Deletion and Duplication Variants around the Long Repeats of Herpes Simplex Virus Type 1 Strain 17

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SUMMARY

Three variants of herpes simplex virus type 1 strain 17 were isolated. One variant had a deletion of 2.5 x 10^6 Mr in IRL/UL and 0.8 x 10^6 Mr in TRL such that sequences were deleted from both long repeats. The deletion in UL removed the 20K and 22K open reading frames. The second variant had a deletion of 3.5 x 10^6 Mr in IRL/UL which again removed the 20K and 22K open reading frames. The third variant had a similar deletion in UL, but in this case the deleted sequences were replaced by sequences from the left end of UL, such that the long repeat was extended by 3 x 10^6 Mr and the overall genome size by 2 x 10^6 Mr. All three variants grew almost normally in vitro. The analysis of 11 isolates with extensive variation in the long repeats outside the 'a' sequence is also reported.

INTRODUCTION

Herpes simplex virus (HSV) is a double-stranded DNA virus, with a genome approximately 100 x 10^6 Mr (150 kb) (Becker et al., 1968) consisting of two unique sequences (U_L and U_S) each bounded by a set of inverted repeats (IR_L/TR_L and IR_S/TR_S) (Sheldrick & Berthelot, 1974; Delius & Clements, 1976; Fig. 1 a). Sequences and hence genes contained within the repeats are diploid. The genes coding for V_mxE110 (IE1) (Clements et al., 1979; Perry et al., 1986) and a 43.5K Mr protein ICP34.5 (Chou & Roizman, 1986) are located in the long repeat. The short repeat contains the entire gene (IE3) coding for V_mxE175 (Clements et al., 1979; McGeoch et al., 1986) and also the 5' untranslated leader and intron of the genes (IE4 and IE5) coding for V_mxE68/12, whose coding sequences are entirely within U_S.

Analysis of single plaque isolates has led to the isolation of HSV variants exhibiting genome rearrangements. This allows the identification of (i) genes whose products are non-essential in tissue culture, (ii) those regions of the genome exhibiting instability and in the long term those sequences involved in rearrangements and (iii) the nature of viable genome structures, especially in relation to the size and necessity of the repeats. Such studies have shown that variants containing only one copy of R_L or R_S are viable in tissue culture (although their ability to grow in vitro has not been explored); thus one copy of the genes contained within the repeats suffices for growth in vitro. In some of the deletion mutants adjacent unique sequences and their encoded protein products have been deleted with no effect on viral viability in vitro (Davison et al., 1981; Brown et al., 1984; Harland & Brown, 1985; Longnecker & Roizman, 1986; Umene, 1986; Brown & Harland, 1987; MacLean & Brown, 1987).

Umene (1986) and Brown & Harland (1987) have reported deletions of sequences at the right end of U_S, accompanied by replacement with sequences from the left end of U_S. This leads to extended short repeats and, because the inserted sequences are larger than the deleted ones, larger genomes. Brown & Harland (1987), from the same transfection, also isolated a genome with a similar deletion but without replacement of the deleted sequences. They speculated that this variant might be involved in the formation of the two variants with the extended short repeats which they described, the alterations in repeat and unique length being concordant with the postulate of evolutionary contraction/expansion of the repeats involving both homologous
and illegitimate recombination (McGeoch, 1984; Whitton & Clements, 1984; Davison & McGeoch, 1986).

Most of the deletions in HSV reported to date involve one of the repeats and the adjacent unique sequences, suggesting that the repeats may act as hotspots for illegitimate recombination. Different tandem short repeats are known to be present throughout the genome, especially in R₁ (Rixon et al., 1984), and have been proposed to favour unequal recombination. Banks of tandem repeats are believed to be responsible for the variation in size of certain restriction fragments, which is seen between strains and between single plaque isolates within a strain (Lonsdale et al., 1980). Variation is also found in the size of the terminal and joint fragments which contain the ‘a’ sequence but this is due to variability in the number of ‘a’ sequences present (Davison & Wilkie, 1981).

In the wild-type stock of HSV-2 strain HG52 24% of plaque isolates were found to have various deletions in R₁ (Harland & Brown, 1985), whereas in HSV-1 strain 17 only one deletion variant has been isolated from over 5000 plaques (MacLean & Brown, 1987). In this paper we report the isolation of three deletion variants of HSV-1 strain 17, identified through analysis of 80 single plaques generated in one recombination experiment. These may be closely related. From the same experiment we also isolated 11 variants that showed more variation in the size of HpaI r and/or o (Fig. 3) than has been previously identified (Lonsdale et al., 1981). These HpaI fragments span the long repeats but exclude the ‘a’ sequences. These 14 variants allow us to elucidate further the role of the long repeat of HSV-1 and the results have implications for the evolution of HSV.

**METHODS**

**Cells.** Baby hamster kidney clone 13 cells (BHK C13) (Macpherson & Stoker, 1962) were propagated in Eagle’s medium containing twice the normal concentration of vitamins and amino acids, 5% (v/v) tryptose phosphate broth and 10% (v/v) calf serum (ETC10).

**Viruses.** Virus stocks were grown and titrated in BHK C13 cells as described previously (Brown et al., 1973). The parental strain was HSV-1 Glasgow strain 17 (Brown et al., 1973) which contains four XbaI sites at 0.07, 0.29, 0.45
and 0.63 map units (Wilkie, 1976; Fig. 2a). Isolation and characterization of the mutant 1707 (previously known as KB11/45) has been described (Cook & Brown, 1987). It lacks two of the parental XbaI sites at 0.07 and 0.63 map units but contains an extra XbaI site at 0.74 map units (Fig. 2a).

Restriction enzyme analysis of virus genomes. Restriction enzyme analysis of single plaque isolates was carried out by a modification of the technique of Lonsdale (1979). Five \( \times 10^5 \) BHK C13 cells were infected at an m.o.i. of 10 p.f.u./cell in the presence of \( ^{32} \)P, in phosphate-free Eagle's medium containing 1% (v/v) calf serum and incubated at 31 °C for 48 h. Viral DNA was extracted with SDS and phenol and ethanol precipitation. The DNA was treated with various restriction enzymes using the manufacturer's recommended conditions. Digests were analysed by electrophoresis on agarose gels of appropriate concentrations (0.5 to 0.8%) in TBE buffer (89 mM-Tris-borate, 89 mM-boric acid, 2 mM-EDTA). Gels were air-dried and exposed to Kodak X-ray film. When the restriction enzyme profiles indicated a different pattern from the wild-type, three rounds of plaque purification were carried out prior to further restriction enzyme analysis.

Recombinant DNA plasmids. HSV fragments used as hybridization probes were all cloned into pAT153. The plasmids used were pMC9 the insert in which extends from the KpnI site to the HpaI site (map positions 0.729 to 0.762), pMC16 containing HpaI r (map positions 0.762 to 0.791), plasmid BamHI c (map positions 0.079 to 0.164) and plasmid BamHI b (map positions 0.74 to 0.808) (Fig. 1, 2 and 7). These were kindly supplied by Dr C. M. Preston and Dr A. J. Davison.

Preparation of plasmid DNA. Plasmid DNA was obtained by the method of Birnboim & Doly (1979) and further purified by banding in caesium chloride-ethidium bromide gradients (Maniatis et al., 1982).

Preparation of infected cell DNA. BHK C13 cell monolayers (4 \( \times 10^6 \)) were infected at an m.o.i. of 5 p.f.u./cell at 31 °C. After 48 h the supernatant was removed and the cells were incubated with 2 ml lysis buffer (0.6% SDS, 10 mM-EDTA, 10 mM-Tris-Cl pH 7.5, 500 μg/ml pronase) for 4 h at 37 °C. Sodium chloride was then added to a final concentration of 200 mM and infected cell DNA was isolated by phenol extraction and ethanol precipitation. Restriction enzyme digestions were carried out on 5% of the total sample using the manufacturer's recommended conditions (Stow & McMonagle, 1983).

Southern blotting. Restriction enzyme-digested DNA was electrophoresed on agarose gels (0.8 to 1.2%) in TBE buffer containing ethidium bromide (0.5 μg/ml) and then transferred to nitrocellulose sheets (BA85; Schleicher & Schüll) by the method of Southern (1975). These were prehybridized at 75 °C in 6 × SSC (1 × SSC is 15 mM-sodium citrate and 150 mM NaCl), 5 × Denhardt's buffer (1 × DB is 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone and 0.02% Ficoll), 0.1 mg/ml salmon sperm DNA and 10 mM-Tris–HCl pH 7.5, and then hybridized to nick-translated DNA at 75 °C (Rigby et al., 1977). Following extensive washing at 60 °C in 2 × SSC, 0.1% SDS and 5 mM-Na2HPO4, pH 7, the sheets were air-dried and exposed for autoradiography.

Virus growth properties. One-step growth experiments were carried out as described by Brown & Harland (1987). Confluent BHK C13 monolayers (2 \( \times 10^6 \) cells) were infected at an m.o.i. of 5 p.f.u./cell and incubated at 37 °C; samples were harvested at 0, 2, 4, 6, 10, 12 and 24 h post-infection and titrated as normal. Longer term growth experiments involved infecting cells at 0.001 p.f.u./cell, harvesting samples at 0, 2, 4, 12, 24, 48 and 72 h and titrating as before.

RESULTS

Isolation of the variant 1708 containing five XbaI sites

In order to generate a variant containing five XbaI sites, recombination experiments were carried out using the wild-type strain 17 (with four XbaI sites at 0.07, 0.29, 0.45 and 0.63 map units; Fig. 2a) and the mutant 1707 (with three XbaI sites at 0.29, 0.45 and 0.74 map units; Fig. 2a). BHK C13 cells were infected at an m.o.i. of 5 p.f.u./cell with each virus. After 24 h at 37 °C, virus was harvested from the infected cells, titrated, single plaques were picked and stocks were prepared from 50 mm Petri dishes. DNA was labelled \( \text{in vivo} \) with \( ^{32} \)P and preparations were subjected to restriction enzyme analysis by XbaI to identify recombinant genomes. A number of recombinants were isolated some of which gave a pattern indicating the presence of five XbaI sites at 0.07, 0.29, 0.45, 0.63 and 0.74 map units. One of these, 1708, is regarded as the prototype. Fig. 2(a) indicates where a single crossover generating this genome structure would have occurred. Fig. 2(b) shows the pattern following XbaI digestion. The d band was cleaved into two bands, d1 and d2; d1 was 1 M of about 9 × 10^6 M, and d2 was 0.5 M of about 7 × 10^6 M. The a joint fragment (d + [S]), was reduced by about 9 × 10^6 M and comigrated with b and c.

A HpaI–XbaI digestion confirmed the presence of the extra XbaI site in 1708 compared to 17 and showed that this site occurred in HpaI s (data not shown). A BglII–XbaI digestion also confirmed the presence of the extra XbaI site in 1708 and showed it to be in BglII j (Fig. 3a, b). The four XbaI sites in strain 17 were contained within BglII j, m, d and g respectively (Fig. 3a;
Fig. 2. (a) XbaI maps for the DNA of HSV-1 strains 17 (above) and 1707 (Cook & Brown, 1987) (below). The region marked 1 between the 0.63 site in 17 and 0.74 site in 1707 is where recombination must occur to give 1708 with five XbaI sites. (b) Autoradiograph of XbaI restriction digests of HSV-1 DNA labelled with $^{32}$P in vivo (0.5% agarose). Lane 1, 17; lane 2, 1708 with $d_1$ and $d_2$ referring to the products of cleavage of $d$ at the XbaI site at 0.74 map units. (c) Autoradiograph of XbaI restriction digestion products of HSV-1 DNA labelled with $^{32}$P in vivo (0.5% agarose). Lane 1, 17; lane 2, 1704; lane 3, 1706; lane 4, 1705.

3b, lane 2). Thus BgIII j ($9.8 \times 10^6$ Mr) was cleaved by XbaI to two $j'$ bands of around $7 \times 10^6$ Mr and $2.8 \times 10^6$ Mr, which comigrated with BgIII k and below BgIII p respectively, while the $j'$-containing junction fragments $b$ and $e$ comigrated with BgIII c and $f$ respectively; BgIII m ($4 \times 10^6$ Mr) was cleaved by XbaI to two $m'$ bands of around $2.1 \times 10^6$ Mr and $1.9 \times 10^6$ Mr, migrating below $p$; BgIII d ($17 \times 10^6$ Mr) was cleaved by XbaI to two $d'$ bands of around $13 \times 10^6$ Mr and $4 \times 10^6$ Mr, which comigrated with $f$ and $n$ respectively; and BgIII g ($11 \times 10^6$ Mr) was cleaved by XbaI to two $g'$ bands of around $6 \times 10^6$ Mr and $5 \times 10^6$ Mr, migrating with and below BgIII l respectively.

A BgIII--XbaI digestion of 1707 (Fig. 3a; Fig. 3b, lane 3) resulted in cleavage of BgIII m and $d$ and also of BgIII f due to the presence of the 0.74 XbaI site. BgIII f ($13 \times 10^6$ Mr) was cleaved by XbaI to two $f'$ bands of around $8.5 \times 10^6$ Mr and $4.5 \times 10^6$ Mr, migrating above k and m respectively; the $f'$-containing L-S junction fragments $a$ and $c$ had also undergone deletion and migrated between $b$ and $c$ and above $d$ respectively. A BgIII--XbaI digestion of 1708 (Fig. 3a; Fig. 3b, lane 4) resulted in cleavage of BgIII j, m, $d$, $g$ and $f$ indicating the presence of all five XbaI sites.

**Isolation of several variants in TRL/IRL from one recombination experiment**

Eighty plaques were analysed from one recombination experiment carried out to isolate a virus with five XbaI sites. None appeared to be a recombinant, but three demonstrated large deletions on XbaI digestion; these variants were designated 1704, 1705 and 1706. Digestion of the DNA of each of the 80 isolates with HpaI in addition indicated a further 11 whose HpaI o and r fragments varied by up to $0.4 \times 10^6$ Mr from the standard size (Fig. 4 and 5). The migration
HSV deletion mutants

Fig. 3. (a) BglII map of HSV-1 strains 17, 1707 and 1708 (identical) above the line, showing the position of the XbaI sites (X) in each genome below the line, thus indicating which BglII bands are cleaved by XbaI. (b) Autoradiograph of HSV-1 DNA labelled in vivo with $^{32}$P (0.5% agarose). Lane 1, 17 cleaved with BglII; lane 2, 17 cleaved with BglII-XbaI; lane 3, 1707 cleaved with BglII-XbaI; lane 4, 1708 cleaved with BglII-XbaI. The nomenclature of the BglII bands is given in lane 1. In lanes 2, 3 and 4, the products of XbaI cleavage of the BglII bands are marked by the letter of the fragment from which they are derived plus a prime symbol (').

Fig. 4. BglII, HpaI and BamHI maps for the DNA of HSV-1 strain 17.

differences in HpaI o and r represented respectively variation in regions of TR$_L$ and IR$_L$ distal from the ‘a’ sequence; this variation was considerably more extensive than normally observed. Variation in HpaI o and r occurred independently. Southern blotting with HpaI r as a probe confirmed that the variable fragments were indeed HpaI o and r and not HpaI p and q which comigrated with o and r respectively in the case of wild-type virus (data not shown).

Analysis of variant 1704

One variant (1704) exhibited changes indicating a $0.8 \times 10^6$ $M_r$ deletion in TR$_L$ and a $2.5 \times 10^6$ $M_r$ deletion in IR$_L$/UL. On XbaI digestion (Fig. 2c, lane 2), the g band showed a
Fig. 5. Autoradiograph of HpaI restriction digests of HSV-1 DNA labelled in vivo with $^{32}$P (0.8% agarose). Lanes 1 to 11 represent different plaque isolates exhibiting variation in HpaI o and/or r; lane 12 represents strain 17.

0.8 x 10^6 Mr, deletion, and the d band a 2.5 x 10^6 Mr, deletion (causing it to comigrate with the e band). The g- and d-containing L-S junction fragments b and a respectively were also reduced in size. [Note that the g band is usually seen as a doublet; this is considered to be due to variation in the number of ‘a’ terminal sequences (Davison & Wilkie, 1981).]

Digestion with BgIII (Fig. 4; Fig. 6a, lane 2) revealed a 0.8 x 10^6 Mr, deletion in j and a 2.5 x 10^6 Mr, deletion in f causing this to comigrate with i. The j-containing L-S junction fragments b and e were also deleted as were the f-containing joints a and c, with the latter comigrating with d.

Digestion with BamHI (Fig. 4; Fig. 6b, lane 2) showed that the e band was reduced by 0.8 x 10^6 Mr, and comigrated with f, while the b band was reduced by 2.5 x 10^6 Mr, and comigrated with j. Variation in the mobility of the terminal s and joint k fragments was due to variation in the number of ‘a’ sequences. The x band situated within the short repeat also exhibited variation between isolates (Davison & Wilkie, 1981). Within the stock of 1704 the x band existed as two size classes.

Digestion with HpaI (Fig. 4; Fig. 6c, lane 2) revealed that o was reduced by approximately 0.8 x 10^6 Mr, and migrated below s, s was unaltered, v and r were absent and m was unaltered. These findings were confirmed by Southern blotting of HpaI-digested DNA with one probe containing $\text{HpaI } r$ and another containing $\text{HpaI } v$ and s. A 0.4 x 10^6 Mr, band was identified that hybridized to both probes, indicating it to be the fused remnants of $\text{HpaI } v$ and r (data not shown). Probing of a Southern blot of BamHI–HpaI–SmaI-digested DNA with pMC9 (Fig. 1; Fig. 7, lane 2) confirmed that the 1240 bp $\text{HpaI } v$ fragment was absent, but all the Sinai fragments of $\text{HpaI } s$ were present. A novel band of around 700 bp corresponded to the fused remnants of $\text{HpaI } v$ and r (as discussed above).

Overall this variant had a deletion of 0.8 x 10^6 Mr, in TR_L and a 2.5 x 10^6 Mr, deletion in IR_L/UL. This is illustrated in Fig. 1(a).
HSV deletion mutants

Analysis of variant 1705

Analysis of this variant indicated that it had lost approx. $3.5 \times 10^6 \text{ M}_r$ from IR_{UL}. On XbaI digestion, the d band was smaller by greater than $3 \times 10^6 \text{ M}_r$, causing it to comigrate with the f band. The d-containing L–S junction fragment a was also reduced in size (Fig. 2c, lane 4). On BglII digestion, the f band had a $3.5 \times 10^6 \text{ M}_r$ deletion causing it to comigrate with j. The f-containing L–S junction fragments a and c were also reduced in size, now comigrating with b and d respectively (Fig. 4; Fig. 6a, lane 4). On BamHI digestion, the b band was found in a form that comigrated with l, indicating a deletion of $3-5 \times 10^6 \text{ M}_r$ (Fig. 4; Fig. 6b, lane 4). Variation in the mobility of k, s and x was seen, with x again existing as two size classes. On HpaI digestion, s, v and r were absent, m was unaltered, but a novel band of around $1.8 \times 10^6 \text{ M}_r$ running below s was present (Fig. 4; Fig. 6c, lane 4). Southern blotting of HpaI-digested DNA with probes containing HpaI s and another containing HpaI v and s confirmed these findings and indicated that the novel band represented a fusion of remnants of HpaI s and r.

The endpoint of the deletion in HpaI s was mapped by Southern blotting of a BamHI–HpaI–SmaI digest with pMC9 (Fig. 7). The locations of the fragments are illustrated in Fig. 1. The 1240 bp HpaI v fragment was absent, as was the 297 bp fragment, but the 344 bp fragment was present. A novel band of approximately 650 to 700 bp was detected; this corresponded to the fused remnants of the 297 SmaI fragment and HpaI r. Therefore, the deletion extended from within HpaI r into the 297 bp fragment and terminated around the 3' end of IE gene 2.

Overall this variant had a deletion of $3.5 \times 10^6 \text{ M}_r$ in IR_{UL}. This is illustrated in Fig. 1.
Analysis of variant 1706

This variant had a $1 \times 10^6$ Mr deletion in the right end of U$_l$, adjacent to IR$_l$, which had been replaced by $3 \times 10^6$ Mr, from the opposite end of U$_l$ such that the repeated region was extended by $3 \times 10^6$ Mr, and consequently the overall genome size was increased by $2 \times 10^6$ Mr.

On XbaI digestion (Fig. 2c, lane 3) the g band, which is usually 0.5 M, became 1 M. The 0.5 M d band was absent and was replaced by a novel 1 M band of $14 \times 10^6$ Mr, migrating below f. The d-containing L–S junction fragment a was absent and the g-containing L–S junction fragment b was increased in intensity. This suggested that there was a deletion in the right end of U$_l$ while the left end of U$_l$ was now repeated at the right end. As a result there were two copies of g and also of its joint fragment b, both now being 1 M. The d band was absent and had been replaced by a $14 \times 10^6$ Mr, 1 M band consisting of the d remnant fused with the duplicated c sequences.

On BglI digestion (Fig. 4; Fig. 6a, lane 3), the j band remained 0.5 M indicating that the inserted sequences did not extend as far as the j/k site. The f band was increased in size by $2.3 \times 10^6$ Mr, and migrated just below e. The f-containing L–S junction fragments a and c were also increased in size, c comigrating with b.

On BamHI digestion (Fig. 4; Fig. 6b, lane 3), the b band was absent and the e band was 2 M. A novel band of $2.7 \times 10^6$ Mr, migrating above o was observed. That this was a fusion between part of BamHI c and part of BamHI b was confirmed by blotting with BamHI c and b which both hybridized to this band (Fig. 1, 8 and 9). The k and s bands again exhibited altered mobility. On HpaI digestion (Fig. 4; Fig. 6c, lane 3), the o band was 2 M, the s, r and v bands were absent and a novel band of $4.7 \times 10^6$ Mr, above k was present. This was confirmed to be a fusion between the s and l bands by its ability to hybridize to BamHI b and c (Fig. 1, 8 and 9).

A Southern blot of a BamHI–HpaI–SmaI digest with pMC9 (Fig. 1; Fig. 7, lane 4) enabled mapping of the left end of the deletion in HpaI s. Again the 1240 bp HpaI v fragment was absent, as was the 297 fragment, but the 344 fragment was present. A novel fragment of around 400 bp was present and this corresponded to the remnant of the 297 bp fragment fused to the SmaI fragment at the right end of the deletion in BamHI c. As in the case of variant 1705, the deletion
Fig. 9. Autoradiograph of a Southern blot in which nick-translated BamHI b and c (Fig. 1a and 8) were hybridized to (a) BamHI-digested 17 and 1706 DNAs: lane 1, 1706 probed with BamHI b; lane 2, 17 probed with BamHI b; lane 3, 1706 probed with BamHI c; lane 4, 17 probed with BamHI c. (b) HpaI-digested 17 and 1706 DNAs: lane 1, 17 probed with BamHI b; lane 2, 1706 probed with BamHI b; lane 3, 1706 probed with BamHI c; lane 4, 17 probed with BamHI c. The novel BamHI and HpaI bands which hybridize to both BamHI b and c are marked ▶ (Fig. 8).

extended close to the 3' end of IE gene 2. A model showing where recombination would have occurred to give 1706 is shown in Fig. 8. The repeats are extended by $3 \times 10^6$ Mr and consequently the genome was larger by $2 \times 10^6$ Mr. The restriction sites and fragments defining the long repeats in 1706 are also illustrated.

Virus growth properties

One-step growth experiments were carried out over a 24 h period in BHK C13 cells (Fig. 10a). 1708 showed the wild-type growth characteristics of strain 17. All three deletion variants, 1704, 1705 and 1706, although growing at a slower rate produced a similar 24 h yield. Similar results were obtained in growth experiments carried out at low m.o.i. and allowing multiple rounds of replication (Fig. 10b). High titre stocks (> $10^9$ p.f.u./ml) were obtained from the variants indicating that the growth impairment was overcome in time. All the variants gave similar titres at 38.5 °C and 31 °C indicating that they were not temperature-sensitive (data not shown).

DISCUSSION

The analysis of single plaque isolates allows the elucidation of genomic rearrangements within a virus population; this is not normally possible in studies of pooled virus populations. Harland & Brown (1985) have shown that the frequency of variation between genomes in stocks of different strains of HSV-1 and HSV-2 differs considerably. At one extreme of variation 24% of wild-type plaque isolates of an HSV-2 strain HG52 stock showed deletions in R1. At the other extreme MacLean & Brown (1987) found only one deletion variant among 5000 plaques of HSV-1 strain 17. Most of these deletions have occurred within and/or adjacent to the repeated regions.
of the genome, and may be due to illegitimate recombination events possibly involving the banks of tandem repeats which are found in these regions of the HSV genome.

Although some of the deletion variants pre-existed in our wild-type stocks, most have been isolated from ‘manipulated’ virus populations. Manipulations such as transfection and restriction enzyme treatment (Brown et al., 1984; Harland & Brown, 1985; Brown & Harland, 1987; MacLean & Brown, 1987), genomic disruption (Longnecker & Roizman, 1986), or the use of high m.o.i. in recombination experiments (Umene, 1986), may serve to increase the instability of the genomes, leading to an increased rate of generation of rearrangements in naturally unstable regions of the genome.

During the course of a recombination experiment, 80 single plaque isolates were analysed by restriction enzyme analysis. Out of these, 11 showed extensive variation (up to several hundred base pairs) in the long repeats of the genome (not including the ‘a’ sequence). The variation is much more extensive than has been previously identified in this region (Lonsdale et al., 1980 and unpublished observations); it could be due to variation in the copy number of the tandemly reiterated short sequences found in $R_L$ (Rixon et al., 1984; Perry, 1986). Much less variation in their copy number is found between different plasmid clones (Davison & Wilkie, 1981; Rixon & McGeoch, 1984; F. J. Rixon, personal communication).

The variation in $T_R_L$ and $I_R_L$ occurred independently and thus these variants have $T_R_L$ which differ in size from $I_R_L$, a finding supporting that previously made by Lonsdale et al. (1980). A further three isolates had extensive rearrangements in $I_R_L/T_R_L$. These three deletion variants were designated 1704, 1705, 1706. Variant 1704 had a $0.8 \times 10^6 M_r$ deletion in $T_R_L$ and a $2.5 \times 10^6 M_r$ deletion in $I_R_L/U_R_L$, extending up to $6 \times 10^6 M_r$ into $U_L$. No coding sequences are known to be removed from the long repeat, as the deletions start downstream of the 3' end of IE1. The $I_R_L/U_R_L$ deletion removes two open reading frames for putative polypeptides of 20K and 22K (Perry, 1986). Because of the sizes of the deleted fragments, there must be an overlap

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Fig. 10. (a) One-step growth curves of HSV-1 strains 17 (○), 1704 (●), 1705 (△), 1706 (■) and 1708 (□) in BHK C13 cells. Cells were infected at an m.o.i. of 5 p.f.u./cell. After adsorption for 60 min at 37 °C, the monolayers were washed twice in phosphate-buffered saline, overlaid with ETC10 and incubated at 37 °C (see Methods). Cultures were harvested at the times indicated and virus titres were measured by plaque assay on BHK C13 cells. (b) Long-term growth curves of 17 (○), 1704 (●), 1705 (△), 1706 (■) and 1708 (□) in BHK C13 cells. The method was as above except the infecting m.o.i. was 0.001 p.f.u./cell and the interval times differed (see Methods).
between the TRL and IRL deletions with $0.2 \times 10^6 M_r$ to $0.8 \times 10^6 M_r$ of sequence missing from both long repeats. As far as we know this is the first time that part of the long repeat outside that coding for $V_{mw}IE110$ (Stow & Stow, 1986; Sacks & Schaffer, 1987) has been found to be totally lost. Variant 1704 synthesized normal amounts of $V_{mw}IE63$ (data not shown). The IE2 gene is located upstream of the deletion, and thus our previous suggestion that the 20K and 22K polypeptides may have a role to play in the expression of $V_{mw}IE63$ (MacLean & Brown, 1987) is discounted.

Variant 1705 has a deletion of $3.5 \times 10^6 M_r$ in IRL/UL. The deletion again terminates downstream of IE1 and removes the 20K and 22K open reading frames. It extends just downstream of the 3' end of IE2 but leads to decreased synthesis of $V_{mw}IE63$ (data not shown).

Variant 1706 is considerably more complex. 1706 appears to have a deletion in UL similar to that of 1705, but the deleted sequences are replaced by sequences from the left end of UL, thus leading to an extended long repeat. Approximately $1.0 \times 10^6 M_r$ has been deleted from the right end of UL, and has been replaced by $3.0 \times 10^6 M_r$ from the left end of UL. Thus the long repeat has been extended by $3 \times 10^6 M_r$ and the genome by $2 \times 10^6 M_r$. IE gene 1 is unaffected, the 20K and 22K polypeptides have been deleted as have sequences up to the 3' end of IE2. $V_{mw}IE63$ synthesis is again decreased (data not shown). The genes in the left $3 \times 10^6 M_r$ terminal portion of UL are now present in diploid amounts and their expression may be increased. No known polypeptides are encoded in this part of the genome, but several open reading frames have been identified (Perry, 1986).

How did the extended repeat arise and are any of these variants likely to be related? All three variants arose in the same experiment and considering that only one strain 17 deletion variant has been previously isolated, it seems likely that 1704, 1705 and 1706 may have a common origin. We speculate that the right end of the IRL deletions in 1704 and 1705 are identical and that they arose from the same progenitor molecule, with the deletion extending further to the left in 1705. The deletion in TRL in 1704 could have arisen in the process of recombination between a wild-type and a deleted repeat.

The model which has been previously proposed for the expansion or contraction of the repeats (McGeoch, 1984; Whitton & Clements, 1984; Davison & McGeoch, 1986) could explain the origin of 1706. Recombination between two wild-type viruses with UL in opposite orientation would occur homologously in the repeat and illegitimately possibly through short similar sequences in the unique portion of the genome, to give an extended repeat arising from one end of UL with the other end becoming deleted (Fig. 8). If the endpoint of the deletion in 1705 and 1706 is the same then recombination between IRUL of 1705 and TRUL of wild-type virus in the opposite orientation would also result in 1706. The other potential product of this recombination, in which the left end of UL is deleted and the right end duplicated, has not been isolated. This is the first reported occurrence of an extended long repeat in HSV although Umene (1986) and Brown & Harland (1987) have postulated a similar mechanism in the derivation of a genome with an extended short repeat.

The occurrence of the three deletion variants, as well as genomes with extremely variable repeat lengths, in a virus which was not known previously to be prone to rearrangements raises the question of why these arose. They may have been a result of the high m.o.i. in the recombination experiment. However, previous and subsequent recombination experiments have not yielded any variants. It is possible that the cells used affected the outcome as it is known that HSV recombination frequencies vary on different batches of BHK C13 cells (Taylor, 1976). If the particular cell batch used was highly recombinogenic, this might have led to high frequencies of HSV recombination, both homologous and illegitimate. The deletion variants were isolated from a cross designed to generate a recombinant with five $XbaI$ sites, but among the 80 plaques tested this recombinant was not isolated; this would appear to refute the postulate that the cells were highly recombinogenic.

A more favoured explanation is that one variant genome occurred which because of instability went through several rounds of rearrangement prior to the formation of stable genomes; thus one initial event could potentially lead to the isolation of several variants in a population. The relationship between these variants and the sequences involved in such
rearrangements could be determined by DNA sequence analysis and comparison to the wild-type sequence.

The growth properties of all three variants as assayed by one-step and multistep growth experiments are impaired compared to strain 17. Given the ability to raise high titre stocks (10^9 p.f.u./cell), the impairment appears to affect only the rate of maturation, confirming that two full copies of R^2 and the right end of U^1 are non-essential in vitro (Brown et al., 1984; Harland & Brown, 1985; MacLean & Brown, 1987). The growth properties of the variants in vivo have not yet been explored. Study of the reduced synthesis of V_{mIE63} in variants 1705 and 1706 will be reported in a future paper.

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REFERENCES


BROWN, S. M. & HARLAND, J. (1987). Three mutants of herpes simplex virus type 2: one lacking the genes US10, US11 and US12 and two in which R^2 has been extended by 6 kb to 0.91 map units with loss of U^1 sequences between 0.94 and the U^1 TR^2 junction. *Journal of General Virology* 68, 1–18.


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RIXON, F. J. & MCGEOCH, D. J. (1984). A 3' co-terminal family of mRNAs from the herpes simplex virus type 1 short region: two overlapping reading frames encode unrelated polypeptides one of which has a highly reiterated amino acid sequence. Nucleic Acids Research 12, 2473–2487.


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