and *v*), pGZ66 (*Bam*HI *a′* clone) and pGZ67 (*Bam*HI *b′* clone). It can be seen in the *Eco*RI digest of HG52 that the *Bam*HI *g* fragment hybridized to *Eco*RI *m*, *k*, *f* and *h* (end fragments) and the four joint fragments *b*, *c*, *d* and *e* (Fig. 5*d*, lane 1). In the HG52X163X12 digest the *m* band was missing and the joints containing *m*, i.e. *d* and *e*, were not in their normal position; *d* ran above *f* and *e* comigrated with *f* (Fig. 5*d*, lane 2). The terminal fragment containing part of *m* and part of *o* was small and migrated through the gel. In the

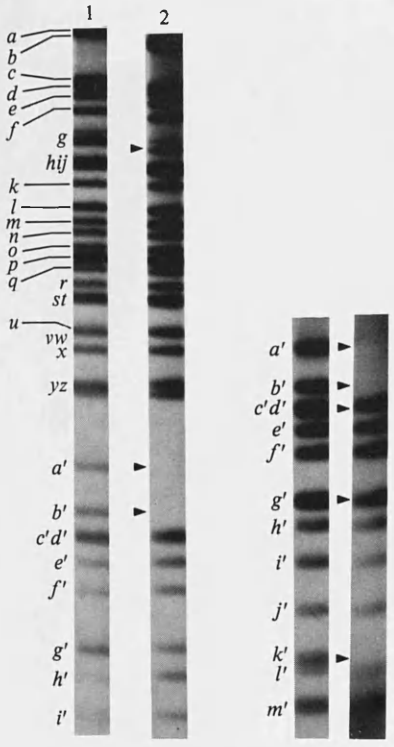

Fig. 4. Autoradiograph of a *Bam*HI digest of viral DNA ^{32}P-labelled *in vivo* from HG52 (lane 1) and HG52X163X12 (lane 2). Letters refer to specific *Bam*HI fragments; ►, missing fragments.

*Bam*HI digest of HG52 (Fig. 6*d*, lane 1), *Bam*HI *g* hybridized to *u*, *v* and the joint *g* fragment. There was some cross-hybridization with the *Bam*HI *p* fragment which is adjacent to *Bam*HI *v* (Fig. 1). Similar cross-hybridization has been reported previously (Brown *et al.*, 1984). In HG52X163X12 there was hybridization to *u*, *v* and *g*, cross-hybridization to *p* and hybridization to two bands that ran above *p* (Fig. 6*d*, lane 2). The *g* band which ran in the normal position is made up of *u* from IR_S plus *v* from IR_L. The bands running above *p* were new joints made up of either *v*, plus the new terminal fragment. The new joint fragments had approximate mol. wt. of $2{\cdot}6 \times 10^6$ and $2{\cdot}7 \times 10^6$; *v* has a mol. wt. of approximately 2×10^6. Therefore the new terminal fragment was about $0{\cdot}6 \times 10^6$. The lack of hybridization to a small fragment suggests that sequences from the terminal *u* made little contribution to the new terminal fragment.

When the *Bam*HI *b'* fragment was used as a probe with the EcoRI digest of HG52, hybridization was to the *o* fragment only (Fig. 5*c*, lane 1). In HG52X163X12 there was no hybridization to *o* but to a band below *o* and to two bands near the top of the gel (Fig. 5*c*, lane 2). The band below *o* is interpreted as consisting of the remainder of *o* plus the remainder of *m*, i.e. the new terminal fragment with approximate mol. wt. $0{\cdot}6 \times 10^6$; the bands at the top of the gel are therefore the new joints *d'* and *e'* containing this new fragment. The sizes would suggest that at most there is $0{\cdot}1 \times 10^6$ mol. wt. of the *o* fragment retained.

When BamHI *b'* was hybridized to a *Bam*HI digest of HG52 only the *b'* fragment was detected (Fig. 6*c*, lane 1). With HG52X163X12 there was no hybridization at the *b'* position but to a band of approximate mol. wt. $0{\cdot}7 \times 10^6$ below *b'* (Fig. 6*c*, lane 2). This is interpreted as part of *b'* and part of *u* (with *k'*, *a'*, *m'* and *g'* deleted) making a new terminal fragment. At the top of the gel hybridization is to the new *g* joint fragments.

When the *Bam*HI *a'* fragment was hybridized to an *Eco*RI digest of HG52 there was positive hybridization of *Eco*RI *m* and *k* and the joints containing *m* and *k*, i.e. *b*, *c*, *d* and *e* (Fig. 5*b*, lane

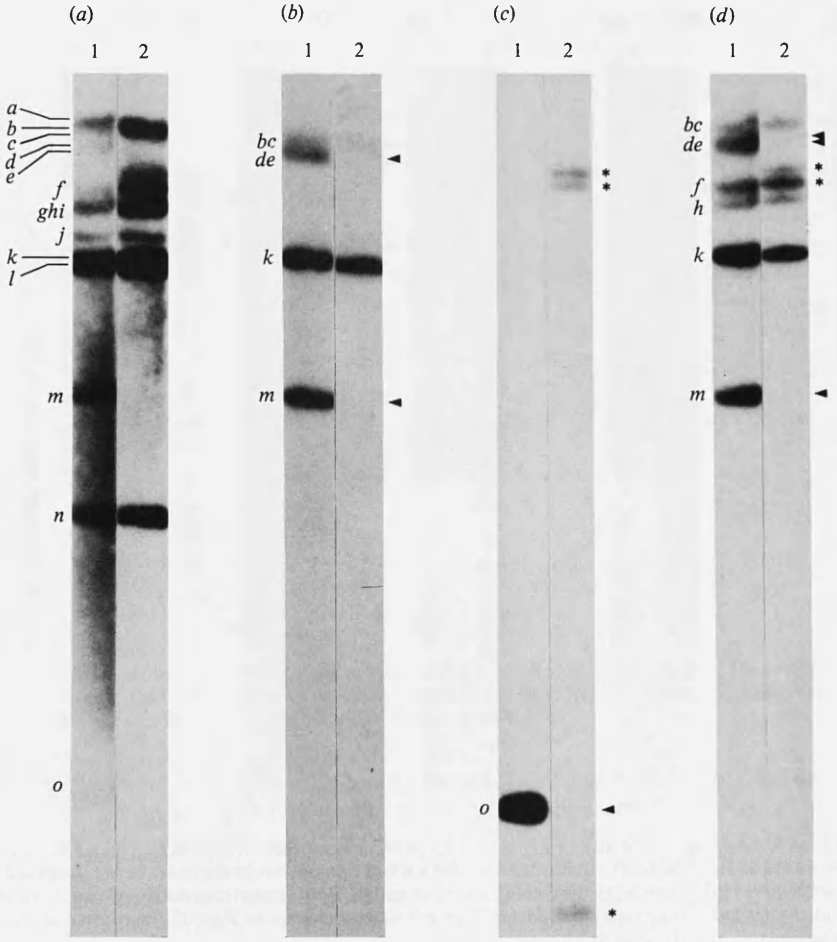

Fig. 5. Autoradiograph of nitrocellulose blot strips containing *Eco*RI restriction fragments of HG52 (lanes 1) and HG52X163X12 (lanes 2) to which nick-translated DNA probes have been hybridized. The probes were HSV-2 DNA (*a*), the recombinant plasmid pGZ66 (*Bam*HI *a'*) (*b*), the recombinant plasmid pGZ67 (*Bam*HI *b'*) (*c*), and the recombinant plasmid pGZ1 (*Bam*HI *g*) (*d*). The letters denote specific *Eco*RI fragments; ►, missing fragments; *, new fragments.

1). With HG52X163X12 there was no hybridization to *m*, *d* and *e* but positive hybridization to *k*, *b* and *c* (Fig. 5*b*, lane 2). When *a'* was the probe with a *Bam*HI digest of HG52 (Fig. 6*b*, lane 1) there was hybridization to *a'* and *z*. In HG52X163X12 there was no hybridization to *a'* and the two bands in the *z* position were due to heterogeneous size classes of the *z* fragment. This is known to be a variable region. When individual plaque stocks of HG52 were digested with *Bam*HI there was obvious variation in the size of the *z* fragment (data not shown). The hybridization results show that the *Bam*HI *a'* fragment sequences in the short terminal repeat had been completely deleted.

Taken together, the Southern blotting results confirm the deletion in HG52X163X12 as deduced from the restriction endonuclease analysis and show that the deletion extends from 0·94 to 0·994 m.c.

Two mutants with deletions and insertions in the short region of the HSV-2 genome

HG52X163X14

It can be seen in Fig. 7(*a*) that the isolate HG52X163X14 exhibited an *Xba*I profile in which the *i* fragment was missing while the *j* band was normal. The *Eco*RI digest showed the *m* band,

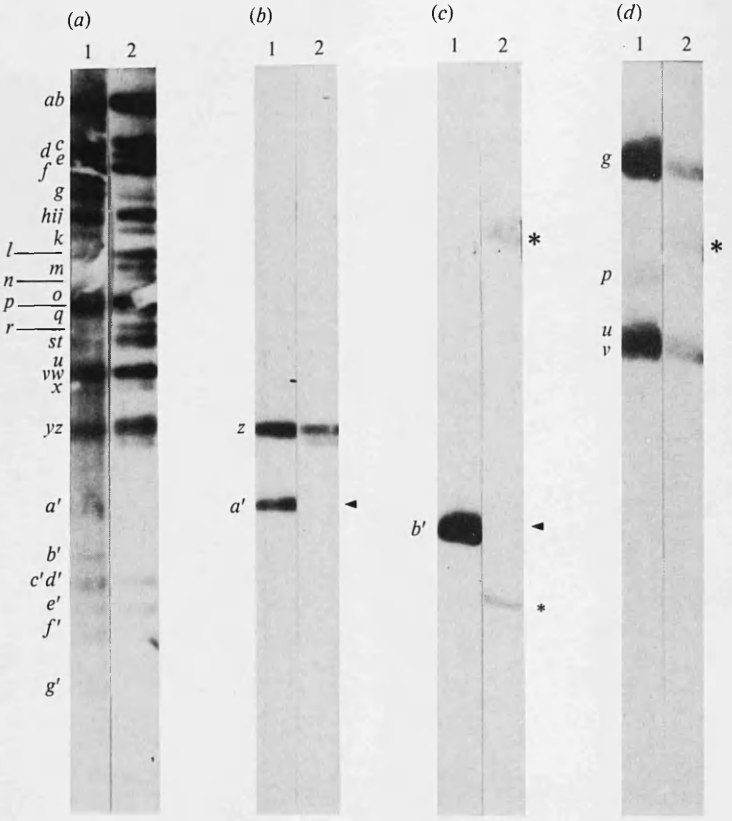

Fig. 6. Autoradiograph of nitrocellulose blot strips containing *Bam*HI restriction fragments of HG52 (lanes 1) and HG52X163X12 (lanes 2) to which nick-translated DNA probes have been hybridized. The probes were HSV-2 DNA (*a*), the recombinant plasmid pGZ66 (*b*), the recombinant plasmid pGZ67 (*c*) and the recombinant plasmid pGZ1 (*d*). The letters denote specific *Bam*HI fragments; ►, missing fragments; *, new fragments.

the *o* band and the two joints containing *m*, i.e. *d* and *e*, to be missing with their new positions on the gel not apparent (Fig. 7*b*). The data suggested that the molecule had a deletion starting within the short unique sequences and extending over most of the terminal repeat sequences. A new 1 M fragment of approximately $0{\cdot}5 \times 10^6$ was detected at the bottom of the *Eco*RI gel. When *Bam*HI *b'* was hybridized to an *Eco*RI digest there was no hybridization to the *o* fragment but positive hybridization to the $0{\cdot}6 \times 10^6$ mol. wt. fragment. (Due to the amount of Southern blotting necessary to unravel the structure of the mutants HG52X163X14 and HG52X163X21 we are not showing the data.) As the *k* fragment and joints containing *k*, i.e. *b* and *c*, were present, the molecule must be capable of inversion. As expected, on *Hin*dIII digestion the joints containing the short terminal *k* fragment, i.e. *c* and *d*, were missing but unexpectedly a new 1 M band of approximately 4×10^6 could be seen between *m* and *n* (Fig. 7*c*). When *Bam*HI *b'* was hybridized to a *Hin*dIII digest of HG52X163X14 there was no hybridization to *k* and the joints containing *k* (*b* and *c*) but positive hybridization to the band running between *m* and *n*. Similarly on *Bgl*II digestion the terminal *k* fragment and the *a* and *e* joints were missing while a new molar band of $2{\cdot}7 \times 10^6$ mol. wt. appeared below *o* (Fig. 7*d*). When *Bam*HI *b'* was hybridized to a *Bgl*II digest of HG52X163X14 there was no hybridization to *k*, *a* or *e* but positive hybridization to the band running below *o*. Also when *Hin*dIII *l* was hybridized to a *Bgl*II digest there was normal hybridization to *l* and *q* and to the band running below *o*. Hybridization with *Bam*HI *a'* indicated missing *k*, *a* and *e* fragments but the presence of *m* and the two *m*-containing joints.

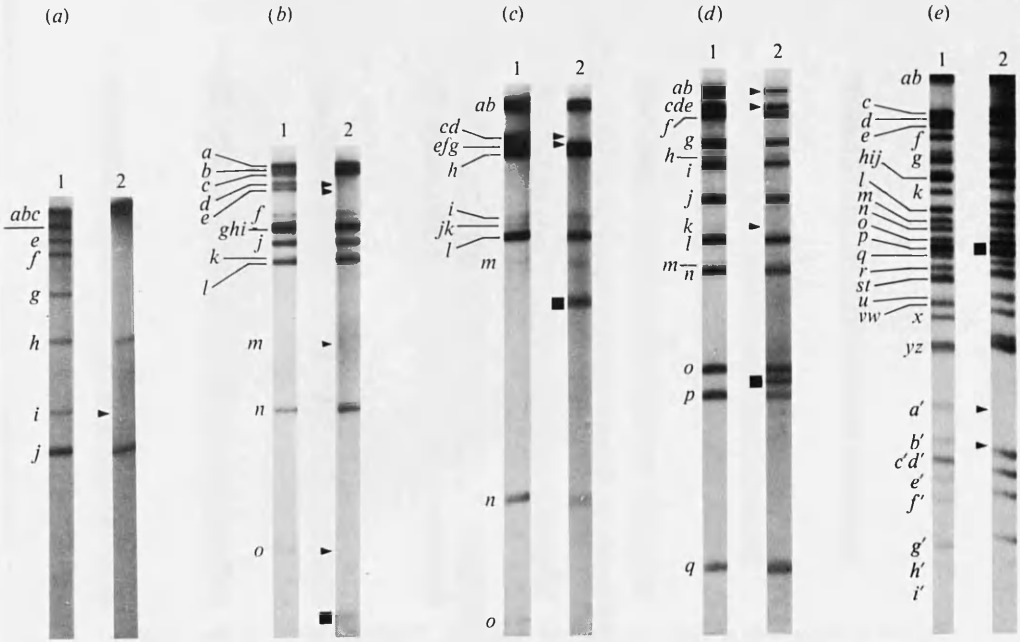

Fig. 7. Autoradiographs of restriction digests of viral DNA ^{32}P-labelled *in vivo*. (*a*) *Xba*I, (*b*) *Eco*RI, (*c*) *Hin*dIII, (*d*) *Bgl*II and (*e*) *Bam*HI digests of HG52 (lanes 1) and HG52X163X14 (lanes 2). Letters refer to specific fragments; ■, new fragments ►, missing fragments.

Therefore the *Bam*HI *a′* information was totally deleted. The *Bam*HI profile (Fig. 7*e*) had the *a′*, *b′* and *k′* (not seen on this gel) fragments absent and the molarities of *e′* and *f′* appeared increased. There was also a new band running with *q*. When *Bam*HI *a′* was hybridized to a *Bam*HI digest of HG52X163X14, *a′* was missing and *z* was normal. When the probe was *Bam*HI *b′* there was no hybridization in the *b′* position but positive hybridization to a larger fragment running with *q*. (The position of this fragment can be seen in Fig. 7*e*.) When *Bam*HI *g* was the probe, the smaller terminal copy of *u* was missing, *v* was present and *g* present. Using *Hin*dIII *l* as the probe there was normal hybridization to *Bam*HI *l*, *c′*, *d′*, *e′* and *f′* and novel hybridization to the band running with *q*.

We interpret the data as follows. The mutant HG52X163X14 has lost *Xba*I *i*, most of *Hin*dIII *k*, *Eco*RI *m* and part of *o*, most of *Bgl*II *k* and all of *Bam*HI *a′* and *k′*, the terminal copies of *m′*, *g′* and *u* and part of *b′*. However, part of the deleted sequences was replaced by DNA giving rise to a new 1 M *Hin*dIII fragment of approximately 4×10^6 mol. wt., a new 1 M *Bgl*II band of $2{\cdot}7 \times 10^6$ mol. wt and a new 1 M *Eco*RI band of approximately $0{\cdot}5 \times 10^6$ mol. wt. There is no evidence for removal of sites from, or addition of sites to, another part of the genome giving rise to the new *Hin*dIII, *Bgl*II and *Eco*RI sites.

The restriction endonuclease analysis in conjunction with the Southern blotting data for this mutant suggests the following. HG52X163X14 has entirely lost the sequences contained in *Xba*I *i*; it has retained the *Xba*I *j* fragment and hence the *h*/*j* *Xba*I site but has replaced the *Xba*I *i* sequences by the internal *Xba*I *h* sequences such that the short region of this variant now has two copies of the information contained in *Xba*I *h*. As most of the sequences in *h* are repeated terminally and internally it means a loss of the unique sequences contained in *Xba*I *i* and their replacement by a second copy of the sequences between the end of the internal repeat and the *Xba*I *h*/*j* site but in the opposite orientation. This model would be compatible with the other restriction endonuclease profiles. The new 1 M *Hin*dIII band is bounded by the *l*/*k* site and the *m*/*l* site in inverted orientation repeated to the right of the *l*/*k* site; the new small 1 M *Eco*RI fragment is bounded by the *n*/*o* and the *k*/*n* site in inverted orientation repeated to the right of the

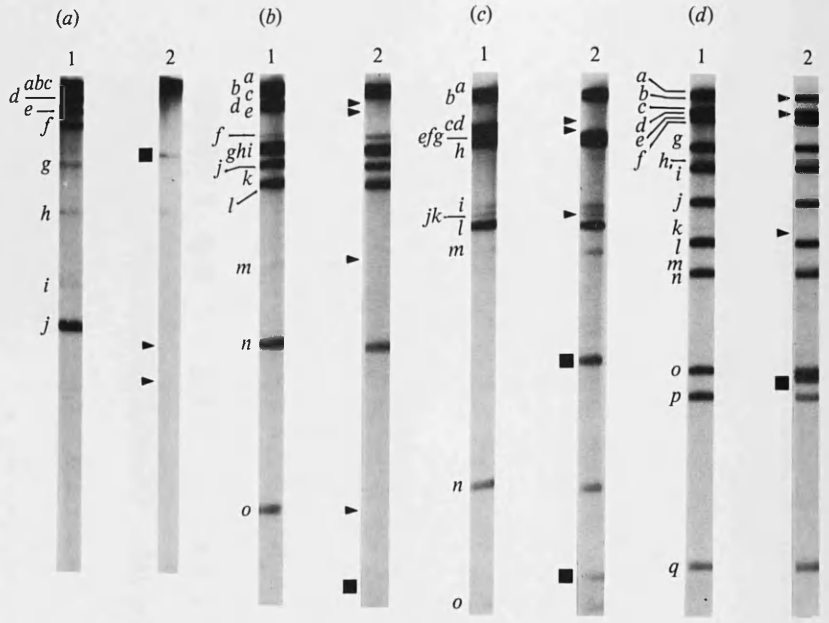

Fig. 8. Autoradiographs of restriction digests of viral DNA ^{32}P-labelled *in vivo*. (*a*) *Xba*I, (*b*) *Eco*RI, (*c*) *Hin*dIII and (*d*) *Bgl*II digests of HG52 (lanes 1) and HG52X163X21 (lanes 2). ■, New fragments; ►, missing fragments.

n/*o* site; the new 1 M *Bgl*II fragment is bounded by the *l*/*k* site and the *q*/*l* site in inverted orientation repeated to the right of the *l*/*k* site; the new 1 M *Bam*HI fragment is formed by the *d*′/*b*′ site and the *f*′/*l* site in inverted orientation repeated to the right of the *d*′/*b*′ site. The proposed model is shown later in Fig. 10(*a*). HG52X163X14 therefore has an extended inverted repeat in the short region of the genome. The repeats extend from 0·83 to 0·91 m.c. and from 0·94 to 1·2 m.c. The repeat is extended by 6 kb on either side of the unique sequences and the whole genome has increased by 3 kb.

HG52X163X21

The *Xba*I profile of the mutant HG52X163X21 showed a loss of the 1 M *j* and 0·5 M *i* bands, and a new 0·5 M band running above *g* (Fig. 8*a*). The position of the band running above *g* was the same as that in the mutant HG52X163X3 in which the *h*/*j* *Xba*I site at 0·91 m.c. had been removed (Fig. 2*a*). An *Eco*RI digest (Fig. 8*b*, lane 2) demonstrated that the *o* and *m* bands had gone, while the *n* band remained; thus the *n*/*o* site was present and the *m*/*o* site had been deleted. The *k* band was present and the joints containing *k* (*b* and *c*) were also present indicating that the molecule was not fixed in a particular orientation. The joints containing *m* (*d* and *e*) were not in their normal position. A small 1 M band of approximate mol. wt. $0{\cdot}4 \times 10^6$ migrated to below *o*. Southern blotting of an *Eco*RI digest gave a similar picture to that of HG52X163X14, i.e. *Bam*HI *b*′ hybridized to the $0{\cdot}4 \times 10^6$ mol. wt. fragment indicating that it was composed in part of the sequences contained in *Bam*HI *b*′. The *Hin*dIII molar *l* band was present and therefore the *l*/k site had not been removed. The *Hin*dIII *k* fragment contains the *Xba*I *i* fragment but as the *k* band comigrated with *j* it was not possible to determine any alteration in the *k* fragment (Fig. 8*c*, lane 2). The joints containing *k* (i.e. *c* and *d*) were not observable above *e*, *f* and *g* and their new position could not be determined. A new 1 M band of $3{\cdot}0 \times 10^6$ mol. wt. was seen migrating between *m* and *n* and a new 1 M band of mol. wt. approximately $1{\cdot}0 \times 10^6$ mol. wt. just above *o*. The hybridization data showed positive hybridization of *Bam*HI *b*′ to the band of $1{\cdot}0 \times 10^6$ mol. wt. running above *o* and when *Hin*dIII *k* was the probe there was also positive hybridization to the small $1{\cdot}0 \times 10^6$ mol. wt. fragment. A *Bgl*II digest (Fig. 8*d*, lane 2)

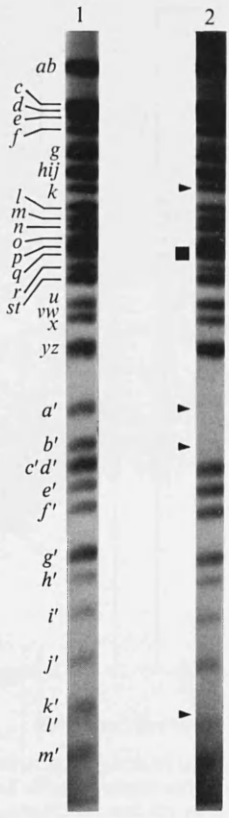

Fig. 9. Autoradiographs of *Bam*HI digests of viral DNA ^{32}P-labelled *in vivo* from HG52 (lane 1) and HG52X163X21 (lane 2). Letters refer to specific *Bam*HI fragments; ►, missing fragments.

demonstrated that the short terminal *k* fragment had gone and the *a* joint containing *k* was missing. The *e* joint was also missing from the *c*, *d*, *e* group. A new molar band of approximately $2{\cdot}8 \times 10^6$ was seen running below *o*. *Bam*HI *b'* hybridized to the band below *o* and there was no hybridization to *k*, *a* and *e*. Also *Hin*dIII *l* hybridized to the band below *o* and *Bam*HI *a'* failed to hybridize to *k*, *a* and *e*. A *Bam*HI digest showed the *a'*, *b'* and *k'* fragments to be missing: the molarities of *e'* and *f'* were increased and there appeared to be a band running with *p* (Fig. 9). Hybridization showed an absence of *Bam*HI *a'* information. *Bam*HI *b'* hybridized to the band running with *p* and when *Bam*HI *g* was the probe the terminal copy of *u* was missing but *v* and *g* were present.

The data indicate that HG52X163X21 had been deleted within U_S and TR_S with loss of the *a'*, *k'*, *m'* and *g'* *Bam*HI fragments as well as part of *b'* and all of the terminal *u* fragment. As was the case with HG52X163X14 here too some of the deleted sequences have been replaced by an insertion giving rise to a new 1 M *Bgl*II, two new 1 M *Hin*dIII and two new 1 M *Eco*RI fragments.

Our interpretation of the restriction endonuclease and Southern blotting data is as follows. The mutant HG52X163X21 has entirely lost the sequences within *Xba*I *i*, has retained an *Xba*I site at 0·94 m.c., has lost the 0·91 *Xba*I site such that *Xba*I *h* and *j* have fused to give the band running above *g* (Fig. 8*a*) and has replaced the information in *Xba*I *i* with *Xba*I *h* sequences. As for HG52X163X14, this means deletion of unique sequences between 0·94 m.c. and the start of TR_S and replacement of these sequences by a repeat copy in the opposite orientation of the sequences contained between 0·91 m.c. and the start of IR_S. The model is shown in Fig. 10(*b*) and the derivation of the novel *Hin*dIII, *Eco*RI, *Bgl*II and *Bam*HI fragments is indicated. Compared to HG52X163X14, the novel *Bgl*II and *Bam*HI fragments are $0{\cdot}1 \times 10^6$ mol. wt. larger and the

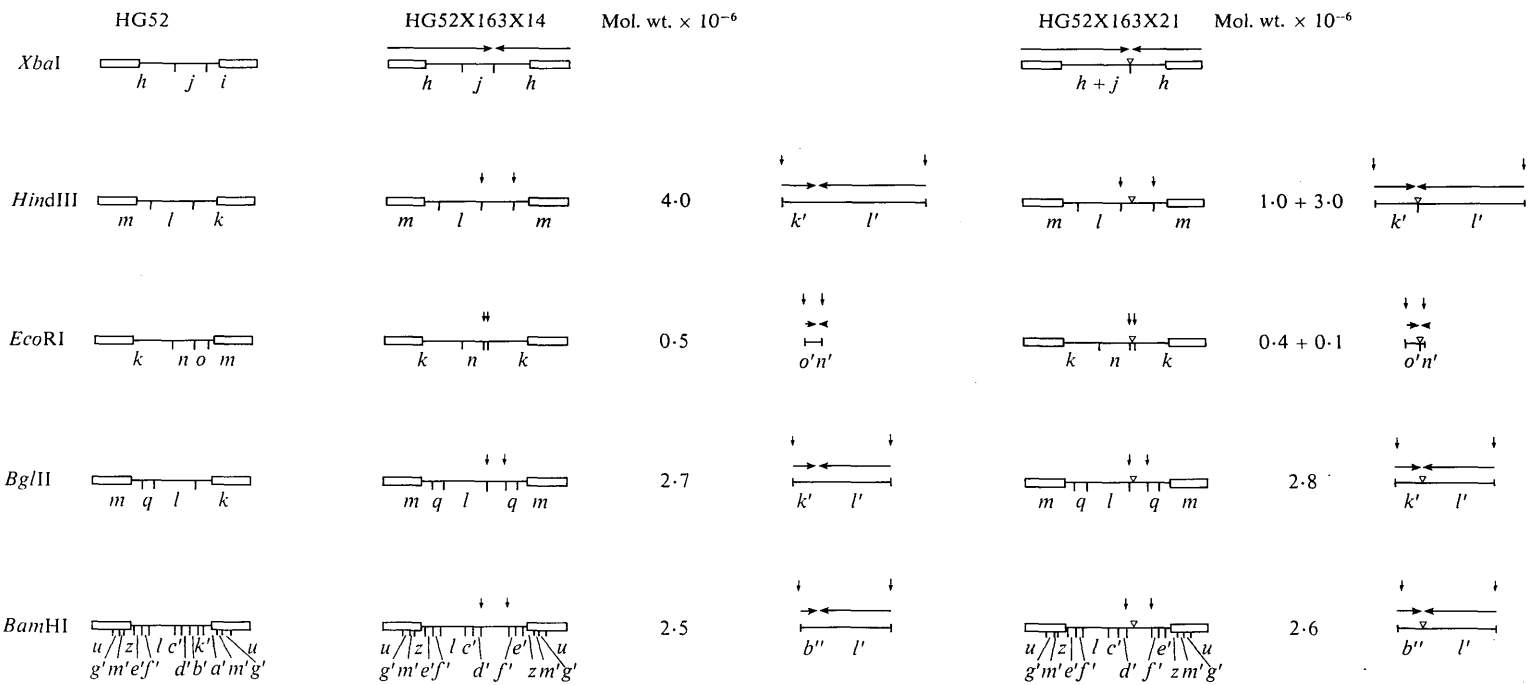

Fig. 10. Proposed models for the structure of HG52X163X14 and HG52X163X21 compared to HG52, showing the positions of the various restriction endonuclease sites. The fragments between the vertical arrows are the new observed fragments whose size and composition are shown on the right of each part. The open triangles denote the position of inserts containing additional *Hin*dIII and *Eco*RI sites.

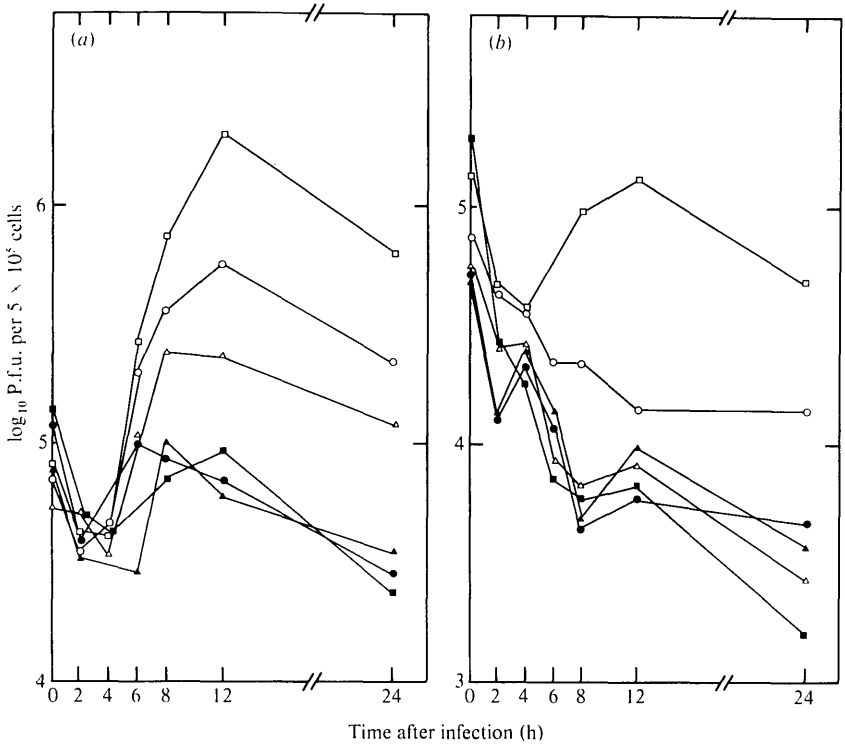

Fig. 11. One-step growth curves in BHK21/C13 cells at (*a*) 37 °C and (*b*) 38·5 °C. After adsorption for 45 min, the monolayers were washed twice, overlaid and incubated at the appropriate temperature. Cultures were harvested at the times indicated and the virus titre was measured by plaque assay on BHK21/C13 cells. □, HG52; ○, HG52X163; △, HG52X163X3; ●, HG52X163X12; ▲, HG52X163X14; ■, HG52X163X21.

Table 1. *growth of parental and deletion mutants**

Virus	Titre at 31 °C (p.f.u./ml)	Titre at 38·5 °C (p.f.u./ml)
HG52X163	$7{\cdot}7 \times 10^8$	$>5 \times 10^8$
HG52X163X3	1×10^6	$3{\cdot}3 \times 10^5$
HG52X163X12	$1{\cdot}9 \times 10^8$	10^3 (c.p.e.)
HG52X163X14	1×10^8	4×10^6 (very small plaques)
HG52X163X21	$1{\cdot}3 \times 10^8$	10^2 (c.p.e.)

* See text for experimental details.

*Eco*RI fragment is $0{\cdot}1 \times 10^6$ mol. wt. smaller. However on *Hin*dIII digestion of HG52X163X21 two novel 1 M bands of 3×10^6 and 1×10^6 mol. wt. were present compared to a single 1 M band of 4×10^6 mol. wt. on *Hin*dIII digestion of HG52X163X14. This can be explained by postulating an insert of approximately 150 bp around the 0·94 *Xba*I site. The insert must contain or generate a new *Hin*dIII site and the smaller size of the *Eco*RI fragment compared to that of HG52X163X14 suggests an additional *Eco*RI site generating the $0{\cdot}4 \times 10^6$ mol. wt. fragment plus a $0{\cdot}1 \times 10^6$ fragment which would run off the gel.

The structures of HG52X163X14 and HG52X163X21 were originally determined from DNA analysis of stocks derived one passage on from single plaques obtained after DNA transfection. Following three rounds of stringent plaque purification and growth of large-scale virus stocks, the

DNA profiles of the stocks were exactly the same as those from the original single plaques. The two mutants with major rearrangements of the short unique region of the genome are therefore viable, stable and capable of growth in tissue culture without helper virus.

Biological properties of the deletion mutants

High titre stocks of the three plaque-purified deletion mutants were obtained by low multiplicity infection of BHK21/C13 cells at 31 °C over 3 days. The viruses grew at a comparable rate to that of the parental HG52X163 and the wild-type HG52. However on titration in BHK21/C13 cells at 31 °C and 38·5 °C all three mutants were found to be impaired to different extents at the higher temperature (Table 1). Neither HG52X163X12 nor -X21 gave distinct plaques at 38·5 °C but showed marked cytopathic effect at the two lowest dilutions. The mutant HG52X163X14 gave minute but countable plaques at 38·5 °C but with a titre reduced by nearly 100-fold compared to that at 31 °C. It appears therefore that HG52X163X12 and -X21 are temperature-restricted and to some extent HG52X163X14 is also temperature-restricted. The parental HG52X163 grew equally well at 31 °C and 38·5 °C. The mutants and parental virus gave comparable titres at 37 °C. Marker rescue experiments with fragments from the deleted regions are underway to determine whether the wild-type phenotype can be recovered.

The results of one-step growth experiments are shown in Fig. 11(*a*) and (*b*). Cells were infected at 5 p.f.u./cell and grown at 37 °C; the yields at intervals over 24 h, titrated at 37 °C, are shown in Fig. 11(*a*). It can be seen that the wild-type HG52 gave the expected growth pattern as did HG52X163 although with a somewhat lower yield. However HG52X163X3 grew appreciably less well and HG52X163X12, HG52X163X14 and HG52X163X21 showed markedly impaired growth, reaching maximum titres at very early times and thereafter declining. The results of growth experiments carried out at 38·5 °C, with the yields titrated at 37 °C, are shown in Fig. 11(*b*). It can be seen that HG52 itself was somewhat impaired in growth at the elevated temperature. This property of HG52 has been noted before (D. Dargan, personal communication). The parental HG52X163 was more impaired although this was not apparent in the burst from the 24 h infection illustrated in Table 1. However the three mutants with deletions in U_S/TR_S and the *Xba*I site deletion mutant exemplify what could only be described as death curves.

DISCUSSION

To isolate restriction endonuclease site deletion mutants of HSV-1 and HSV-2 it has been necessary to determine the DNA structure of a large number of single plaque stocks. This revealed that the elite stocks of virus already contained a number of variants with extensive deletions in one or other of the long repeat regions of the genome (Brown *et al.*, 1984; Harland & Brown, 1985). The frequency of occurrence of such deletions varied between different strains of HSV, but our initial speculation that the deletions may have resulted from restriction endonuclease digestion prior to transfection was not borne out. Genomic deletions occurred in stocks of virus which had been neither treated with restriction endonucleases nor transfected. The high frequency of occurrence of long repeat deletions in strain HG52 firmly ruled out the suggestion that such deletions were a consequence of heterotypic repetitive regions (Davison *et al.*, 1981). We feel it is more probable that deletions originate from distinct regions of instability in the DNA, for example banks of tandemly reiterated short sequences like those found in the long repeat near the U_L/IR_L junction, in the middle of IR_L, near the IR_L/IR_S joint and in U_S of HSV-1 (D. J. McGeoch, personal communication).

The main aim in initiating this study was the isolation of mutants lacking restriction endonuclease sites in order to use the sites as non-selected markers in the study of recombination. To this end we have now removed the HSV-2 *Xba*I site at 0·91 m.c. through either a single base change or a small undetectable deletion or insertion. The variant HG52X163X3 thus lacks both the 0·7 m.c. and 0·91 m.c. *Xba*I sites. The DNA sequence around 0·91 m.c is not yet known for HSV-2, but in HSV-1 this site would be within the gE (US8) gene (McGeoch *et al.*, 1985).

In endeavouring to isolate restriction site mutants thorough DNA analysis of single plaque

stocks has revealed the presence of mutants with extensive DNA alterations not necessarily related to the removal of *Xba*I sites. We have demonstrated the existence of a viable stable mutant, HG52X163X12, in which a deletion has removed about 5×10^6 mol. wt. (7·5 kb) of sequence extending from approximately 0·94 to 0·994 m.c. The mutant retains the '*a*' sequence(s) to allow inversion. This area of the genome in HSV-2 has not been completely sequenced but the equivalent region in HSV-1 contains the genes US10, 11 and 12 within U_S (McGeoch *et al.*, 1985) and one copy of ORI_S and of IE gene 3 in TR_S (Stow & McMonagle, 1983; Murchie & McGeoch, 1982). Transcripts equivalent to US10, 11 and 12 have been identified in HSV-2 strain HG52 (Whitton, 1984). The HSV-2 polypeptide V_{mw}IE182 has been identified as being equivalent to V_{mw}IE175, the product of IE gene 3, in HSV-1 (Morse *et al.*, 1978) and a polypeptide of mol. wt. 12·5K in HSV-2 has been shown to be equivalent to V_{mw}IE12 in HSV-1 (Marsden *et al.*, 1982).

In HSV-1 the US10 gene encodes a polypeptide of mol. wt. 33K previously identified by Lee *et al.* (1982) by *in vitro* translation. The US11 gene encodes a polypeptide of theoretical mol. wt. 18K thought to be equivalent to the 21K and 22K proteins which have been shown to have a preferential affinity for '*a*' sequences (Dalziel & Marsden, 1984). The US12 gene encodes the immediate early protein V_{mw}IE12 (Watson *et al.*, 1979; Marsden *et al.*, 1982).

The functions of the 33K mol. wt. protein and the V_{mw}IE12 polypeptide are unknown. Because of its affinity for '*a*' sequences it has been suggested that the 21K mol. wt. polypeptide may have a role in either genome inversion or packaging or both (Dalziel & Marsden, 1984). The V_{mw}IE175 polypeptide is known to be essential (Preston, 1979; Dixon & Schaffer, 1980; Watson & Clements, 1980). The function of ORI_S as opposed to ORI_L is unknown and an absolute requirement for both copies of ORI_S has not been demonstrated.

Isolation and propagation of HG52X163X12 which lacks the HSV-2 genes equivalent to US10, 11 and 12, demonstrates that the products of these three genes are not essential for HSV-2 lytic growth in BHK21/C13 cells. It is interesting that the mutant is competent *in vitro* with only one copy of IE gene 3 and of ORI_S. We have previously shown that one copy of IE gene 1 suffices for lytic growth of HSV-2 (Harland & Brown, 1985).

The products of the US10 and US11 genes are difficult to identify by SDS–PAGE. The abundance of the US11 gene products, i.e. 20K and 20·5K mol. wt., is much lower in HSV-2 than in HSV-1 which increases the difficulty of identification (Marsden *et al.*, 1978). An antiserum or monoclonal antibody against the HSV-2 product is not available to us and our HSV-1 anti-peptide antiserum does not cross-react with the HSV-2 product. Preliminary SDS–PAGE analysis indicates that the 20K/20·5K mol. wt. polypeptide is not produced by HG52X163X12. From this and from the unambiguous restriction endonuclease and Southern blotting results we postulate that the HSV-2 equivalents of V_{mw}33K and V_{mw}IE12·5 are not made either. It would appear that the 20K/20·5K mol. wt. HSV-2 polypeptide cannot be an absolute requirement for packaging or inversion of viral DNA molecules.

As expected from the calculation of size, the deletion does not extend into the coding region for gE (US8) in HG52X163X12, which has been shown to make gE in normal amounts in SDS–PAGE analysis of sulphated polypeptides (Hope & Marsden, 1983). Due to the lack of an antiserum or antibody against the HSV-2 equivalent of the 10K (US9) mol. wt. polypeptide we have been unable to determine whether synthesis of this polypeptide is affected.

The deletions in both HG52X163X14 and HG52X163X21 have been defined; like HG52X163X12 these two mutants are also deleted in genes US10, 11 and 12. They both produce gE (unpublished results) but whether the deletion affects US9 cannot be determined at present. The deleted DNA has not been inserted elsewhere in the genomes of HG52X163X14 and HG52X163X21.

The results from restriction endonuclease analysis and Southern blotting experiments with both HG52X163X14 and HG52X163X21 suggest the models proposed in Fig. 10(*a*) and (*b*). The variants have novel short regions of the HSV-2 genome in that both have two copies of the sequences between the IR_L/IR_S joint and 0·91 m.c. The two copies are in the opposite orientation and flank the normal unique sequences between 0·91 and 0·94 m.c. thus extending the length of the inverted repeats in the short region by 6 kb on either side. This means that each

mutant has two copies of the genes US1, 2, 3, 4 and the coding region of US5. The only differences between the two variants are retention of both *Xba*I sites in HG52X163X14, additional *Hin*dIII and *Eco*RI sites in HG52X163X21, and slight differences in the molecular weights of the novel restriction fragments generated by the duplication. The origin of the *Hin*dIII and *Eco*RI sites around 0·94 map units in HG52X163X21 is unknown, but it is possible that they originated from the reiteration of *Hin*dIII *l*/*k* and *Eco*RI *n*/*o* sites.

Whether the three mutants HG52X163X12, -X14 and -X21 have arisen independently or are sequentially related is open to speculation. It could be proposed that HG52X163X12 and -X14 may have been generated one from the other and that -X21 may be the result of a recombination event between -X3 and -X14. Whether these mutants are the result of a progressive process initiated by *Xba*I cleavage or partial cleavage, or whether the variants already existed within the elite stock of HSV-2 (HG52) is not known. It seems more than coincidental that the genome alterations have arisen at the *Xba*I sites. It could be envisaged that cleavage at such a site leaves a sticky end which can ligate by chance to the complementary sticky end of any other *Xba*I fragment. Most such rearrangements will be inviable but in the case of HG52X163X14 and HG52X163X21 such an event generates molecules with extended inverted repeats which do not render the variants inviable.

Whitton & Clements (1984) proposed that an expansion of R_S might come about by non-homologous recombination. They found that in HG52 the first translation initiation codon of IE5 had the A of ATG as the first base in U_S and this defines the R_S/U_S junction. Comparison with HSV-1 led to the speculation that R_S could expand and that the ATG acts as a stop to expansion. Our mutants may have arisen by a similar mechanism involving recombination between short stretches of sequence homology at different positions in U_S but the extent of the expansion up to the *Xba*I sites may point to the novel rearrangements being due at least in part to the *Xba*I treatment of the DNA from which the mutants were derived.

However our work has clearly established that DNA analysis of purified single plaque stocks as opposed to large-scale DNA preparations allows first the isolation of variants at a relatively high frequency and second the identification of genes with dispensable functions.

The finding that the three deletion mutants were temperature-restricted at 38·5 °C was quite unexpected. They also showed impairment of growth in single-cycle growth experiments carried out at 37 °C. However virus stocks grown from low multiplicity infections over 3 days at 31 °C gave yields of virus comparable to HG52 and the parental HG52X163. The disparity when growth was at different temperatures and using different initial infecting multiplicities (0·003 p.f.u./cell at 31 °C: 5 p.f.u./cell at 37 °C) implies that the low yield of infectious virus per cell at 31 °C is overcome by infection of surrounding cells over a 72 h period. The low burst at 37 °C from a higher m.o.i. is further limited by lack of susceptible cells. It may also be that the virus produced by the deletion mutants is unstable, a property not apparent at 31 °C. When single-cycle growth experiments were carried out at 38·5 °C some impairment of growth was exhibited by HG52. The parental HG52X163 showed some growth but the *Xba*I site mutant and the three deletion mutants did not grow at all. Even taking into account the poor growth of HG52X163 and HG52X163X3 at 38·5 °C, we feel that the impairment of growth at 37 °C in conjunction with lack of growth at 38·5 °C demonstrated by the three deletion mutants may be a consequence of the deleted genes. The fact that the three mutants have the same region deleted would lend support to this supposition. The most appropriate explanation would be that one or more of the lost gene products is necessary in conjunction with other gene product(s) for growth or stability of the virus at 38·5 °C. The stability of the viruses at the three temperatures is being investigated. We have demonstrated however that the genes US10, 11, 12, one copy of IE gene 3 and one copy of ORI_S can be deleted without any serious impairment of lytic function. Whether these genes are necessary for viral functions *in vivo* is now under investigation.

As this manuscript was being submitted for publication Longnecker & Roizman (1986) and Umene (1986) published papers reporting similar deletions in HSV-1.

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Isolation and Characterization of Deletion Mutants of Herpes Simplex Virus Type 2 (Strain HG52)

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SUMMARY

We provide evidence that: (i) two variants lacking the *Xba*I site at map coordinate 0·7 have been selected following *Xba*I treatment of the DNA of herpes simplex virus type 2 strain HG52; (ii) one of these mutants had lost the 0·7 restriction site due to a deletion of approximately 150 base pairs and in the other the site loss was due to a similar sized sequence insertion; (iii) following *Xba*I treatment, four variants with deletions ranging in size from 1·5 kb (in both TR_L and IR_L) to 9 kb in IR_L were isolated; (iv) substantial deletions in the long terminal repeat regions of HG52 are present with a frequency of 24% of genomes in the elite stock, a variant with a 3·75 kb deletion in IR_L making up 10% and one with a 1·5 kb deletion in both IR_L and TR_L making up 14%; (v) two of the variants isolated after *Xba*I treatment of viral DNA were identical to the deletion prototype within the elite stock, suggesting that these variants were not generated as a result of *Xba*I treatment but pre-existed in the viral DNA pool; (vi) the deletion variants were stably maintained during routine stock propagation, were viable and could be propagated as cloned populations; (vii) the deletions did not have a marked deleterious effect on the one-step growth kinetics of the virus.

INTRODUCTION

We have recently shown that it is possible to isolate mutants lacking particular *Xba*I restriction endonuclease (RE) sites from herpes simplex virus type 1 (HSV-1; Glasgow strain 17) and an intertypic recombinant (R12-5) (Brown *et al.*, 1984). The method of isolation used was an enrichment selection technique, dependent on genomes with fewer than the standard number of restriction sites having a greater survival probability than the majority population. During the course of the analysis it became apparent that virus genomes lacking specific *Xba*I sites (RE site mutants), as well as molecules with deletions of up to 9 kb in either TR_L or IR_L (terminal long repeat and internal inverted long repeat) had been isolated from the intertypic recombinant R12-5 virus stock. As such deletion mutants were not detected in a small sample of untreated (i.e. not previously subjected to *Xba*I digestion) virus genomes, it was thought that they might have been produced as a consequence of the procedures involved in the isolation of the RE site mutants.

In parallel with the study of HSV-1 and R12-5, we have similarly isolated mutants of HSV-2 (strain HG52) lacking an *Xba*I restriction endonuclease site. The genome structures of two independently isolated mutants lacking the *Xba*I site at map coordinate 0·7 are described. In addition, genome analysis of the survivors from *Xba*I digestion identified a number of variants having deletions in the long terminal repeats (both TR_L and IR_L). As the frequency of isolation of these deletion mutants was relatively high (3·5%), and as deletions had already been shown to occur in the HSV-2 terminal repeat of the intertypic recombinant R12-5, it was decided to investigate the untreated strain and analyse a number of independently isolated single plaque stocks of HG52 for the presence of TR_L/IR_L deletions.

Surprisingly, out of 50 plaque stocks picked, 12 were shown to have deletions in IR_L. This paper describes the structure of the isolated deletion mutants and compares the frequency of

such mutants in HG52 with the frequency of occurrence in HSV-1 strain 17 (Brown *et al.*, 1973), HSV-1 strain KOS (Aron *et al.*, 1975), HSV-2 strain 186 (Esparza *et al.*, 1974), HSV-2 strain 333 (Seth *et al.*, 1974), a cloned HG52 stock (*ts*1) (Timbury, 1971), and a freshly recloned stock of wild-type HG52 derived from a single plaque with an undeleted internal long repeat. The growth properties of the deletion mutants in terms of one-step growth characteristics (Dargan & Subak-Sharpe, 1984) have been studied and the synthesis of polypeptides (Marsden *et al.*, 1978) coded by the deleted regions of the genome have also been analysed.

The implications of HSV-2 strain HG52 being able to lose up to 9 kb from one long repeat, and to sustain deletions of some equivalent sequences from both TR_L and IR_L of its genome while retaining viability are discussed.

METHODS

Growth of virus. Virus stocks were grown and titrated as described previously (Brown *et al.*, 1973). Parental strains of virus used were HSV-2 strain HG52 (Timbury, 1971), HSV-2 strain 186 (Esparza *et al.*, 1974), HSV-2 strain 333 (Seth *et al.*, 1974), *ts*1 from HG52 (Timbury, 1971), HSV-1 Glasgow strain 17 (Brown *et al.*, 1973) and HSV-1 strain KOS (Aron *et al.*, 1975).

Preparation of virion DNA. Virion DNA was prepared according to the method of Wilkie (1973) and Stow & Wilkie (1976) as described by Brown *et al.* (1984).

Restriction endonuclease digestion of viral DNA. Digestion of DNA at 50 to 100 μg/ml was carried out at 37 °C in 0·006 M-Tris–HCl pH 7·5, 0·006 M-$MgCl_2$, 0·006 M-2-mercaptoethanol, 0·02 M-KCl and 1 mg/ml bovine serum albumin using a two- or fivefold excess of *Xba*I (2 or 5 units/μg DNA). DNA restriction enzyme profiles were obtained by electrophoresis through 0·5% agarose gels, which were stained with ethidium bromide.

Transfection of virus DNA. Intact and *Xba*I-digested DNA (1 to 2 μg/plate) was transfected onto BHK21/C13 monolayers (4×10^6 cells) using the calcium phosphate infectivity assay technique (Stow & Wilkie, 1976). Single plaques obtained from transfections were isolated, grown into individual stocks and titrated.

Restriction enzyme analysis of virus genomes. Restriction enzyme analysis was carried out using the Linbro well technique (Lonsdale, 1979). Whenever the restriction enzyme profiles indicated mixtures of virus, three rounds of further plaque purification of the individual virus stocks were carried out before analysis.

DNA–DNA hybridization. DNA fragments from *Bam*HI digests of HG52 were transferred from agarose gels to nitrocellulose sheets (BA-85, Schleicher & Schüll) and hybridized with nick-translated DNA by the method of Southern (1975). Nick-translated DNA was made from total HSV-2 DNA (HG52) and a recombinant plasmid (pGZ1) containing HSV-2 *Bam*HI fragment *g* (Davison & Wilkie, 1981). Pre-soaking and hybridization were carried out at 75 °C in 3 × SSC, 5 × Denhardt's buffer, 1 × salmon sperm DNA, 10 mM-Tris–HCl pH 7·5. After extensive washing at 60 °C in 2 × SSC, 0·1% SDS and 5 mM-Na_2HPO_4 pH 7, the sheets were dried and autoradiographed.

One-step growth experiments. One-step growth analyses of mutants and control virus were carried out as described by Brown *et al.* (1984).

Preparation of virus stocks for restriction enzyme analysis. Elite virus stocks of HG52 (passage 1), strain 186, strain 333, *ts*1 and the HSV-1 strains 17 and KOS were titrated on BHK21/C13 cells in the presence of 5% human serum. From each stock, 50 well-separated single plaques were picked after washing of the cell monolayers three times with PBS (phosphate-buffered saline) containing 5% calf serum (PBSCa). The single plaques were picked into 0·5 ml PBSCa, sonicated and 0·2 ml was used to infect monolayers of BHK21/C13 cells on 50 mm Petri dishes (4×10^6 cells). The infected monolayers were incubated at 37 °C for 2 to 3 days until cytopathic effect (c.p.e.) was confluent. The cells were then harvested into the medium, centrifuged at 2000 r.p.m. for 10 min and resuspended in 1 ml of the medium. After sonication, the samples were stored at − 70 °C until used for Linbro well infection. A re-cloned stock of HG52 was grown from a single plaque shown not to be deleted in IR_L. This re-cloned stock was also treated as above.

Preparation and analysis of immediate early polypeptides. Confluent human foetal lung (HFL) cells (2×10^6 cells) were treated with cycloheximide in 2% calf serum at a concentration of 100 μg/ml 15 min prior to infection at a m.o.i. of 100 p.f.u./cell and incubated at 38·5 °C for 1 h. The infected cells were then washed twice with E Met/5 (4 parts methionine-free Eagle's medium and 1 part Eagle's medium) containing 2% calf serum, overlaid with 2 ml E Met/5 containing 100 μg/ml cycloheximide and incubated for 5 h at 38·5 °C. Fifteen min before labelling, actinomycin D was added at 2·5 μg/ml. The medium was then removed and the monolayers were washed four times with prewarmed complete PBS (A + B + C). Each wash was left in contact with the cells for approximately 1 min. [^{35}S]Methionine was added at a concentration of 200 μCi/ml in PBS and the plates were incubated for 1 h at 38·5 °C. After washing once with PBS, the infected cells were harvested into 0·35 ml boiling mix diluted 1 : 3 in water (Marsden *et al.*, 1976). The samples were run on 7·5% SDS–polyacrylamide gels (Preston *et al.*, 1978).

Separation of nuclei and cytoplasm for immediate early polypeptide analysis. After infection and labelling as above, the infected cells were harvested into 10 ml ice-cold PBS and pelleted at 2000 r.p.m. for 10 min. The pellet was resuspended in 1 ml lysis buffer (0·15 M-NaCl, 10 mM-Tris–HCl pH 7·8, 1·5 mM-$MgCl_2$, 0·65% NP40) with a vortex mixer before being left on ice for 30 min. The nuclear pellet was disrupted in 0·35 ml 1 : 3 boiling mix.

RESULTS

Isolation of HSV-2 genomes lacking XbaI *restriction endonuclease cleavage sites*

The selection technique used was as described for the isolation of RE mutants of HSV-1 (Glasgow strain 17) and the intertypic recombinant R12-5 (Brown *et al.*, 1984). DNA from HSV-2 strain HG52 at a concentration of 100 μg/ml was digested with *Xba*I under various conditions and then transfected onto BHK21/C13 monolayers. Untreated HG52 DNA (1 μg) transfected onto a 50 mm confluent monolayer of BHK21/C13 cells (4 × 10^6 cells) gave in excess of 500 plaques/plate after 4 to 5 days incubation at 37 °C. Similar transfection of DNA (1 μg/plate) which had been digested with *Xba*I at 5 units/μg DNA for 2 h at 37 °C failed to give any plaques. Reduction of the *Xba*I concentration to 1 unit/μg DNA combined with incubation for 1 or 2 h but with otherwise similar treatment and transfection gave 12 plaques on average. Digestion with 1 unit of *Xba*I/μg DNA which reduced DNA infectivity 40-fold was subsequently employed as the standard treatment from which a reasonable number of plaques could be isolated and analysed for *Xba*I site loss. One-hundred plaques each were picked from plates transfected with DNA which had been digested with 1 unit *Xba*I/μg DNA for either 1 or 2 h at 37 °C. Only two of these 200 plaques were found to contain genomes lacking *Xba*I sites and each had lost the same site at map coordinate (m.c.) 0·7. This represents an isolation frequency of 1% compared to the previously found frequencies of *Xba*I RE mutants (following treatment with 5 units *Xba*I/μg DNA for 3 h at 37 °C) in HSV-1 Glasgow strain 17 of 3·6% and of 5·8% in the intertypic recombinant R12-5 (Brown *et al.*, 1984).

There are four *Xba*I cleavage sites in the HSV-2 genome: two in the long unique region and two in the short unique region. Complete digestion gives rise to fragment *c* (m.c. 0 to 0·45, mol. wt. 45 × 10^6), fragment *d* (m.c. 0·45 to 0·7, mol. wt. 25 × 10^6), fragment *g* (m.c. 0·7 to 0·82, mol. wt. 12 × 10^6), fragment *h* (m.c. 0·82 to 0·9, mol. wt. 8 × 10^6), fragment *j* (m.c. 0·9 to 0·944, mol. wt. 4·4 × 10^6) and fragment *i* (m.c. 0·944 to 1, mol. wt. 5·6 × 10^6) plus four fusion fragments [$a=(c+h)$, mol. wt. 53 × 10^6; $b=(c+i)$, mol. wt. 50·6 × 10^6; $e=(g+h)$, mol. wt. 20 × 10^6; $f=(g+i)$, mol. wt. 17·6 × 10^6]. The *Xba*I, *Hpa*I, *Hin*dIII, *Eco*RI and *Bam*HI maps of HSV-2 (strain HG52) are shown in Fig. 1 (Cortini & Wilkie, 1978).

One of the isolated variants, HG52X94, gave an *Xba*I profile with a band in the *a, b, c* position and bands in the normal *h, i* and *j* positions. Four bands were missing, i.e. *d, e, f* and *g*. No new bands were immediately visible although the intensity of the band in the *a, b, c* position was markedly increased (Fig. 2, lane 1). This suggested that this genome had lost the *Xba*I site at m.c. 0·7, giving rise to a new fused terminal fragment ($d+g$) of mol. wt. 36 × 10^6 and two new joint fragments, $d+g+h$ of mol. wt. 44 × 10^6 and $d+g+i$ of mol. wt. 41·6 × 10^6. These three fragments would run at the top of the gel and would account for the increased intensity of the *a, b, c* band (mol. wt. 40 × 10^6). *Hin*dIII and *Hpa*I digests of this mutant detected no changes in the DNA profile compared to the parental HG52, suggesting that the *Xba*I site loss at 0·7 was not due to an extensive deletion which would have resulted in alterations in mobility of the large *Hin*dIII *a* and *Hpa*I *e* fragments (data not shown). However, a *Bam*HI digest of HG52X94 showed that the site loss at m.c. 0·7 was due to a deletion of about 150 base pairs. The *Bam*HI *k* fragment which contains the *Xba*I site at m.c. 0·7 had an altered mobility equivalent to a 0·1 × 10^6 change in mol. wt. compared to the parental HG52 *k* fragment (Fig. 3, lanes 1 and 2).

Another variant, HG52X163, had an *Xba*I profile indistinguishable from HG52X94 (Fig. 2, lane 3) although the method of isolation precluded the two mutants from being clonally related. Again, the *Xba*I profile suggested that HG52X163 had lost the *Xba*I site at m.c. 0·7 and the *Hin*dIII and *Hpa*I profiles were indistinguishable from the parental HG52 pattern. The *Bam*HI digest of this isolate also showed a *k* fragment with an altered mobility, but in this case instead of the site loss being due to a deletion, the mol. wt. of the *k* fragment had increased in size from 3·1

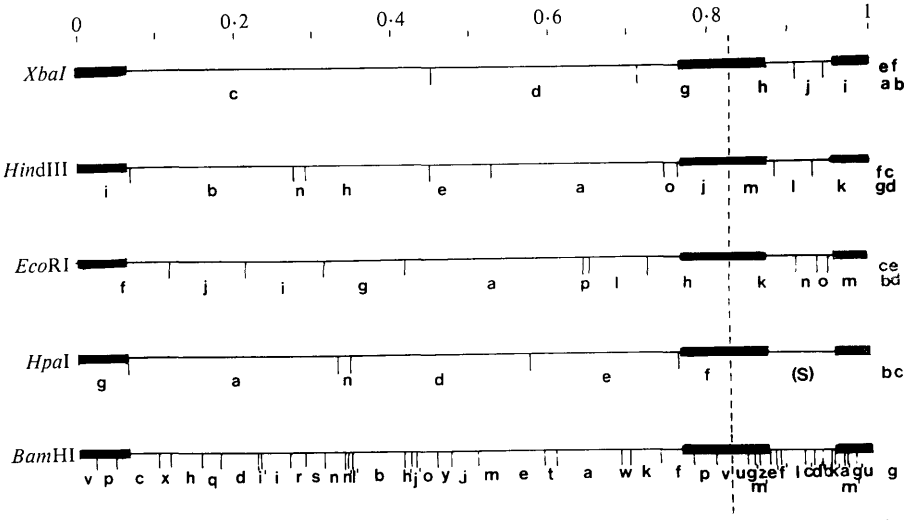

Fig. 1. Restriction endonuclease maps for the DNA of HSV-2 strain HG52 from Cortini & Wilkie (1978). The origin of the joint fragments is as follows. *Xba*I: $a = c + h, b = c + i, e = g + h, f = g + i$. *Hin*dIII: $c = i + k, d = j + k, f = i + m, g = j + m$. *Eco*RI: $b = f + k, c = h + k, d = f + m, e = h + m$. *Hpa*I: $b = f$ + [S], $c = g$ + [S]. *Bam*HI: $g = v + u$.

$\times 10^6$ to $3{\cdot}2 \times 10^6$, suggesting an insertion of about 150 base pairs and a consequent loss of the *Xba*I site (Fig. 3, lane 3). The only other apparent difference in the *Bam*HI profile of HG52X163 compared to HG52 was an alteration in the mobility of the *u* fragment rendering it indistinguishable from the *v*, *w* band: an alteration in mol. wt. of $0{\cdot}1 \times 10^6$. Southern blot hybridization of a *Bam*HI digest with the pGZ1 plasmid ($g + u$) (Davison & Wilkie, 1981) was carried out to determine whether the inserted sequences in the *k* fragment could be from the IR_S *Bam*HI *u* fragment. No hybridization of the pGZ1 plasmid with the *k* band was detectable. The source of the insertion into the *k* fragment remains at present unknown.

Isolation of variants of HSV-2 strain HG52 with deletions in the long terminal repeat regions of the genome

During *Xba*I analysis of the 200 virus clones obtained after transfection with *Xba*I-digested HG52 DNA, it became apparent that several variants had been isolated with altered mobility of the *Xba*I *g* fragment (m.c. 0·7 to 0·82) and consequent mobility changes in the *e* and *f* joint fragments ($g + h$ and $g + i$). The mobility changes suggested deletions in the *g* fragment and therefore possibly in IR_L. As we had already encountered deletions in the long terminal repeat of the intertypic recombinant R12-5, the HG52 variants with suspected deletions were subjected to further analysis. The position of the *Xba*I sites in HG52 precludes identification with *Xba*I of deletions in TR_L, as the size of the *c* fragments (mol. wt. 40×10^6) would not allow small deletions to be observed. Rather than screen all 200 plaques with another enzyme that would allow detection of deletions in TR_L, it was decided initially to concentrate on analysis of the variants already observed in *Xba*I *g*. Seven of the 200 plaque stocks digested with *Xba*I showed altered mobility of the *g*, *e* and *f* bands. Three of these have so far been analysed in detail and are designated HG52XD85, HG52XD86 and HG52XD192.

The altered mobility of the *Xba*I *g*, *e* and *f* bands of HG52XD86 indicated a deletion in *Xba*I *g* of mol. wt. about 3×10^6 or 4·5 kb (Fig. 4, lanes 3 and 4). This was confirmed by *Hpa*I digestion of this variant which showed an altered mobility of the *Hpa*I *f* fragment (m.c. 0·75 to 0·82) and therefore confined the deletion to the internal long repeat leaving the long unique region of the genome intact (data not shown). The size of the *Hpa*I joint *c* fragment (f + [S]) prevented the alteration being observed in this band. A *Bam*HI digest of HG52XD86 (Fig. 4, lanes 1 and 2)

indicated that the *f* fragment (m.c. 0·725 to 0·775) was missing and that one copy of the *p* fragment (0·775 to 0·8) was also missing. As the *v* fragment (0·8 to 0·82) co-migrated with the *u* fragment, the digest did not allow identification of a loss of one copy of *v*. However, the intensity of the *g* joint fragment (*v* + *u*) suggested that the internal repeat copy of *v* was present. A new band of $4{\cdot}4 \times 10^6$ mol. wt. above *f* is thought to be a fusion fragment made up from part of *f* and part of *p* due to the deletion covering the *Bam*HI *f*/*p* site (Fig. 4, lane 2). A normal *f* + *p* joint fragment would have a mol. wt. of $6{\cdot}9 \times 10^6$. We conclude that the deletion in HG52XD86 is 2·5 $\times 10^6$ mol. wt. (3·75 kb) in size and includes the *Bam*HI *f*/*p* site at m.c. 0·775. Most or all of the deleted sequences were from IR_L but they may also have included the U_L/IR_L junction. Precise coordinates cannot be determined without finer analysis. The deletion must include some or all of the coding region for the V_{mw}IE118 protein (m.c. 0·785 to 0·81).

The *Xba*I profile of the isolate HG52XD192 indicated a deletion in the *g* fragment of mol. wt. about 1×10^6 (1·5 kb). The *e* and *f* joint fragments were also reduced in size compared to the equivalent bands in the parental HG52 (Fig. 5, lanes 1 and 2). The *Hpa*I profile of this isolate showed not only the expected deletion in the *f* fragment (m.c. 0·75 to 0·82) but also a deletion of similar size in the *Hpa*I *g* fragment (m.c. 0 to 0·064) (data not shown). This was confirmed by a *Hin*dIII analysis which showed a deletion in both *Hin*dIII *i* (0 to 0·07) and *j* (0·75 to 0·82) with new *i* and *j* bands running between the *l* and *m* bands and consequent mobility changes in the four joint fragments (*c*, *d*, *f*, *g*) (Fig. 5, lanes 3 and 4). These data indicated that HG52XD192 was deleted in both TR_L and IR_L by about 1×10^6 mol. wt. To determine the location of the deletion in each repeat, a *Bam*HI digest and a Southern blot hybridization of a *Bam*HI digest with nick-translated HG52 DNA and the plasmid pGZ1 (*Bam*HI *v* + *u*) were carried out.

The *Bam*HI digest (Fig. 5, lanes 5 and 6) showed that the *Bam*HI *p* fragment was present in normal 2-molar amounts and the *Bam*HI *c* and *f* fragments were normal in size. The *Bam*HI *g* joint fragment (*v* + *u*) was missing and it appeared that it was running in two places, just above the *l* and *m* bands. The gel also showed that the *v* fragment was missing and the *u* fragment present (Fig. 5, lane 6). The data indicate that HG52XD192 has deletions within the *Bam*HI *v* fragment in both TR_L and IR_L and that the *v*/*p* site was retained in both repeats. Southern blot hybridization with pGZ1 (*Bam*HI *g*, i.e. *v* + *u*) demonstrated that in HG52XD192 the *v* fragment was missing and that there was hybridization to two smaller fragments running at different positions considerably below *v*. At the position of the *Bam*HI *g* fragment, there was no hybridization but positive hybridization was detected to two fragments (mol. wt. $2{\cdot}8 \times 10^6$ and $2{\cdot}9 \times 10^6$) running in positions equivalent to *Bam*HI *m* and *l* (Fig. 6, lane 2). These are assumed to be new joint fragments consisting of the remnant of *v* in each long repeat joined to *u*. The difference in intensity of the two *v* and two *g* bands may suggest that in each case the higher mol. wt. band was due to an additional '*a*' sequence. The deletion in TR_L is thought to be of approx. mol. wt. $1{\cdot}1 \times 10^6$ and in IR_L $1{\cdot}3 \times 10^6$. This could mean that the same sequences have been deleted in part or wholly from TR_L and IR_L and that information encoded in this region is completely non-essential for the viability of the virus in tissue culture. However, it could also be that the 1·5 kb deleted in each *v* fragment may be in adjacent regions so that in the total genome all of the *v* fragment sequences are retained. It seems highly probable that the deletions in *v* overlap and at least some sequences have been lost completely, as the total mol. wt. of the *v* fragment is 2×10^6. The new joints (*g* fragments) have been cloned and fine structure and heteroduplex mapping is being carried out to delimit the deletion in each *Bam*HI *v* fragment.

Another isolate, HG52XD85, on initial *Xba*I digestion also seemed to lack *Xba*I *g* but it appeared as if a mixture of genomes was in the stock population. Three rounds of further plaque purification identified two main genome classes. These were designated HG52XD85/5 and HG52XD85/4.

*Xba*I digestion of HG52XD85/5 showed that the *g* fragment co-migrated with *h*, indicating a deletion of about 4×10^6 mol. wt. The *e* and *f* joints were similarly deleted (Fig. 7, lane 3). The *Hpa*I digest (Fig. 7, lane 9) showed a missing *f* fragment and a new fragment running half way between *g* and *h*. A new joint fragment was also found running between the *e* and *c*/*d* bands. This essentially limited the deletion to IR_L. Confirmation was provided by a *Hin*dIII digest which showed a missing *j* fragment and a new fragment running midway between *n* and *m*. Two new

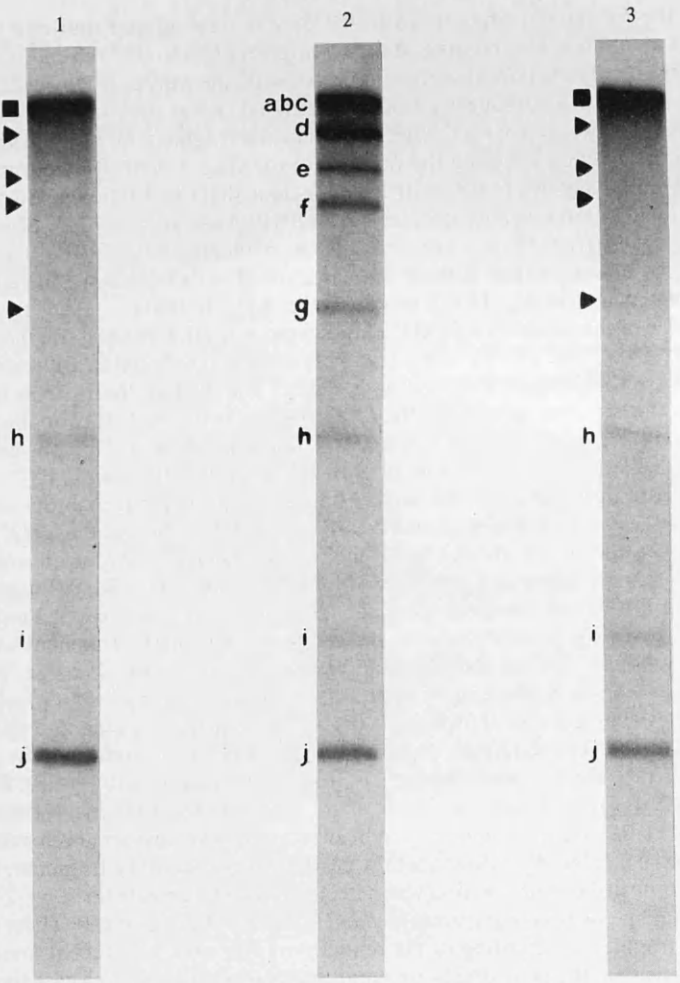

Fig. 2. Autoradiographs of *Xba*I restriction digests of viral DNA (^{32}P-labelled *in vivo*) of the RE site mutant HG52X94 (lane 1), strain HG52 (lane 2) and the RE site mutant HG52X163 (lane 3). Letters refer to specific *Xba*I fragments; ■, new fragments; ▶, missing fragments.

joint fragments composed of the new *j* fragment with *m* and with *k* ran between the *i* and *h* bands (Fig. 7, lane 6). When HG52XD85/5 was digested with *Bam*HI (Fig. 7, lane 12) the profile showed reduced intensity of the *p* fragment with a new band running above *k* (mol. wt. 3·2 × 10^6). These mobility changes suggest a deletion in *Bam*HI *f* and *p* spanning the *f*/*p* site. The 1-molar band running above *k* was a fusion fragment composed of the remainder of *f* plus the remainder of *p*. The mutant HG52XD85/5 therefore has a deletion of 5·5 kb including the *Bam*HI *f*/*p* site, which is mostly confined to IR_L but, as in the case of HG52XD86, the U_L/IR_L junction may be within the deleted sequence.

The HG52XD85/4 isolate showed on *Xba*I digestion (Fig. 7, lane 1) a deletion of 9 to 9·3 kb in that the *Xba*I *g* fragment was running marginally above the *i* fragment (mol. wt. 5·6 × 10^6). The *Hpa*I *f* fragment was also missing (Fig. 7, lane 7), with the new *f* fragment smaller than 2 × 10^6 and having run off the bottom of the gel. The *Hin*dIII digest (Fig. 7, lane 4) disclosed a missing *j* fragment and the two new joint fragments equivalent to *d* (*j* + *k*) and *g* (*j* + *m*), one running just above *m* (mol. wt. 5·2 × 10^6) and one above *j* (mol. wt. 6·4 × 10^6). It follows that in

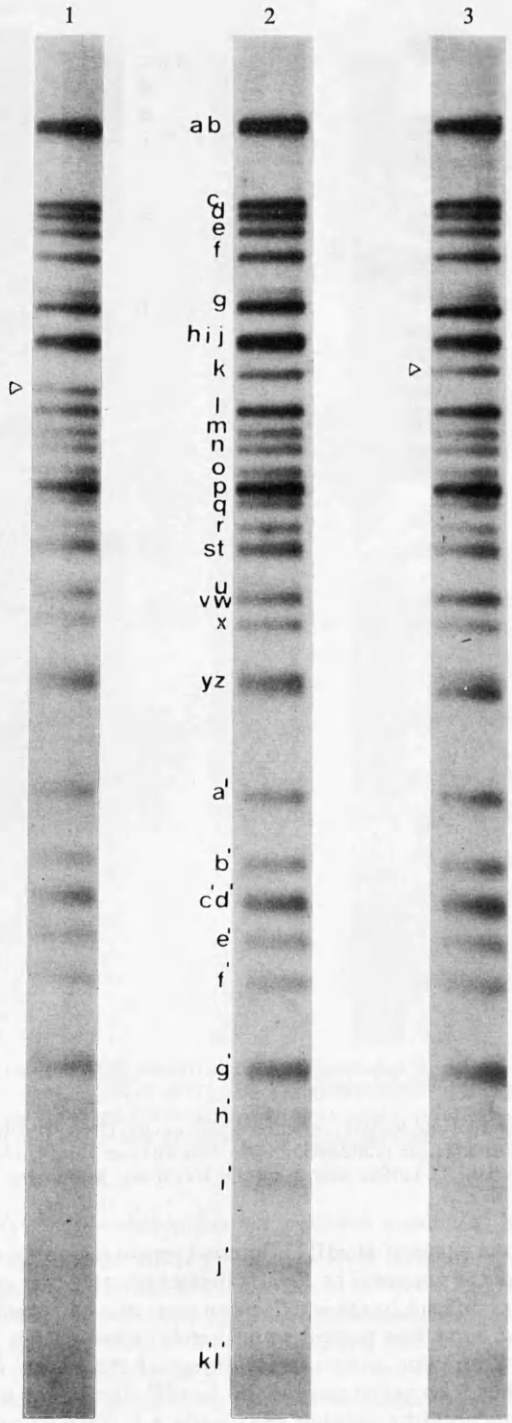

Fig. 3. Autoradiographs of *Bam*HI restriction digests of viral DNA (^{32}P-labelled *in vivo*) of the RE site mutant HG52X94 (lane 1), strain HG52 (lane 2) and the RE site mutant HG52X163 (lane 3). Letters refer to specific *Bam*HI fragments; ▷, new fragments.

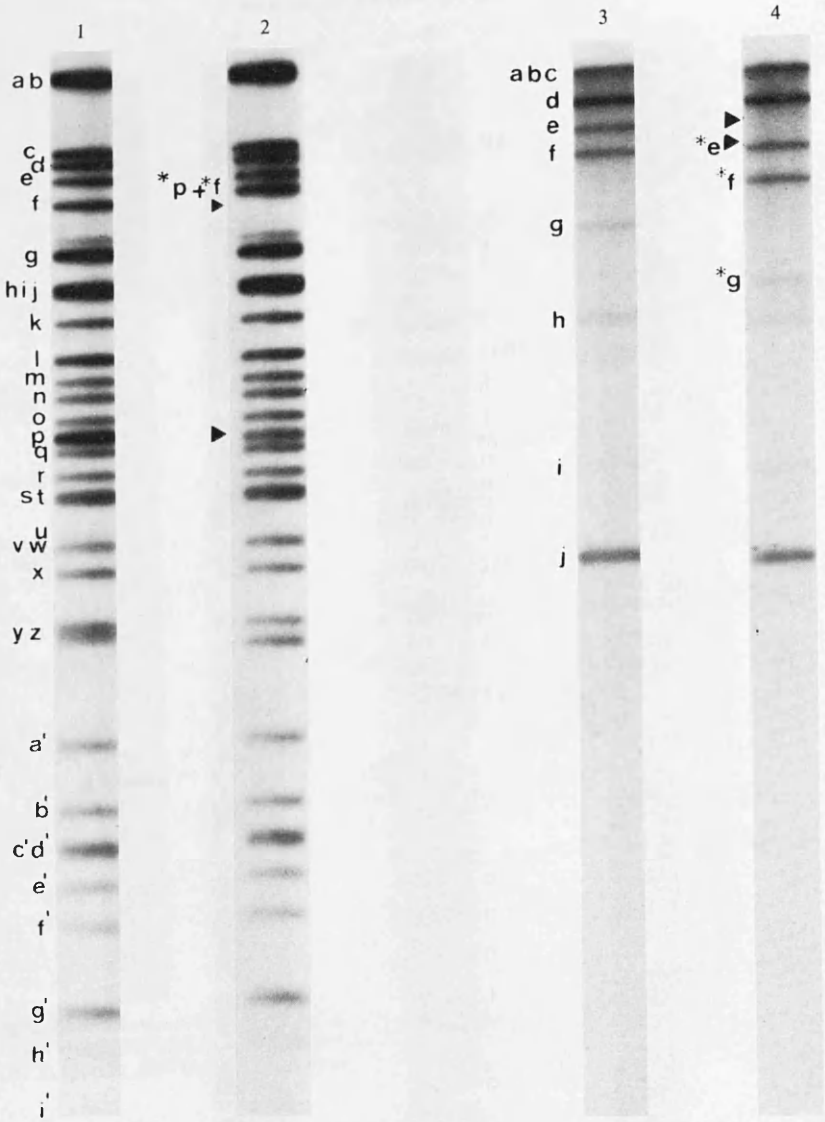

Fig. 4. Autoradiographs of *Xba*I and *Bam*HI restriction digests of viral DNA ^{32}P-labelled *in vivo*. Lanes 1 and 2, *Bam*HI digests of HG52 (lane 1) and HG52XD86 (lane 2); lanes 3 and 4, *Xba*I digests of HG52 (lane 3) and HG52XD86 (lane 4). Letters refer to specific fragments; ▶, missing fragments; *, new fragments.

HG52XD85/4 practically the whole of *Hin*dIII *j* (internal repeat region) had been deleted. The *Hin*dIII *o* fragment appeared as normal. The *Bam*HI digest indicated that one of the two copies of *p* was missing, but it was difficult to see whether one copy of *v* had also been deleted. The *f* band had gone but a new band was present immediately below *f* (Fig. 7, lane 10). Taken together, we interpret the restriction endonuclease digests of this isolate to indicate that the molecule has been deleted by 9 kb encompassing the *Bam*HI *f*/*p* site, the total *Bam*HI *p* and *v* fragments excluding '*a*' sequences [the molecule still inverts, e.g. *Hin*dIII *c* and *f* joint fragments (Fig. 7, lane 4) are still visible]. The new joint fragment composed of part of *f* + *u* ran marginally below *f* with a mol. wt. of 4·3 × 10^6. The deletion therefore extends from approximately 0·76 to 0·82 map units, including the total coding region for V_{mw}IE118 and the U_L/IR_L junction.

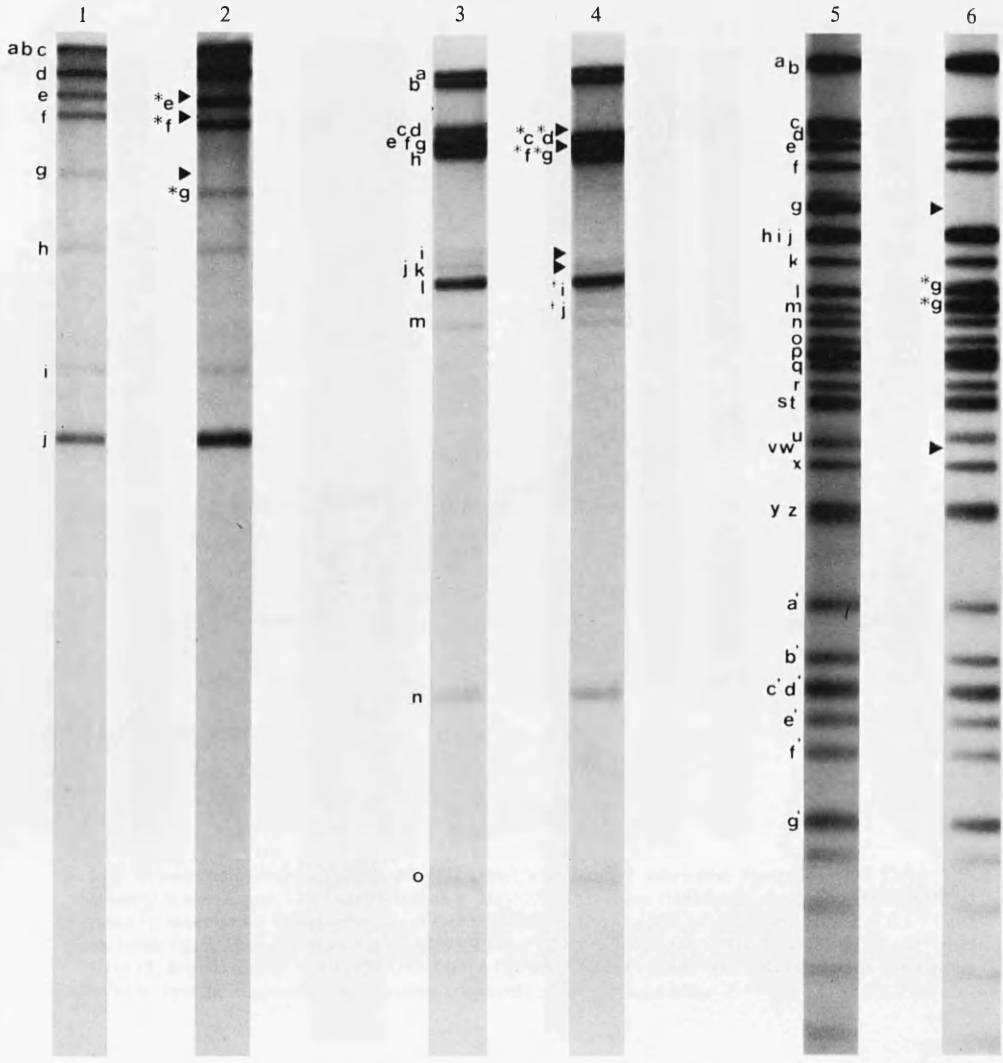

Fig. 5. Autoradiographs of *Xba*I, *Hin*dIII and *Bam*HI restriction digests of viral DNA ^{32}P-labelled *in vivo*. Lanes 1 and 2, *Xba*I digests of HG52 (lane 1) and HG52XD192 (lane 2); lanes 3 and 4, *Hin*dIII digests of HG52 (lane 3) and HG52XD192 (lane 4); lanes 5 and 6, *Bam*HI digests of HG52 (lane 5) and HG52XD192 (lane 6). Letters refer to specific fragments; ▶, missing fragments; *, new fragments.

Genome deletions in virus stocks not previously exposed to Xba*I treatment*

As the frequency of genome deletions isolated from *Xba*I-treated HG52 stock virus was relatively high (3·5%), it was decided to analyse stocks of HSV-1 and HSV-2 which had not been treated with *Xba*I for the occurrence of terminal repeat deletions. A stock of HG52 was grown from the elite stock, i.e. elite stock + P1 (passage one). The elite stock had been produced as follows. An isolated single plaque was purified by successive plaque purifications in BHK21/C13 cells. The resulting virus was propagated in three stages to produce the elite stock kept in this Institute.

The elite stock was passaged once in BHK cells and this formed the starting material (HG52P1) which was plated on BHK21/C13 cells. After 2 days incubation at 37 °C in medium containing human serum, 50 well-separated single plaques (HG52P2) were each picked, put into

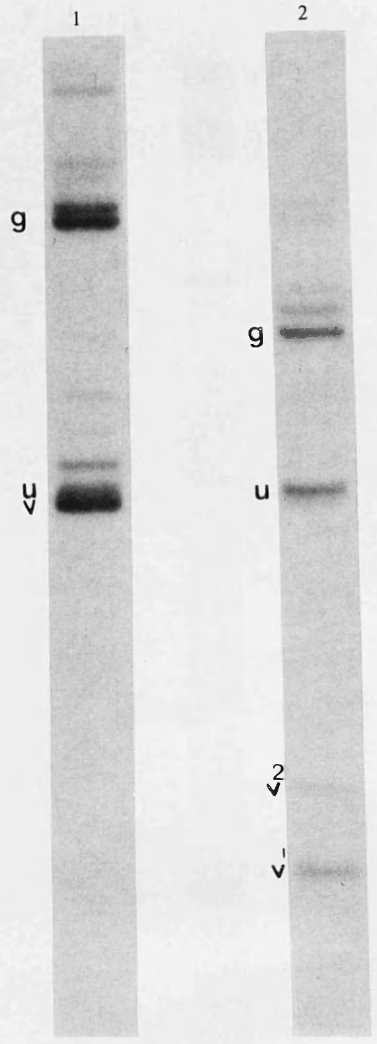

Fig. 6. Autoradiograph of nitrocellulose blot strips containing *Bam*HI restriction fragments of HG52 (lane 1) and HG52XD192 (lane 2) to which nick-translated DNA probes from the recombinant plasmid pGZ1 have been hybridized. Lane 1 shows specific hybridization to the HSV-2 terminal fragments *u* and *v* and the joint fragment *g*. Lane 2 shows normal hybridization to the *u* terminus from the short repeat and hybridization to the new long terminal fragments v^1 and v^2 and the new joint *g* fragment. Hybridization to bands thought to contain larger numbers of *'a'* sequences can be seen.

0·5 ml of PBSCa and after sonication 0·1 ml was plated on to a separate confluent 50 mm monolayer of BHK21/C13 cells. After 2 days incubation the infected monolayers were harvested and the virus (HG52P3) was released by sonication. These separately recloned virus stocks (clones 1 to 50 of HG52P4) were used to infect Linbro wells in the presence of $^{32}P_i$ for restriction endonuclease genome analysis. This procedure was also applied to the analysis of HSV-2 strains 186 and 333 and HSV-1 strains Glasgow 17 and KOS, except that the elite stock was plated directly without P1. (This analysis is therefore of 186P3 clones 1 to 50, 333P3 clones 1 to 50, 17P3 clones 1 to 100 and KOSP3 clones 1 to 50.)

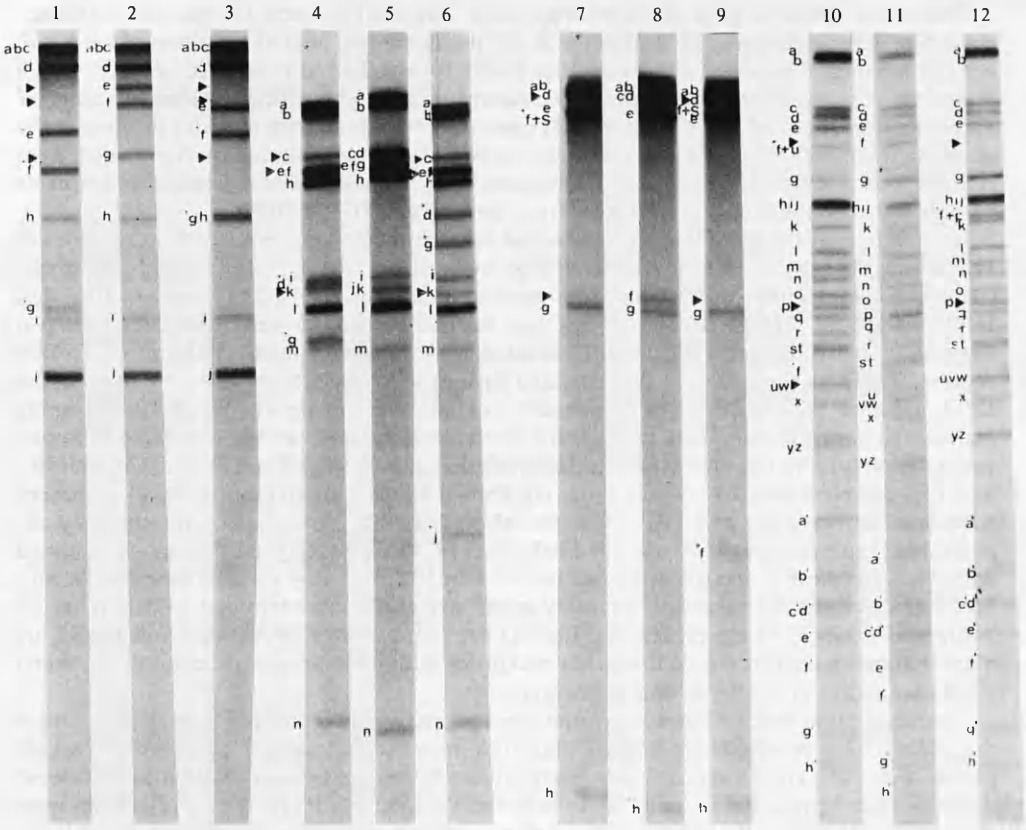

Fig. 7. Autoradiographs of *Xba*I, *Hin*dIII, *Hpa*I and *Bam*HI restriction digests of viral DNA ^{32}P-labelled *in vivo*. Lanes 1 to 3, *Xba*I digests of HG52XD85/4 (lane 1), HG52 (lane 2) and HG52XD85/5 (lane 3); lanes 4 to 6, *Hin*dIII digests of HG52XD85/4 (lane 4), HG52 (lane 5) and HG52XD85/5 (lane 6); lanes 7 to 9, *Hpa*I digests of HG52XD85/4 (lane 7), HG52 (lane 8) and HG52XD85/5 (lane 9); lanes 10 to 12, *Bam*HI digests of HG52XD85/4 (lane 10), HG52 (lane 11) and HG52XD85/5 (lane 12). Letters refer to specific fragments; ▶, missing fragments; *, new fragments.

The DNA of the 50 HG52P4 clone stocks was digested with *Xba*I and *Eco*RI to facilitate identification of deletions in both IR_L and TR_L. Twelve of the 50 clones showed alterations in both the *Xba*I and *Eco*RI profiles. Five of the 12 showed indistinguishable profiles [prototype HG52P4 clone 5 (HG52/5)] and the other seven formed a second group showing indistinguishable profiles (prototype HG52/10), i.e. in addition to the archetype there were two variant genome classes within 50 clones. Variant HG52/5 showed an *Xba*I profile with an increased mobility of the *g*, *e* and *f* bands and an *Eco*RI profile which indicated a deletion in the *Eco*RI *h* fragment. From the change in mobility in the *Xba*I *g* fragment and the *Eco*RI *c* and *e* fragments (joint fragments) the size of the deletion was calculated to be between 2×10^6 and 3×10^6 mol. wt. This was more accurately determined by *Bam*HI digestion which showed a missing *f* fragment, only one copy of the *p* fragment and a new band of mol. wt. $4{\cdot}4 \times 10^6$ running above *f*. The variant HG52/5 is therefore deleted by $2{\cdot}5 \times 10^6$ mol. wt. or 3·75 kb in the *Bam*HI *f* and *p* fragments; the deletion spans the *Bam*HI *f*/*p* site and may include the U_L/IR_L junction and/or part of the coding region for V_{mw}IE118. The size of the deletion in HG52/5 suggests that it was similar to that in HG52XD86, isolated after *Xba*I digestion.

Analysis of HG52/10 gave the following results. The *Xba*I *g*, *e* and *f* bands had mobilities consistent with a deletion in *Xba*I *g* but the *Eco*RI profile showed altered mobilities of the *b*, *c*, *d*, *e*, *f* and *h* bands. This could only mean that HG52/10 was deleted in both the *Eco*RI *f* and *h* fragments. The size of the deletion was calculated for both to lie within the mol. wt. range of approximately 10^6 to $1{\cdot}5 \times 10^6$. The *Bam*HI digest results were consistent with a deletion in the *Bam*HI *v* fragment in both TR_L and IR_L: the normal *g* band had gone and two new bands were running above *m* and *l* respectively. The variant HG52/10 clone was indistinguishable in its various restriction endonuclease profiles from the variant HG52XD192.

It would appear therefore that: (i) substantial deletions in the long terminal repeat regions of HG52 were already present at the relatively high frequency of 24% of genomes in the elite stock; (ii) two different types of deletions were present, such that HG52/5 represented 10% and HG52/10 14% of the elite stock; (iii) the two deletion prototypes were identical to the two variants HG52XD86 and HG52XD192 isolated after *Xba*I treatment of the DNA, which suggests that these variants were not generated during the *Xba*I treatment but pre-existed in the DNA molecule population of the elite stock; (iv) both types of deletion appear to be stably maintained during routine stock propagation, both are viable and can be propagated as cloned populations; (v) a viable variant with a stable deletion of mol. wt. 10^6 to $1{\cdot}5 \times 10^6$ in both IR_L and TR_L has been isolated, although it is not known whether completely identical sequences have been lost from IR_L and TR_L. Two other strains of HSV-2 have been similarly analysed: strain 186 (Esparza *et al.*, 1974) and 333 (Seth *et al.*, 1974). Of 50 plaques of strain 186 analysed with *Xba*I and *Eco*RI, one showed a deletion of 1×10^6 in *Xba*I *g*, *e* and *f* (data not shown). Strain 186 also showed inherent variability in the size of the short terminal repeat. When 50 plaques of strain 333 were picked and their DNA restricted with both *Xba*I and *Eco*RI, no alterations were seen in any of the bands except for additional *'a'* sequences on the terminal *Eco*RI (*m* and *k*) and *Xba*I (*h* and *i*) fragments.

A stock of strain HG52 virus was grown from a plaque whose DNA had been shown to be of the archetype (i.e. not deleted in *Xba*I *g*). This stock in turn was titrated, 50 plaques were picked, grown up and the virus from each was used to infect Linbro wells for restriction endonuclease digestion after being labelled with ^{32}P. In both the *Xba*I and *Eco*RI profiles, all 50 clones were shown to be identical without detectable deletions in the genome. Therefore, deletions in TR_L or IR_L occur at frequencies lower than 2% after four passages from a cloned non-deleted stock.

An additional experiment was carried out to analyse a cloned temperature-sensitive mutant stock (*ts*1) of HG52. Treatment was as before (50 single plaque stocks) and DNA was restricted with *Xba*I and *Eco*RI. No deletions were detected with either enzyme. As *ts*1 was isolated originally from a single plaque, it must not have had deletions in its genome; on subsequent passage to obtain working stocks, if deletions had occurred and been maintained, their cumulative frequency must be below 2%.

As it seemed relevant to compare the frequency of deletions in HSV-1 with those in HSV-2, two strains of HSV-1, i.e. Glasgow strain 17 and KOS (Aron *et al.*, 1975), were subjected to restriction endonuclease analysis. Stocks from single plaques were obtained and analysed in the same manner as those of HSV-2, but using *Xba*I and *Hpa*I neither strain exhibited detectable deletions in its genome. Over 100 plaques of Glasgow 17 and 50 plaques of KOS were subjected to analysis.

Immediate early (IE) polypeptides induced in HFL cells

IE polypeptides induced in HFL cells by the method of Preston *et al.* (1978) were extracted from either whole cell lysates or from purified nuclei. The two variants, HG52XD86 and HG52XD192, were compared with the parental HG52. Fig 8 shows the IE polypeptides from whole cell lysates (*a*) and from nuclei (*b*) analysed by SDS–PAGE. Four HSV-2-coded IE polypeptides could be identified in the HG52 profile, i.e. 182K, 138K, (early/IE) 118K and 64K. HG52XD86 which had been identified as having a deletion of 3·75 kb including the *Bam*HI *f*/*p* site, showed a reduction in the amount of V_{mw}IE118 synthesized both in whole cells (lane 2) and nuclei (lane 8). This would support the conclusion reached from the DNA analysis: one copy of

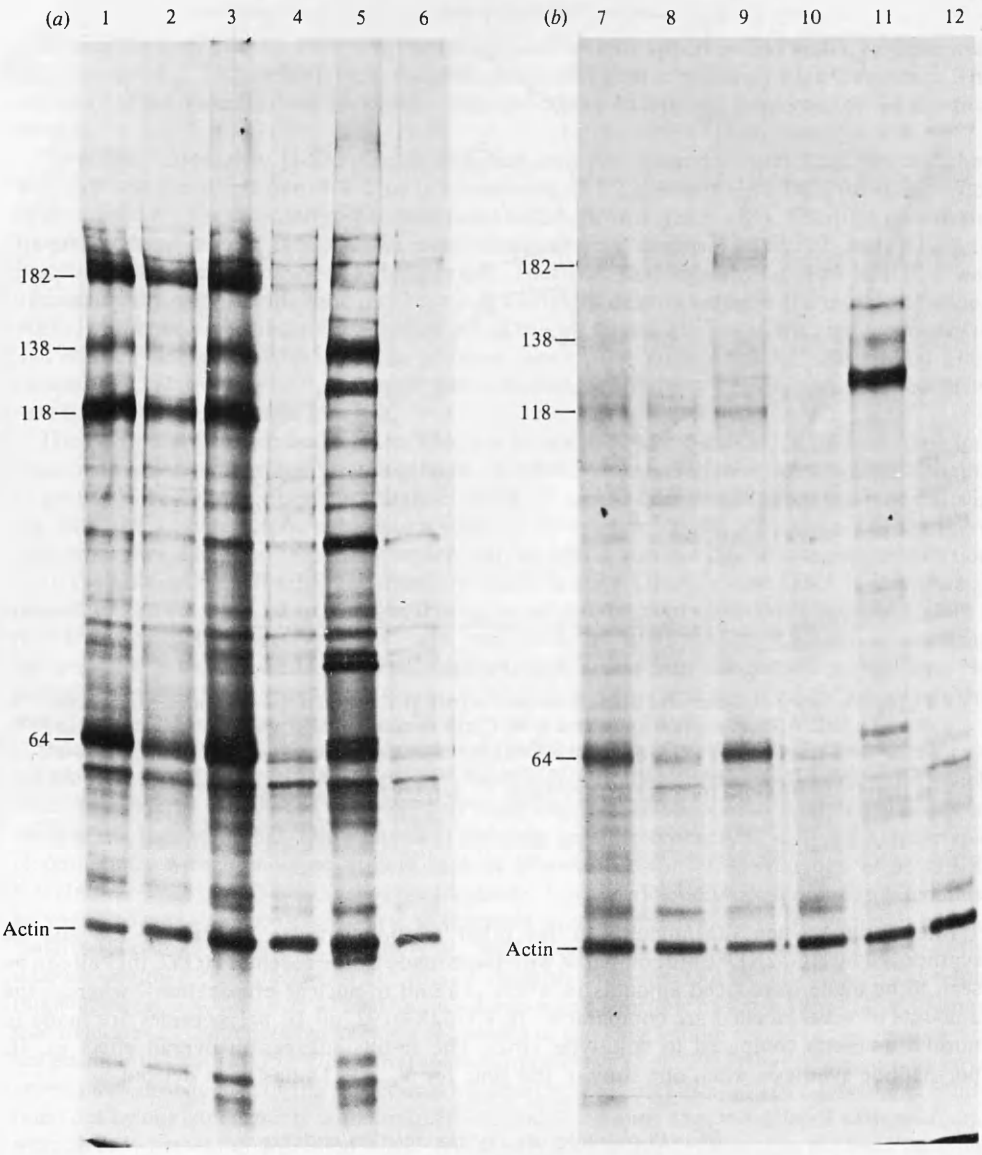

Fig. 8. Autoradiographs of IE polypeptides induced in HFL cells and separated by SDS–PAGE. (*a*) Whole cell extracts; (*b*) nuclear extracts. Lanes 1 and 7, HG52; lanes 2 and 8, HG52XD86; lanes 3 and 9, HG52XD192; lanes 4 and 10, mock-infected; lanes 5 and 11, HG52 without cycloheximide/actinomycin D block; lanes 6 and 12, mock-infected without cycloheximide/actinomycin D block. Numbers to the left of the gels show the apparent molecular weight ($\times 10^{-3}$) of HSV-2 IE polypeptides.

the region coding for V_{mw}IE118 (0·785 to 0·81 map units) has been deleted from this mutant. The mutant HG52XD192 whose DNA was shown to lack both copies of *Bam*HI *v* appeared to synthesize wild-type amounts of V_{mw}IE118. However, until the precise coordinates of the deletions in each copy of the long repeat are determined, it would be premature to claim that one particular deletion affects the coding region for V_{mw}IE118.

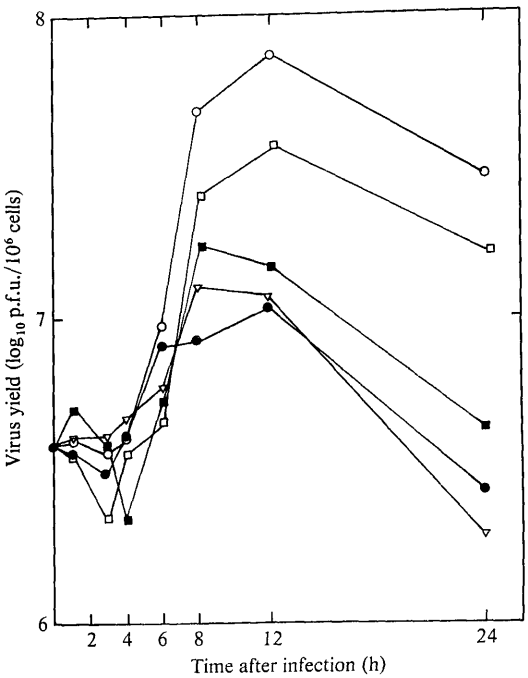

Fig. 9. One-step growth curves of deletion mutants in BHK21/C13 cells. Cells were infected at a m.o.i. of 5 p.f.u./cell. After adsorption for 45 min at 37 °C, the monolayers were washed twice with PBSCa, overlaid with Eagle's medium containing calf serum and incubated at 37 °C (see Methods). Cultures were harvested at the times indicated and the virus titre measured by plaque assay on BHK21/C13 cells. ●, HG52; ▽, HG52XD192; ■, HG52XD85/5; □, HG52XD86; ○, HG52XD85/4.

Interestingly, when the amounts of the other IE polypeptides V_{mw}IE182, 138 and 64 synthesized by HG52XD86 are compared with those made by the parental HG52, they all can be seen to be made in reduced amounts in whole cell and in nuclear preparations, whereas the amounts of actin labelled are comparable. In HG52XD192, all IE polypeptides are made in normal amounts compared to wild-type virus. The results suggest an overall effect on IE polypeptide synthesis when one copy of the gene for V_{mw}IE118 has been deleted.

Growth characteristics of the deletion mutants

Comparative one-step growth experiments were carried out for each of the deletion mutants and the parental HG52 (Fig. 9) over a 24 h period at 37 °C. Each of the variants grew reasonably well, and the final yield at the plateau level was higher than that of HG52. The variants with deletions in one copy of the gene for V_{mw}IE118, i.e. HG52XD86, HG52XD85/5 and HG52XD85/4, grew at the same rate as the wild-type virus but HG52XD192, the variant with the deletion within both copies of *Bam*HI *v*, appeared to grow more slowly in the exponential phase than the others. By 24 h, the yield of virus had fallen off considerably for each of the mutants and the wild-type virus. It should be pointed out that the particle to p.f.u. ratios for HG52, HG52XD86, HG52XD192, HG52XD85/5 and HG52XD85/4 were 47:1, 36:1, 71:1, 114:1 and 105:1 respectively.

We concluded from many growth experiments that deletions of up to 9 kb in one of the long repeat regions of the genome can be tolerated without marked impairment of the lytic growth cycle under normal conditions in BHK cells.

DISCUSSION

During the screening for HSV-2 RE site mutants it became apparent that viable variants with deletions in IR_L, TR_L or both were found in strain HG52 at a relatively high frequency. The objective of our investigation became to study the origin, nature and properties of the deletion mutants.

From 200 independent HG52 plaques screened, only two showed deleted *Xba*I sites and they had both lost the site at m.c. 0·7. This is a frequency of 1%, compared to 3·6% for strain 17 of HSV-1 and 5·8% for the intertypic recombinant R12-5 (Brown *et al.*, 1984). The difference in the frequency may merely reflect differences distinguishing strains HG52, 17 and R12-5 in mutability of different regions of the genome. That the *Xba*I site at m.c. 0·45 in R12-5 was apparently lost very readily (one in 17 plaques analysed) despite being in the middle of a long HSV-2 sequence in this recombinant, does not fit this argument. However, the high frequency of loss of the 0·45 site in R12-5 may be spurious, since after finding the RE site mutant after analysis of 17 plaques no further plaques were screened, the frequency of isolation is based on an unsatisfactorily low sample number.

The two mutants which had lost the *Xba*I site at m.c. 0·7 were unrelated in that each site loss was due to different alterations in the genome: HG52X94 had lost the site due to a small deletion of about 150 base pairs, while the mutant HG52X163 had an addition of about 150 base pairs in the *Bam*HI *k* fragment. The latter had a consistently smaller *Bam*HI *u* fragment but Southern blot experiments showed that the insertion into *Bam*HI *k* was not due to a sequence insertion from the *u* fragment. Whether the insertion is due to HSV DNA or host DNA is therefore at present unknown. It would appear that the region around the *Xba*I site at 0·7 is not essential for a productive lytic infection in BHK cells and that the synthesis or function of essential polypeptides is not affected by a small deletion or insertion into the genome at this site. No polypeptides or mRNA species are known to map in this area and the deletion lies outside the transcript for IE mRNA 2 (0·74 to 0·75 m.c.) which codes for V_{mw}IE65.

Davison *et al.* (1981), analysing the intertypic recombinant Bx1 (28-1) which is heterotypic for the repeat sequences flanking the long unique region of the genome, found subclones which bore deletions in either TR_L or IR_L and were capable of successive complete rounds of replication; they state that "No equivalent deletions have been observed in over 100 intertypic recombinants with homotypic diploid regions (Preston *et al.*, 1978; Marsden *et al.*, 1978; Chartrand *et al.*, 1979, 1980 . . .), thus the frequency at which deletions occur or are isolated must be lower." There being no known case of deletions in repetitive regions of the genomes of field isolates (Hayward *et al.*, 1975; Skare *et al.*, 1975; Lonsdale, 1979), this was suggestive of the deletions being a consequence of heterotypic repetitive regions. Our results with HG52 refute this suggestion. The fact that we also found a deletion in a subclone of strain 186 of HSV-2 shows that deletions in the repetitive flanking sequences of the long unique region of the genome are not peculiar to strain HG52. Our initial speculation (Brown *et al.*, 1984) that deletions may have arisen as a consequence of the procedures involved in *Xba*I digestion of HSV DNA was firmly ruled out by our experiments with strain HG52 and the finding that the deleted variants found were already present in untreated stock. The reason why such deletions have not been detected previously in field isolates or laboratory strains is probably because the DNA from stocks has been analysed and not the DNA from plaque-purified subclones.

The fact that in HG52 deletions are occurring at a frequency of 24% by passage 8 from the original field isolation suggests that spontaneous deletions are relatively common, at least in strain HG52, and that deletions in the long repetitive flanking sequences do not confer a selective disadvantage for growth in BHK cells. The fact that from a single isolated deleted genome pure stocks of virus can be grown in which the deletions are retained suggests that recombination restoring the deleted sequences is rare. We have no evidence in any of our deleted virus strains of reversion to wild-type DNA structure.

The deletions we have analysed range in size from 1·5 kb (HG52XD192) to 9 kb (HG52XD85/4) and cover the long repeat region. Thus, all the information in one copy of the long repeat can be removed without any marked deleterious effect on the lytic growth of the virus.

Sequence analysis of HSV-1 has shown that there are three groups of tandemly reiterated sequences within the internal long repeat region of the genome, one situated near the U_L/IR_L junction, one in the middle and one within *Bam*HI *s* near the IR_L/IR_S joint (D. J. McGeoch, personal communication). Although it would be unwise to extrapolate from HSV-1 to HSV-2, it could be envisaged that these three banks of sequences are potential sites of instability which may be the starting points of deletions in TR_L/IR_L. The fact that we were unable to detect any deletions in the long repeats of HSV-1 strain 17 may only reflect a frequency difference. Deletions within the long internal repeat of R12-5 HSV-1 sequences were detected (Brown *et al.*, 1984).

The variant HG52XD192 in having lost information from the *Bam*HI *v* fragment in both TR_L and IR_L must have lost at least 150 base pairs of equivalent sequences from both copies of the repeat. When a Southern blot of a *Bam*HI digest of this variant was carried out using pGZ1 as probe, two new end fragments and two new joint fragments could be detected. These new fragments differed in mol. wt. by between $0{\cdot}1 \times 10^6$ and $0{\cdot}2 \times 10^6$ from wild-type, showing that a minimum of 150 base pairs within the *Bam*HI *v* fragment of the long repeat is dispensable for normal lytic growth. However, if the differences in the sizes of the two new ends and the two new joints reflect nothing more than additional *'a'* sequences, then a 1·5 kb sequence within *Bam*HI *v* can be deleted and the viability of HSV retained. Sequence analysis of the *Bam*HI *s* fragment from HSV-1 (equivalent to HSV-2 *Bam*HI *v*) has not identified a gene coding, but it could contain control sequences similar to those upstream of the 5′ end of the V_{mw}IE110 message (D. J. McGeoch, personal communication). Transcripts of unknown function have been mapped to this region (F. J. Rixon, personal communication).

The IE polypeptides synthesized by HG52XD86 confirmed that the deletion in the variant affected sequences for the V_{mw}IE118 gene in that the amount of polypeptide synthesized was reduced compared to wild-type HG52. In contrast, the amount of V_{mw}IE118 made by HG52XD192 was the same as wild-type, confirming that the deletion in this variant, although confined to *Bam*HI *v*, is outside the V_{mw}IE118 coding region. Interestingly, in HG52XD86 the amounts of the other IE polypeptides, V_{mw}182, 138 and 64, were also reduced compared to wild-type virus even though the amounts of actin made were comparable. The variants of R12-5 which were deleted in either one copy of the gene for V_{mw}IE110 or V_{mw}IE118 like HG52XD86 showed alterations in the amounts of the other IE polypeptides synthesized (S. M. Brown *et al.*, unpublished). Cognate evidence is provided by Campbell *et al.* (1984) who find that the polypeptides encoded by the HSV-1 *Eco*RI *b* fragment (0·72 to 0·87 map units) have a general stimulatory role in transcription. We are now investigating IE polypeptide synthesis in the other HSV-2 deletion variants and also quantifying IE transcription. It has recently been shown *in vitro* that V_{mw}IE110 in conjunction with V_{mw}IE175 has an enhancing effect on early polypeptide transcription (Everett, 1984).

The function of the inverted repeat regions in the HSV genome is largely unknown, although Thompson *et al.*, (1984) have recently reported that in the HSV-1/HSV-2 intertypic recombinant RE6, the gene(s) conferring neurovirulence are located between m.c. 0·71 and 0·83. However, it is already clear that RE site mutants and the various TR_L/IR_L deletion mutants furnish us with valuable new tools to study both recombination and genomic functions of HSV *in vivo* and *in vitro*.

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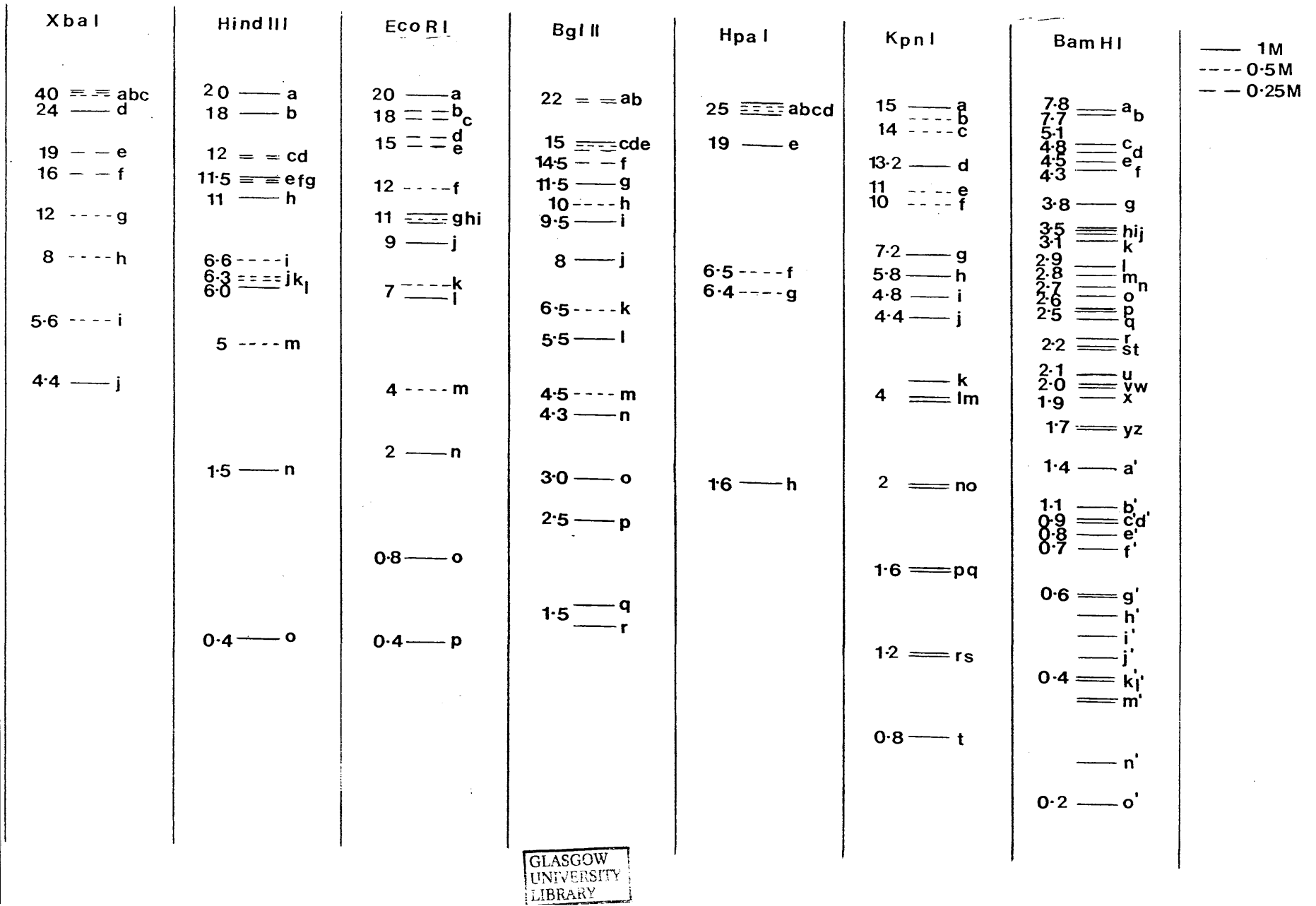
HSV-2



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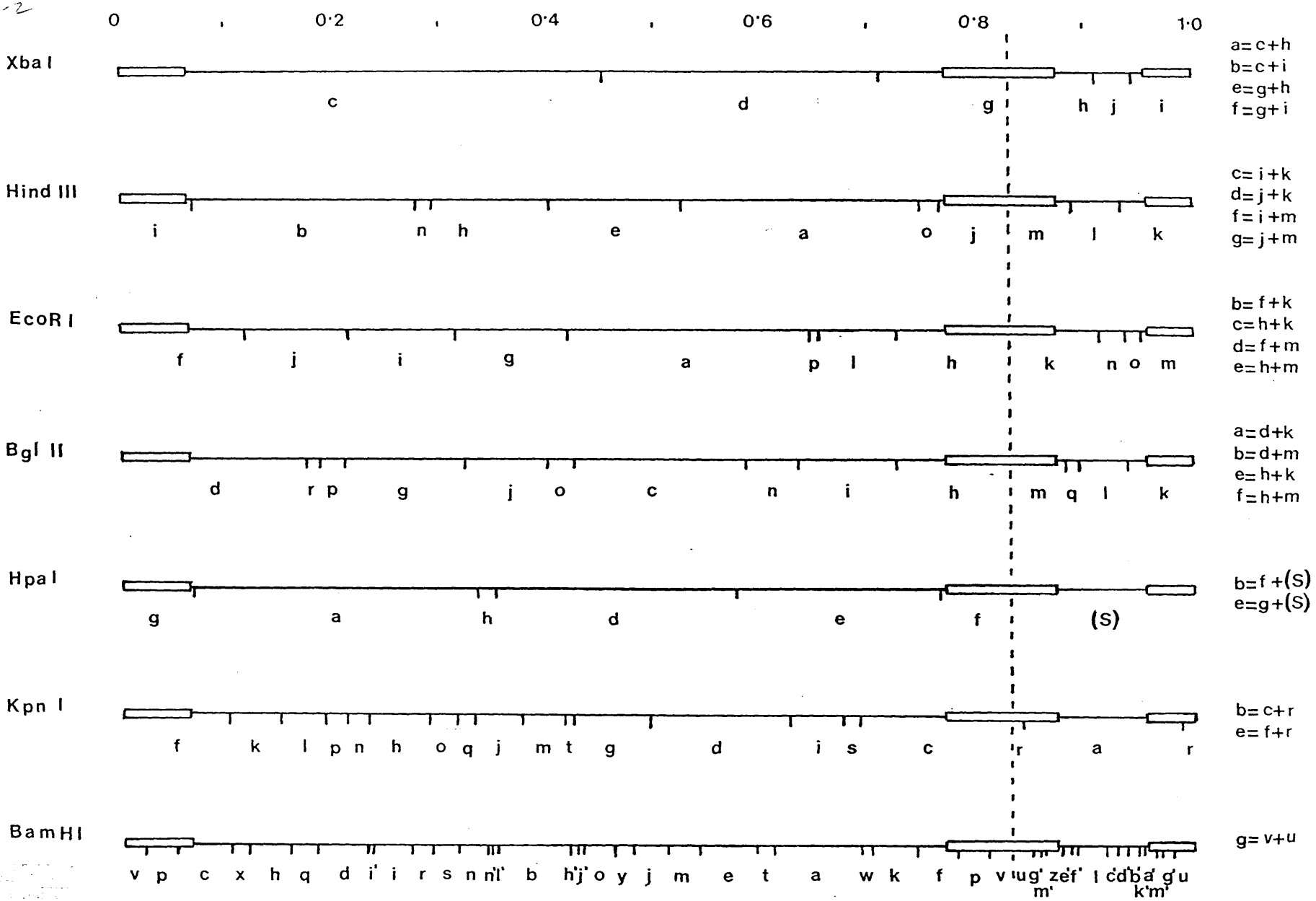

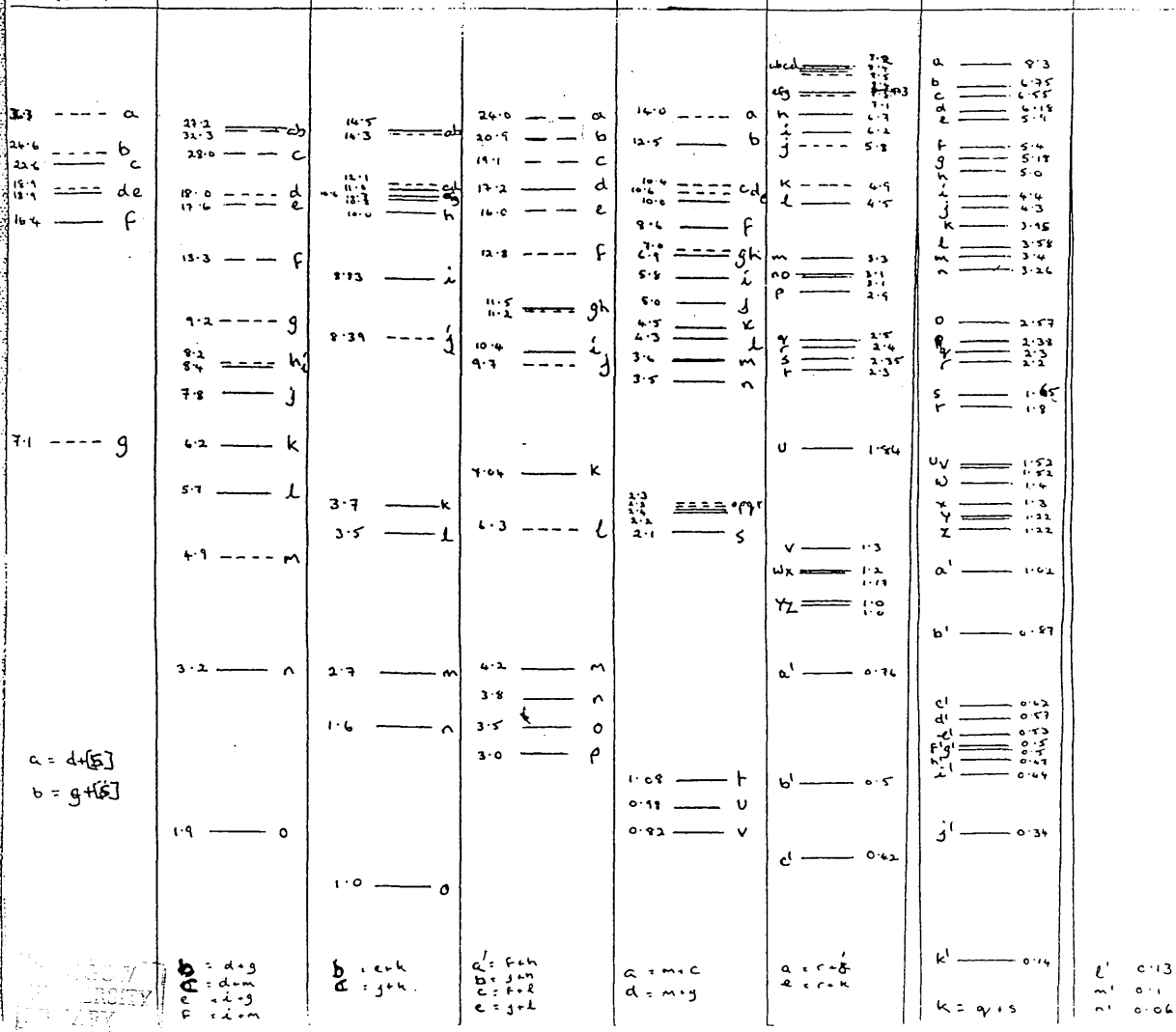
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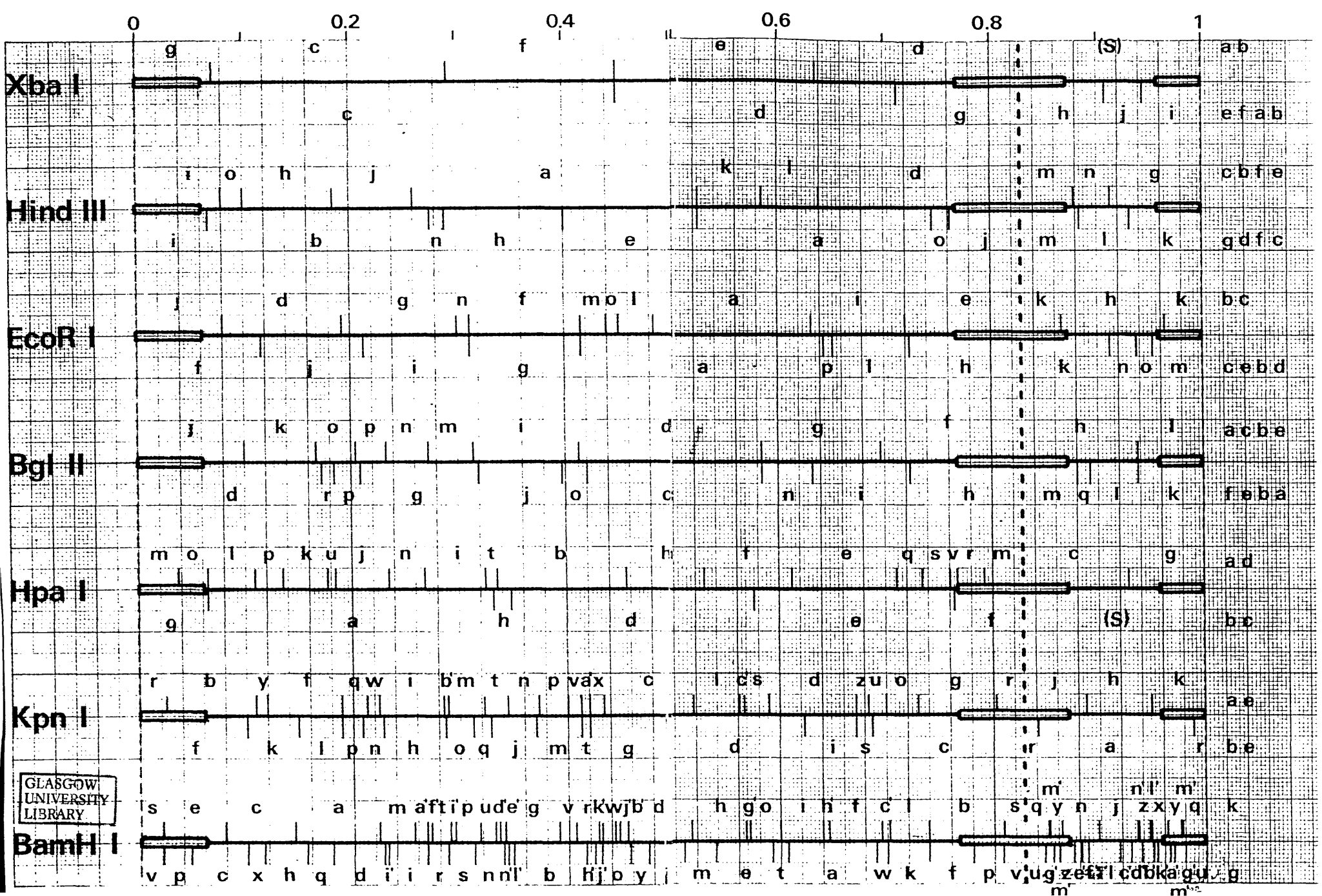
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