Isolation of Restriction Endonuclease Site Deletion Mutants of Herpes Simplex Virus

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(Accepted 15 February 1984)

SUMMARY

We provide evidence that (i) variants lacking individual herpes simplex virus type 1 (HSV-1) XbaI sites can be selected following extensive XbaI treatment of the viral DNA and can be recombined to produce HSV-1 variants lacking two of the four sites normally found, (ii) all XbaI sites can be removed from a viable intertypic recombinant HSV genome, (iii) following XbaI treatment, different mutants with deletions (0.15 to 8.8 kb) in the long repeat (TR_L or IRL) and long unique regions can be readily isolated, as well as mutants with novel XbaI sites, (iv) several mutants with deletions in one of the repeats (TR_L or IRL) have a measurable growth disadvantage in tissue culture.

INTRODUCTION

Conditional lethal mutants of herpes simplex virus (HSV) types 1 and 2 have been invaluable in the analysis of the viral genome. In general these have been temperature-sensitive mutants arising from a single base pair change in the nucleotide sequence of the viral DNA. Deletion or insertion mutants that have larger alterations in the DNA structure are also potentially useful in the biochemical and genetic analysis of the genome. Deletion mutants in the thymidine kinase gene of HSV-1 have already been isolated and analysed (Sanders et al., 1982). Here we report the isolation of restriction endonuclease site deletion mutants of HSV-1 and an intertypic recombinant R12-5 (Chartrand et al., 1981).

Digestion of viral DNA with an excess of the restriction endonuclease XbaI has made it possible to enrich for variants which have either arisen spontaneously within a population of molecules and which lack particular restriction enzyme sites, or which have been induced by the restriction enzyme producing at a low frequency site-specific changes instead of cleaving the double-stranded DNA. The method of enrichment depends on the viable virus genomes remaining after XbaI digestion containing a higher proportion of DNA molecules with fewer restriction endonuclease sites than in the parental population. The survival probability of these molecules is expected to be increased by having fewer than the standard number of sites for the particular endonuclease. By transfecting the digested DNA onto monolayers of C13 cells and screening any resulting plaques, mutants of HSV-1 lacking different XbaI sites have been isolated. In addition, a virus without any XbaI sites has been generated from the parental intertypic recombinant (R12-5) which possesses only one (HSV-2) XbaI site. This method of isolating variants lacking restriction enzyme sites is a modification of the method of isolating deletion and substitution mutants of adenovirus type 5 described by Jones & Shenk (1978), who digested the DNA with EcoRI, ligated the resulting fragments and subsequently isolated a series of deletion mutants. However, in our hands, the ligation of HSV fragments after XbaI digestion did not enhance the isolation frequency of deletion mutants.

One reason for isolating restriction enzyme site deletion mutants is to facilitate the study of recombination, which is still unclear in HSV. We showed previously (Ritchie et al., 1977) that recombination in HSV-1 increases with time, which implies that parental and progeny molecules are involved. It has been inferred from density labelling studies that in pseudorabies virus only parental genomes take part in recombination (Ben-Porat et al., 1982). If there is a
progression with time to genetic equilibrium in HSV recombination, then it follows that there will be a cumulative rearrangement of non-selected markers with a selected set of recombinants. Analysis of supernumerary crossovers, e.g. restriction endonuclease site exchanges, should determine whether this actually occurs. The problem can of course be approached by intertypic recombination, but by analysing intratypic recombinants potential limitations to DNA exchange imposed by non-homology are overcome. In addition, such variants will be useful for superinfection experiments and for study of the HSV genome in latency and transformation. For these reasons we are developing such variants.

The isolated variants lacking XbaI sites have been further characterized to elucidate the nature of the alterations in the genomes. As well as yielding mutants lacking particular sites, we find that this method of selection has generated viruses with additional sites and/or with deletions in their genomes.

METHODS

Growth of viruses. Virus stocks were grown and titrated in baby hamster kidney cells (BHK C13) cultured in Eagle's medium containing twice the normal concentration of vitamins and amino acids and 10% (v/v) calf serum as described previously (Brown et al., 1973). Parental strains of virus used were HSV-1 strain 17 (Glasgow) and the intertypic recombinant R12-5 originally isolated following a co-infection with intact HSV-2 ts12 DNA and unseparated wild-type fragments of HSV-1 DNA (Chartrand et al., 1981).

Preparation of virion DNA. BHK cell monolayers in 80 oz roller bottles were infected with virus at a multiplicity of infection of 0.003 and incubated at 31 °C for 3 days. Infected cells were harvested by centrifugation and cytoplastic virus released by treatment with Nonidet P40; the DNA was extracted from cell-released and cytoplastic virus by treatment with SDS and phenol (Wilkie, 1973; Stow & Wilkie, 1976). HSV DNA was further purified by cesium chloride gradient centrifugation and a quantitative estimation of the DNA was made after electrophoresis with standards, in ethidium bromide-stained agarose gels.

Restriction endonuclease digestion of viral DNA. Restriction endonucleases were obtained from Bethesda Research Laboratories. 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-MgCl₂, 0.006 M-2-mercaptoethanol, 0.02 M-KCl and 1 mg/ml bovine serum albumin, using a two- or five-fold excess of XbaI (2 to 5 units per 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 XbaI-digested HSV DNA (1 to 2 µg/plate) was transfected onto BHK C13 monolayers (4 x 10⁶ cells) using the calcium phosphate–DNA infection technique described by Stow & Wilkie (1976). Single plaques obtained from XbaI-digested HSV DNA were isolated and stocks from individual plaques were grown and titrated.

Restriction enzyme analysis of possible mutants. Restriction enzyme analysis of putative restriction enzyme site deletion mutants was carried out using the Limbro well method described by Lonsdale (1979).

Cells were infected in the presence of ³²P, at an m.o.i. of 0 p.f.u./cell of titrated virus stocks obtained from single plaques; the cells were incubated at 31 °C for 24 to 48 h. ³²P-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 overnight 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. When the restriction enzyme profiles indicated mixtures of mutant and wild-type, three rounds of plaque purification were carried out before further restriction enzyme analysis.

DNA–DNA hybridization. DNA fragments from HindIII, HpaI and BamHI digests of R12-5 were transferred from agarose gels onto nitrocellulose sheets (BA85; Schleicher & Schüll) and hybridized with nick-translated DNA by the method of Southern (1975). Nick-translated DNA was made from total HSV-1 DNA, total HSV-2 DNA, a recombinant plasmid (pGX8) containing HSV-1 BamHI k, and a recombinant plasmid (pGZ1) containing HSV-2 BamHI g (Davison & Wilkie, 1981). Pre-soaking and hybridization were carried out at 75 °C in 15 to 20 x SSC, 10% 50 x Denhardt's buffer, 1% 100 x salmon sperm DNA, 1% 1 M-Tris-HCl pH 7.5 and 73% H₂O; after extensive washing at 60 °C in 10% 20 x SSC, 10% SDS and 0.5% 1 M-Na₂HPO₄ pH 7, the sheets were dried and autoradiographed.

One-step growth experiments. Monolayers of BHK C13 cells were separately infected at an m.o.i. of 5 p.f.u./cell with HSV-1, HSV-2, the intertypic recombinant R12-5 and the HSV-1 and R12-5 variants. After adsorption at 37 °C for 1 h, the cultures were washed twice with phosphate-buffered saline containing 5% calf serum, overlaid with Eagle's medium containing 10% calf serum and incubated at 37 °C. Cultures were harvested and virus was released by sonication at intervals throughout a 24 h period. Virus titres were measured by plaque assay on BHK C13 cells.
RESULTS

Isolation of HSV-1 genomes lacking XbaI restriction endonuclease cleavage sites

Using as a model the method described by Jones & Shenk (1978), DNA from HSV-1 Glasgow strain 17 was cleaved with a twofold or fivefold excess of the restriction endonuclease XbaI. The resulting fragments were either directly transfected on BHK monolayers or transfected after ligation with T4 DNA ligase. The infectivity of the HSV DNA decreased considerably after cleavage with the endonuclease and in the majority of experiments (28/30), no infectious DNA was detectable after XbaI treatment. Ligation of the fragments did not improve the recovery.

Table I gives typical examples of the HSV DNA infectivity before and after XbaI treatment in two experiments in which infectious DNA was recoverable. Single plaque isolates obtained from such experiments were grown into stock, titrated and examined by further XbaI digestion of their 35P-labelled DNAs. The isolation frequency of mutants from virus DNA (of HSV-1 or HSV-2 or an intertypic recombinant) subjected to various degrees of XbaI digestion is shown in Table 2. Survivors were isolated after a 3 h treatment with 5 units of XbaI per μg of HSV DNA. Of 55 plaque isolates examined, two were found to contain genomes lacking XbaI sites. XbaI cleaves HSV-1 at four places in the long unique region of the genome, giving rise to fragment g (0 to 0.07 map units), fragment c (0.07 to 0.29 map units), fragment f (0.29 to 0.45 map units), fragment e (0.45 to 0.63 map units) and fragment d (0.63 to 0.83 map units) plus the two fusion fragments (a = d + [S] and b = g + [S]) (Fig. 1) (Wilkie, 1976).

One of the isolated variants B1/2 gave an XbaI profile with normal a, d, e and f bands and with the b, c and g bands missing (Fig. 2, lane 2). Two new bands were detectable: one co-migrating with a and one running between a and the position of b. The simplest interpretation is that the viral DNA has lost the XbaI site between fragment g and fragment c at 0.07 map units. This accounts for the three missing fragments and the two novel fragments, namely the fused g + c fragment and the fused fragment comprising g + c + [S]. These fragments would have mol. wt. of 29 x 10^6 and 46 x 10^6 respectively and should migrate in the positions of the two new bands.

The second variant B9/6 had bands in the a, b, c, f and g positions but the d and e bands were missing (Fig. 2, lane 3). This suggested a mutant lacking the XbaI site between d and e at 0.636 map units, giving rise to a new fused d + e fragment which would migrate in the position of the a (d + [S]) band. The fused fragment of d + e would have a mol. wt. of 38 x 10^6, virtually

Table 1. Infectivity of HSV DNA

<table>
<thead>
<tr>
<th>Expt.</th>
<th>HSV-1 DNA</th>
<th>HSV-1 DNA + XbaI (2 x)</th>
<th>HSV-1 DNA + XbaI (5 x)</th>
<th>HSV-1 DNA + XbaI + DNA ligase</th>
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</thead>
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<tr>
<td></td>
<td>P.f.u./μg DNA</td>
<td>1000</td>
<td>3</td>
<td>2</td>
</tr>
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</table>

Table 2. Mutant isolation frequency

<table>
<thead>
<tr>
<th>Virus DNA</th>
<th>XbaI treatment</th>
<th>Plaques picked</th>
<th>No. of mutants isolated</th>
<th>Isolation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 (17)</td>
<td>5 3</td>
<td>55</td>
<td>2*</td>
<td>3.6</td>
</tr>
<tr>
<td>HSV-1 (17)</td>
<td>1 1</td>
<td>60</td>
<td>0</td>
<td>&lt;1.2</td>
</tr>
<tr>
<td>HSV-1 (17)</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>&lt;1.2</td>
</tr>
<tr>
<td>R12-5</td>
<td>5 3</td>
<td>17</td>
<td>1</td>
<td>5.8</td>
</tr>
<tr>
<td>HSV-2 (HG52)</td>
<td>1 1</td>
<td>200</td>
<td>2†</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* One mutant lacking XbaI site at 0.07, the other lacking XbaI site at 0.636.
† Both mutants lacking XbaI site at 0.70.
Fig. 1. XbaI map for the DNA of HSV-1 strain 17 (above the line) and HSV-2 strain HG52 (below the line) from Wilkie (1976) and Cortini & Wilkie (1978).

Fig. 2. Autoradiographs of XbaI restriction digests of viral DNA 32P-labelled in vivo, of Glasgow strain 17 ts" syn" (lane 1) and the restriction endonuclease site deletion mutants B1/2 (lane 2), B9/6 (lane 3), X2 (lane 4) and the mutant with an additional XbaI site, C24/6 (lane 5). Letters refer to specific XbaI fragments (Fig. 1). ■, Positions of new fragments; <, positions of missing fragments.

equivalent to the a fragment mol. wt. of $37 \times 10^6$. There is a novel fragment running above the a position which is the fragment formed by $d + e + [S]$ with a mol. wt. of approximately $55 \times 10^6$ (fragments of high mol. wt. are not distinctly resolved on a 0.5% agarose gel).

The two variants had therefore each lost one XbaI site at the opposite extremities of the long unique region of the genome. In order to construct a genome with both these sites missing, cells were doubly infected at equal m.o.i. of each variant and the progeny titrated after 24 h incubation at 37 °C. Fifty progeny plaques were picked and stocks grown. The 32P-labelled DNAs of these isolates were digested with XbaI and run on agarose gels. Taking into account only single crossovers in the region of the genome between XbaI sites at 0.07 and 0.636 and assuming equal probability of a crossover event throughout the genome then it would be expected that a recombinant lacking both the sites at 0.07 and 0.636 map units would be generated by 28% of all the single crossover events. Of the 50 plaques picked, three gave profiles
indicating loss of both XbaI sites. The XbaI profiles of one of the recombinants, X2, is shown in Fig. 2, lane 4. Two others gave identical profiles. As expected, the X2 recombinant has a normal \( f \) band and is lacking the \( b, c, d, e \) and \( g \) bands. There are multiple novel bands in the \( a \) region, consistent with the products expected from fusions between \( g \) and \( c \) \((29 \times 10^6)\), \( d \) and \( e \) \((38 \times 10^6)\), \( g + c + [S] \) \((46 \times 10^6)\) and \( d + e + [S] \) \((55 \times 10^6)\).

**Isolation of a genome with no XbaI sites**

The intertypic recombinant R12-5 has only one XbaI site, the HSV-2 site at \( 0.45 \) map units (Chartrand et al., 1981). DNA stocks of the virus were grown and digested with XbaI using the same procedure as for HSV-1. The infectivity of the DNA dropped from \( 3.4 \times 10^5 \) plaques/\( \mu \)g after XbaI treatment. Seventeen plaques were picked from digested DNA and their \( ^{32} \)P-labelled DNA further restricted with XbaI, but no differences could be detected compared to the parental R12-5 DNA. However, it was considered that even if the XbaI site had been lost, the large size of the fragments would preclude resolution of whole DNA molecules from the two fragments \((45 \times 10^6, 55 \times 10^6)\) generated by XbaI. The isolates were therefore subjected to double digestions with XbaI/HindIII and XbaI/HpaI. In addition the parental R12-5 was separately digested with HindIII and HpaI alone. The HindIII and HpaI agarose gel profiles and maps of R12-5 are shown in Fig. 3.

As shown diagrammatically in Fig. 3(a) R12-5 contains the HindIII \( i, h \) and \( e \) fragments of HSV-2 and the \( o, h, j, m, n \) and \( g \) fragments of HSV-1. It also has a large fused fragment \((0.52 to 0.82 \text{ map units})\) made up of part of the \( d \) (HSV-1 \( 0.64 \) to \( 0.82 \)) fragment and the \( a \) (HSV-2 \( 0.52 \) to \( 0.82 \)) fragment. The map is derived from the \( 0.5% \) agarose gel profile of a HindIII digest of R12-5 DNA (Fig. 3b, lane 2) which contains six new bands: (i) mol. wt. \( 1.2 \times 10^6 \) made up of \( i \) (HSV-2) + \( m \) (HSV-1) (band 3); (ii) mol. wt. \( 16 \times 10^6 \) made up of \( i \) (HSV-2) + \( g \) (HSV-1) (band 2); (iii) mol. wt. \( 30 \times 10^6 \) new fragment \( 0.52 \) to \( 0.82 \) map units (band 1); (iv) mol. wt. \( 35 \times 10^6 \) new fragment + \( m \) (HSV-1) (band 1); (v) mol. wt. \( 39 \times 10^6 \) new fragment + \( g \) (HSV-1) (band 1); (vi) mol. wt. \( 2 \times 10^6 \) new fragment \( 0.25 \) to \( 0.32 \) map units (band 3).

The HpaI fragments of R12-5 are \( l, p, u, j, s, v, r, m, c \) and \( g \) of HSV-1 and \( h, d \) of HSV-2 (Fig. 3a). The \( 0.5% \) agarose gel profile of a HpaI digest of R12-5 (Fig. 3b, lane 7) contains four new bands: (i) mol. wt. \( 9 \times 10^6 \) new molar fragment \( 0.23 \) to \( 0.32 \) map units (band 3); (ii) mol. wt. \( 13 \times 10^6 \) g (HSV-2) + g (HSV-1) 0.25-molar (band 2); (iii) mol. wt. \( 16 \times 10^6 \) g (HSV-2) + c (HSV-1) 0.25-molar (band 1); (iv) mol. wt. \( 13 \times 10^6 \) new molar fragment \( 0.58 \) to \( 0.71 \) map units (band 2). (Some of the terminal fragments although barely visible on the photographic print are distinctly identifiable on the original autoradiograph.)

The crossover positions of HSV-1 to HSV-2 at \( 0.07, 0.26 \) to \( 0.28, 0.64 \) to \( 0.71 \) map units are indicated on the map (Fig. 3a). The HindIII and HpaI profiles of the majority of the single plaque isolates are identical to R12-5 as exemplified by one of them, R12-5X14 (Fig. 3b, lanes 4 and 9). The isolate R12-5X13 is lacking the HSV-2 HindIII \( i \) fragment and the fusion fragment (2) formed by \( i \) (HSV-2) and \( g \) (HSV-1) (Fig. 3b, lane 3). The HpaI profile of R12-5X13 has a missing \( g \) (HSV-2) + c (HSV-1) fusion fragment (band 1 of R12-5) and has two new fragments: one running below the band 2 position of R12-5, and one running just below the HSV-2 \( c \) and \( d \) fragments (Fig. 3b, lane 8). When the fragments from XbaI/HindIII double digests were run on \( 0.5% \) agarose gels (Fig. 4), it was seen that as expected the parental R12-5 lacks the HindIII \( e \) fragment (0.41 to 0.52 map units) of HSV-2 (Fig. 4, lane 1) and contains two new molar fragments. The \( e \) fragment contains the XbaI site at \( 0.45 \) map units. However, the R12-5X13 variant contains the \( e \) fragment (Fig. 4, lane 2), indicating that this fragment has not been cut by XbaI and that the site at \( 0.45 \) is missing in this genome. Similarly, when the agarose gel profiles of the XbaI/HpaI double digests are examined, it can be seen that the parental R12-5 lacks the HpaI \( d \) fragment of HSV-2 (0.34 to 0.54 map units) but contains two new molar fragments which have arisen by XbaI cleavage of this fragment at \( 0.45 \) (Fig. 4, lane 3). R12-5X13 contains the HpaI \( d \) fragment and does not have the two new molar fragments (Fig. 4, lane 4). This substantiates the finding from the XbaI/HindIII digest that R12-5X13 no longer contains the XbaI site at \( 0.45 \) map units. An HSV genome without any XbaI sites has thus been generated for the first time.
Fig. 3. (a) *HindIII* and *HpaI* restriction endonuclease maps for the DNA of HSV-1 strain 17, HSV-2 strain HG52 and the intertypic recombinant R12-5. Restriction sites are shown above the genome for HSV-1 and below the genome for HSV-2. Cross-hatching indicates areas of uncertainty in crossover position. (b) Autoradiographs of restriction digests of viral DNA labelled *in vivo* with $^{32}$P. Lanes 1 to 5, *HindIII* (0.6% agarose); lanes 6 to 10, *HpaI* (0.8% agarose). Lane 1, HSV-2 HG52; letters refer to specific *HindIII* fragments. Lane 2, R12-5; letters refer to specific *HindIII* fragments of HSV-1 and underlined letters to HSV-2 fragments; numbers refer to specific recombinant fragments. Lane 3, R12-5X13; <, missing fragments. Lane 4, R12-5X14. Lane 5, HSV-1 strain 17; letters refer to specific *HindIII* fragments. Lane 6, HSV-2 HG52; letters refer to specific *HpaI* fragments. Lane 7, R12-5; letters refer to HSV-1 fragments and underlined letters to HSV-2 fragments; numbers refer to specific recombinant fragments. Lane 8, R12-5X13; ■, new fragment; <, missing fragments. Lane 9, R12-5X14. Lane 10, HSV-1 strain 17; letters refer to specific *HpaI* fragments.
Fig. 4. Autoradiographs of restriction digests of viral DNA labelled in vivo with $^{32}$P. Lanes 1 and 2, *XbaI/HindIII* double digests (0.5% agarose). Lanes 3 and 4, *XbaI/HpaI* double digests (0.5% agarose). Lane 1, R12-5. Lane 2, R12-5X13; *e* is *HindIII* fragment containing *XbaI* site at 0.45 map units. Lane 3, R12-5. Lane 4, R12-5X13; *d* is *HpaI* fragment containing *XbaI* site at 0.45 map units. >, New fragments; <, missing fragments.

Isolation of variants with altered genomes

In analysing the *XbaI* site deletion variants of HSV-1, it became apparent that an isolate (C24/6) had been recovered whose DNA did not give the wild-type profile when digested with *XbaI*. Nevertheless, it had not lost any of the four *XbaI* sites (Fig. 2, lane 5). The profile shows the normal a, c, d, e and f bands but the g and b bands are not present. Two new bands are visible: one (0.5-molar) running coincidentally with d and e, with a molecular weight of about 20 $\times$ 10$^6$ and one (1.5-molar) running considerably below g with a mol. wt. of 3 $\times$ 10$^6$. This we interpret as being due to an additional *XbaI* site at map coordinate 0.03, which would account for a novel molar and a novel 0.5-molar band of the same size. The new terminal fragment combined with IS] will give a fragment of mol. wt. 20 $\times$ 10$^6$ accounting for the additional band in the d region. This additional site appears to be confined to g in TR$_L$ or IR$_L$ + [S] and not to be duplicated in d in TR$_L$ or IR$_L$ + [S]. If the site had been present in d in TR$_L$ or IR$_L$ + [S] the d and a fragments would have been lost, which has not occurred.

In constructing the *HindIII* and *HpaI* maps of R12-5 and derivatives, it became apparent that four of the 17 isolates from *XbaI* digestion had aberrant *HindIII* and *HpaI* profiles (R12-5X3, R12-5X7, R12-5X13 and R12-5X16). The isolates R12-5X3 and R12-5X7 had identical *HindIII* profiles (Fig. 5a, lanes 2, 3). They had each lost three bands: the HSV-2 i band, the band (no. 2) of mol. wt. 16 $\times$ 10$^6$ comprising i (HSV-2) + g (HSV-1) and the band (no. 3) of mol. wt. 12 $\times$ 10$^6$
comprising \( i \) (HSV-2) + \( m \) (HSV-1). They had simultaneously gained two novel bands: one running above the HSV-2 \( e \) band with a mol. wt. of \( 13 \times 10^6 \) and one running just above the HSV-1 \( g \) band with a mol. wt. of \( 9 \times 10^6 \). This could be due to either (i) the generation of a new \( \text{HindIII} \) site in the HSV-2 \( i \) fragment, leading to the loss of this fragment and the generation of two smaller fragments, one of which would be molar, the other 0.5-molar plus two shortened 0.25-molar joint-spanning fragments, or (ii) the deletion of a piece of DNA of mol. wt. \( 3 \times 10^6 \) within the HSV-2 \( i \) fragment, giving rise to a new 0.5-molar terminal fragment and two shortened 0.25-molar joint-spanning fragments. From the appearance of the band at the \( n/4 \) position it seems more likely that the latter explanation is correct.

When R12-5X3 and R12-5X7 are digested with \( \text{HpaI} \), aberrant profiles are again produced (Fig. 5b, lanes 2, 3). Three bands are missing: the HSV-2 \( g \), the HSV-2 \( g \) plus the HSV-1 \( g \) (band 2) and the HSV-2 \( g \) plus the HSV-1 \( c \) (band 1). Two new fragments are observed: one with a mol. wt. of \( 11 \times 10^6 \) and one of mol. wt. \( 14 \times 10^6 \). Again these profiles could be explained by either an extra \( \text{HpaI} \) site being generated or a deletion of part of the DNA of the HSV-2 \( g \) fragment. The two new fragments formed by fusion of the new terminal fragment with the HSV-1 \( m \) fragment, and the terminal fragment with the HSV-1 \( c \) fragment would have molecular weights consistent with the positions of the two new bands on the gel. The new terminal fragment appears to be running below the low mol. wt. \( o, p, q, r \) HSV-1 group of fragments. As it seems less likely that both a new \( \text{HindIII} \) and a new \( \text{HpaI} \) site have been generated in TRL than that a single deletion has occurred in this fragment, the simplest explanation of these data is the deletion in the TRL region of a \( 3 \times 10^6 \) to \( 4 \times 10^6 \) mol. wt. piece of DNA.

The mutant R12-5X13 which has lost the \( XbaI \) site at 0.45 map units also has a novel profile when \( \text{HindIII} \)-digested DNA is run on an agarose gel (Fig. 3b, lane 3). The HSV-2 \( i \) band is missing, as is the fusion fragment of \( i \) (HSV-2) + \( g \) (HSV-1). The \( \text{HpaI} \)-digested DNA profile of this mutant lacks both the \( g \) (HSV-2) fragment and the \( g \) (HSV-2) + \( c \) (HSV-1) (band 1) fragment and contains a new band below the band 2 position and a new band at the HSV-2 \( c, d \) position (Fig. 3b, lane 8). These changes again suggest a deletion of about \( 4 \times 10^6 \) mol. wt. of DNA in TRL. A new terminal fragment of \( 2.6 \times 10^6 \) mol. wt. would run with the \( \text{HindIII} \) \( n \) fragment (HSV-1) and the \( o, p, q, r \) group of HSV-1 \( \text{HpaI} \) fragments. The two resulting new
HindIII fusion fragments would be of mol. wt. $11 \times 10^6$ and $7 \times 10^6$. Their expected sizes suggest that they would run coincidently with the HSV-2 HindIII e fragment ($11.5 \times 10^6$) and the HSV-1 HindIII h ($7 \times 10^6$) fragment respectively. Similarly, a deletion of $4 \times 10^6$ in TRL would account for the new fragment seen in the HpaI digest. These have molecular weights of $12 \times 10^6$ and $10 \times 10^6$ and would correspond with the fusion of the new terminal fragment with HSV-1 HpaI c ($2.6 \times 10^6 + 9.5 \times 10^6$) and HSV-1 HpaI g ($2.6 \times 10^6 + 7 \times 10^6$) respectively.

A HindIII digest of R12-5X16 disclosed changes in the mobilities of the three fragments at the top of the gel (Fig. 5a, lane 4). These are the fusion fragments from map coordinates 0.52 to 0.82 and the two fusion fragments of this with HSV-1 HindIII m and HSV-1 HindIII g. The large size of these fragments precludes accurate determination of the size changes required to produce the observed increased mobilities; but a deletion of about $5 \times 10^6$ mol. wt. in the 0.52 to 0.82 fusion fragment would account for the changed mobilities of all of these three fragments.

Thus far we have discussed the detection of four altered isolates, all bearing deletions, out of 17 analysed following XbaI treatment of the DNA of R12-5. In order to determine whether the high frequency of alterations in the R12-5 genome was an inherent property of this recombinant, or resulted from the XbaI treatment of its DNA, single plaques were picked from the virus stock and their DNA (which had not been exposed to XbaI treatment) was subjected to HindIII and HpaI digestion. Twenty plaques were analysed but no alterations were detected in either the HindIII or the HpaI profiles. We conclude that deletions in the genome may result from the procedures involved in XbaI digestion.

Nature of the viral DNA alterations

To characterize the alterations in the variants B1/2 and B9/6 more precisely, their DNAs (extracted from nuclei and cytoplasm of infected cells) were cleaved with the restriction endonucleases HindIII, HpaI and BamHI. No alterations were detected in the HindIII- and HpaI-cleaved DNA profiles (not shown). BamHI makes multiple cuts in the HSV genome and the BamHI e fragment (0.02 to 0.08 map units) contains the alteration present in the B1/2 DNA which lacks the XbaI cleavage site at 0.07 map units, whereas the BamHI i fragment (0.58 to 0.64 map units) contains the XbaI cleavage site at 0.636 map units that is missing in B9/6. There is no obvious difference in the migration of the BamHI e fragment of B1/2 (Fig. 6, lane 2). However, the BamHI i fragment of B9/6 migrates faster than the corresponding wild-type fragment (Fig. 6c, lane 3). The difference in relative mobilities of these two BamHI i fragments reflects the difference in their molecular length. We estimate that the variant B9/6 in losing the XbaI site at 0.636 has lost $0.1 \times 10^6$ daltons of DNA which corresponds to a deletion of about 150 base pairs.

The variants R12-5X3, R12-5X7, R12-5X13, R12-5X16 and the parent R12-5 were similarly characterized by cleavage with BamHI (Fig. 6c, lanes 5 to 8). R12-5 DNA shows the expected recombinant profile, except that the terminal HSV-1 s and q fragments and the HSV-2 v fragment are barely detectable. This viral DNA was obtained from nuclei and cytoplasm of infected cells and it was assumed that concatemeric forms of DNA were diluting the terminal fragments. The profile of R12-5 shows both the HSV-2 p fragment and the joint k (HSV-1 s + q; HSV-2 v + HSV-1 q) fragment, indicating that the terminal fragments are in fact being made.

If the lack of terminal fragments were due to concatemeric forms of DNA, then it would be expected that the k fragment would be present in increased amounts; this was not the case. To study this paradox, R12-5 DNA digested with HindIII, HpaI and BamHI was hybridized with nick-translated DNA of HSV-1, HSV-2, pGX8 (HSV-1 BamHI k clone) and pGZ1 (HSV-2 BamHI g clone) in Southern blot experiments. It can be seen from the blot of BamHI-digested DNA (Fig. 7) that the s and q fragments of HSV-1 and the v fragment of HSV-2 are in fact present. There is also hybridization to the HSV-1 k fragment and to a fragment co-migrating with the HSV-1 j fragment (k + 'a' sequences). Thus, the explanation for the lack of any increased amounts of the k fragment in Fig. 6(c), lane 5 must be that additional 'a' sequences on the HSV-2 v fragment and the HSV-1 s fragment are giving larger joint fragments running in the
Fig. 6. (a) BamHI restriction endonuclease map for the DNA of R12-5. Restriction sites are shown below the line for HSV-2 and above the line for HSV-1; ---, crossover positions from HSV-1 to HSV-2.
(b) Diagram to illustrate structure of R12-5 variants. R12-5X3 and R12-5X7: ▼, approximately 4.5 kb deletion affecting HindIII i, HpaI g, and BamHI v and p. R12-5X13: ▼, approximately 6.0 kb deletion affecting HindIII i, HpaI g, and BamHI v and p; ◼, deleted XbaI site at 0.45 map units. R12-5X16: ▲, approximately 9.0 kb deletion including BamHI s and part of BamHI b. ▼, New terminal fragments including BamHI s (HSV-1) and part of BamHI c (HSV-1) plus part of BamHI p (HSV-2). (c) Autoradiographs of BamHI restriction digests of viral DNA labelled in vivo with 32P. Letters refer to specific BamHI fragments; >, altered fragment mobility; ■, new fragments; <, missing fragments. Lanes 1 and 4, HSV-1 strain 17; lane 2, B1/2; lane 3, B9/6; lane 5, R12-5; lane 6, R12-5X3; lane 7, R12-5X13; lane 8, R12-5X16; lane 9, HG52.
**HSV XbaI site deletion mutants**

![Autoradiograph of nitrocellulose blot strips containing BamHI restriction fragments of R12-5 to which nick-translated DNA probes have been hybridized. The probes were HSV-1 DNA (lane 1), the recombinant plasmid pGX8 (lane 2), HSV-2 DNA (lane 3) and the recombinant plasmid pGZ1 (lane 4). Lane 2 shows specific hybridization to the BamHI HSV-1 terminal fragments s and q and to the joint k fragment. The band co-migrating with BamHI (Fig. 6c) is the k fragment plus additional 'a' sequences. Lane 4 shows specific hybridization to the BamHI HSV-2 terminal v, cross-hybridization to the HSV-1 terminus q and hybridization to the joint g fragment (comprising v (HSV-2) and q (HSV-1)). The band above q is due to cross-hybridization to the BamHI HSV-2 p fragment (see text). The band below g is due to cross-hybridization to the HSV-1 k fragment and the band above g is g plus additional 'a' sequences.](image)

There is in addition some hybridization to an additional fragment which was thought to be cross-hybridization to the BamHI p fragment of HSV-2. This fragment is known to be variable and by hybridizing BamHI-digested R12-5 DNA to nick-translated HSV-2 BamHI p, we have shown that it does in fact cross-hybridize with the HSV-2 termini (data not shown). The profile (Fig. 6c, lane 5) also shows a normal HSV-1 m fragment but not the adjacent HSV-1 a' fragment; the HSV-2 i fragment is missing but the HSV-2 r fragment is present. These observations indicate that the crossover from the HSV-1 to the HSV-2 genome is between 0.26 and 0.27 map units. Similarly, the HSV-1 j fragment is absent, but the adjacent c' fragment is present; the HSV-2 a fragment is also absent. A crossover point between HSV-2 and HSV-1 must therefore lie within map coordinates 0.65 and 0.7 map units. These crossover positions plus one at map coordinate 0.07 (derived from HpaI and HindIII mapping) are shown on the BamHI map (Fig. 6a).

The BamHI profiles of R12-5X3 (Fig. 6c, lane 6) and R12-5X7 (which is identical but not illustrated) show that the HSV-2 p fragment is absent in both these isolates. This confirms the conclusion reached from analysis with HindIII and HpaI that both isolates are deleted in TRI. Although it cannot be determined from the gel, it is assumed that the BamHI v fragment is also missing. The deletion cannot extend into the HSV-2 c fragment as no new large fusion fragment is detectable in these mutants. The HSV-1 k fragment is present, indicating that the deletion is not duplicated in IR1. The band running in the HSV-1 j position also seems to be less intense, in keeping with the deletion of HSV-2 v. The deletion in TRI must be between 2 x 10^6 and 4 x 10^6 mol. wt., which would be in agreement with the calculation of 3 x 10^6 from the HindIII and HpaI digests.
Fig. 8. One-step growth curves of mutants in BHK21 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 with phosphate-buffered saline with 5% calf serum, overlaid with Eagle's medium with calf serum and incubated at 37 °C (see Methods). Cultures were harvested at the times indicated and the virus titre was measured by plaque assay on BHK21 C13 cells. (a) HSV-1 mutants: ●, Glasgow strain 17; ○, B9/6; ■, X2; □, B1/2. (b) R12-5 mutants: ○, R12-5; ■, R12-5X16; □, R12-5X7; ▲, R12-5X13. ●, HSV-2 strain HG52.

The cleavage site recognized by XbaI at 0.45 map units is missing in R12-5X13. No alterations in the mobilities of the BamHI HSV-2 o (0.43 to 0.45 map units) or y (0.45 to 0.47 map units) fragments are apparent in the R12-5X13 profile (Fig. 6c, lane 7). This suggests that loss of the XbaI site is probably not due to a deletion unless it is very small. The HindIII and HpaI profiles of R12-5X13 indicated that it was deleted in TR1. This is confirmed by the loss of the BamHI HSV-2 p fragment and presumably the HSV-2 v fragment. The HSV-1 joint k fragment is present and the intensity of the j band is reduced compared to R12-5. The band running below the j fragment is again thought to be due to additional a sequences on the HSV-1 s + q fusion fragment.

The BamHI profile of R12-5X16 shows that this variant has lost the HSV-1 b fragment (mol. wt. 6.7 x 10^6) but has two new fragments: one of mol. wt. 5.2 x 10^6 running above the HSV-2 c fragment and one of mol. wt. 2.7 x 10^6 running above the HSV-2 o fragment. The HSV-1 k fragment is present, which indicates that the HSV-1 s and q fragments are present. Our interpretation of the data is as follows: this mutant is deleted in IR1 by approximately 5.9 x 10^6 mol. wt. of DNA which includes the BamHI s and part of the b fragment. This would give a fragment of 2.8 x 10^6 (the new b fragment) and a fragment of 5.1 x 10^6 comprising the new fragment plus HSV-1 q. The 2.8 x 10^6 fragment must be running coincidently with the HSV-2 m fragment. The fragment running above the HSV-2 o fragment and the absence of the HSV-2 p fragment are interpreted as being due to the R12-5X16 molecule crossing back within the HSV-2 p fragment to HSV-1 e (Fig. 6a). The new fragment of mol. wt. 2.7 x 10^6 consists of part of the HSV-2 p and part of the HSV-1 e fragment. As a consequence of this extra crossover the HSV-1 s fragment is still present in TR1 of R12-5X16 which accounts for the HSV-1 k fragment being present in this molecule despite the deletion of s in IR1. The structures of the R12-5 variants are shown diagrammatically in Fig. 6(b).

**Growth characteristics of the mutants**

Having identified deletions, we investigated whether they resulted in any change of biological fitness. This was done by a comparative study of one-step growth experiments over a 24 h period at 37 °C. It can be seen from Fig. 8 that all the mutants replicated reasonably well. Fig. 8(a) compares the growth of wild-type HSV-1 strain 17 with the mutants B1/2, B9/6 and X2.
The three mutants replicated almost as well as wild-type virus, although B9/6 was consistently slower. Fig. 8(b) compares the growth of the mutants R12-5X7, R12-5X13 and R12-5X16 with the parental R12-5 and the wild-type HSV-1 strain 17 (see Fig. 8a) and HSV-2 (HG52). As expected, HG52 grew appreciably less well than HSV-1 wild-type; the parental R12-5 recombinant grew better than the three variants; R12-5X13 and R12-5X7 grew more slowly, whereas R12-5X16 grew at the same rate. The yields of virus at 24 h from R12-5X7 and R12-5X13 (which invariably grew more slowly) infections are comparable to R12-5, although in most experiments the 24 h yield was marginally lower (six experiments). The mutant R12-5X16, although viable, showed a consistently poor one-step growth pattern, indicating inefficient replication in terms of the yield of p.f.u./cell. We conclude that all the mutants show some impairment, but the base changes and/or deletions present are situated in regions of the genome that are sufficiently dispensable to allow at least some virus replication to occur under BHK cell culture conditions.

DISCUSSION

Herpes simplex virus undergoes recombination during the course of a productive intratypic or intertypic infection in cultured cells (Brown et al., 1973; Brown & Ritchie, 1975a, b; Subak-Sharpe et al., 1973, 1975). The mechanism by which there is exchange of information between molecules in the mating pool is little understood (Ritchie et al., 1977). Intertypic recombination between HSV-1 and HSV-2 (Timbury & Subak-Sharpe, 1973) has allowed gene/polypeptide mapping (Marsden et al., 1978) and has delimited areas of DNA exchange across the genome (Preston et al., 1978). The extent of non-homology (45%) between HSV-1 and HSV-2 as estimated from nucleic acid hybridization may set limits to genomic regions in which recombination can take place. One way of determining to what extent 'normal' DNA homology allows uniform interchange of genetic information between genomes is to analyse restriction enzyme site exchange in intratypic crosses involving otherwise essentially homologous genomes.

This situation can later be compared with intertypic recombination involving the same outside markers and over a defined intertypic region, with the rest of the genome again stemming from one type only. In this latter analysis, however, the effect of relative selective fitness of intertypic recombinants will have to be taken into account.

As a first step towards such recombination analysis, it is necessary to generate mutants lacking specific restriction enzyme sites. This paper describes the isolation and characterization of the first such HSV mutants. We have used a modification of the method of selection described by Klein & Murray (1979) for lambda and Jones & Shenk (1978) for adenovirus type 5, where, following EcoRI cleavage and ligation of resulting fragments, deletion, base change and substitution mutants were isolated. However, with HSV-1 and the intertypic recombinant R12-5, we found that ligation of fragments after XbaI digestion did not improve the yield of viable virus. Although the number of cuts made by XbaI in HSV-1 is small, we assume that the size of the fragments and the probability of assembling the fragments in the correct order makes the ligation step inefficient. All the mutants we have described have been isolated by selecting and enriching for molecules which presumably pre-exist within the genome pool. This is not very efficient, as the envisaged selective advantage stems merely from a reduction of the number of restriction endonuclease-sensitive sites, in our case from four to three. For this method to work, it is essential to start with DNA that is highly efficient in transfection so that after an excess of endonuclease digestion there are still intact infectious molecules. The method is imperfect in that we have not yet evaluated or controlled all the factors that determine optimum numbers of survivors after endonuclease digestion. For HSV-1 the frequency of isolation of mutants with deleted XbaI sites was 3.6%. Minimally this suggests that these mutants were present in the stock virus population at a frequency of 1:400. This is in keeping with our failure to isolate a mutant from 20 plaques picked without XbaI treatment and 60 plaques picked after only 1 h treatment with 1 unit of XbaI (Table 2). The corresponding figures for the isolation of a mutant lacking one XbaI site (R12-5) were 5.8% and 1:340. The frequency of isolation of temperature-sensitive mutants of HSV using a variety of mutagens is 1:10^3 (Coates, 1982). Ts mutations are usually due to single base pair alterations; this also appears to be true for three out of four of our
restriction site mutants. Thus, the difference in the isolation frequency probably reflects both differences in the stringency of selection conditions when screening for a particular characteristic, i.e. temperature sensitivity, or resistance to digestion, and also differences in the pre-existing mutant frequencies in the population. The relatively high frequency of occurrence of XbaI site mutants suggests that enrichment selection may prove a useful general method of obtaining virus with deleted sites for particular restriction endonucleases. This proposition has yet to be tested with other enzymes.

B1/2 and B9/6 of ts+ syn+ and R12-5X13 of R12-5 were each isolated lacking a single XbaI site. Two of these (B1/2 and R12-5X13) have apparently arisen by virtue of a single base pair change in the restriction endonuclease recognition sites at 0.07 and 0.45 respectively, although a very small undetected deletion has not been ruled out. The other mutant B9/6 was shown to be due to a deletion of about 150 base pairs containing the XbaI site at 0.636. The very minor alterations in the XbaI site at 0.07 map units and 0.45 map units constitute insufficient evidence to conclude that substantial deletions from these regions of the genome cannot be tolerated if viability is to be maintained. We do not know any gene that maps at 0.07, and of only one polypeptide with mol. wt. 28000 (Vmw 28) which may be encoded at 0.45. The deletion of 0.1 x 10^6 daltons of DNA at the XbaI site at 0.636 map units (B9/6) suggests that this region of the genome is not essential for a productive lytic infection. The map coordinates of a number of HSV-1 polypeptides including Vmw87, 65, 58, 45 (Marsden et al., 1978) and ICP25, 21, 6 and gC-1 (Morse et al., 1978; Halliburton, 1980; Ruyechan et al., 1979) are at least partially contained in the region of the genome from 0.6 to 0.64 map units. Wild-type levels of one of these polypeptides, Vmw65, appears to be essential for normal virus growth (D. J. Dargan, personal communication). The glycoprotein gC-1 is non-essential (Manservigi et al., 1977) and the functions of the other polypeptides mapping in this region of the genome are not known. However, as B9/6 shows no defect in its lytic cycle, it can be assumed that the small deletion in this mutant is not affecting the synthesis or function of essential polypeptides. When more precise information concerning the XbaI site locations becomes available from sequence data, the role of these regions in HSV replication can be reassessed.

The observation of the novel additional XbaI site in mutant C24/6 at 0.03 in TRL but not present in IRL is interesting. Thirty plaque-purified subclones of this variant showed identical patterns, suggesting that the single base change which lies in the immediate-early (IE) gene coding for the IE Vmw110 polypeptide (0.02 to 0.045 and 0.785 to 0.81) (L. J. Whitton, personal communication) cannot be tolerated in the homozygous condition at 37°C.

Three of the variants of the intertypic recombinant R12-5 exhibited deletions in TRL (R12-5X3, R12-5X7 and R12-5X13) and one a deletion in IRL (R12-5X16). The variants R12-5X3 and R12-5X7, although having no altered XbaI sites, have deletions of between 3 x 10^6 and 4 x 10^6 mol. wt. of DNA in TRL. The mutant R12-5X13 with the missing XbaI site at 0.45 map units is similarly deleted (4 x 10^6) in TRL. All three are missing both BamHI sites delimiting the HSV-2 BamHI p segment. The isolate R12-5X16 has been shown to be deleted in virtually the whole of IR (5.9 x 10^6). As R12-5 is HSV-2 in TRL and HSV-1 in IRL it means that in R12-5X3, R12-5X7 and R12-5X13 most or all of the gene for the IE Vmw118K polypeptide has been deleted and that in R12-5X16 the gene for the HSV-1 equivalent IE Vmw110K polypeptide has been deleted.

Although the three mutants with the deletion in TRL grow appreciably more slowly than the parental R12-5, the final yields of virus in a one-step growth cycle are comparable. The isolate R12-5X16 is exceptional. It is impaired in terms of yield of infectious virus and the whole HSV-1 IR region is missing, but there has been further crossover substituting HSV-1 for HSV-2 information in the TRL region. The IE polypeptides made by this recombinant are under investigation. The conclusion that only one copy of the equivalent genes in TRL and IRL is sufficient for successive complete cycles of virus replication has been reached by Davison et al. (1981). Working with the intertypic recombinant B x 1 (28-1) they found in their in-depth analysis two subclones of this virus which were deleted in either TRL or IRL. These subclones of B x 1 (28-1) were shown to be viable in terms of plaque production but they were not analysed in terms of their efficiency in single-cycle growth experiments. In a further investigation
(unpublished results) we have found them also to be impaired in single-cycle growth experiments.

Analysis of 20 subclones of R12-5 not treated with XbaI did not reveal any deletions. The difference between $B \times 1$ (28-1), where two deletions were found in five subclones, and R12-5 in this respect may reflect the different composition of these recombinants. $B \times 1$ (28-1) is largely fixed in one orientation of $U_1$. Isolation of mutants lacking all XbaI sites in HSV-1 and HSV-2 is underway.

We thank Nigel Stow for his willing advice and are especially grateful to Andrew Davison for his contribution to the analysis of the data and for supplying the pGX8 and pGZ1 plasmids.

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(Received 6 December 1983)