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 XbaI site at map coordinate 0.7 have been selected following XbaI 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 XbaI treatment, four variants with deletions ranging in size from 1.5 kb (in both TR_L and IR_L) to 9 kb in IR_R 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_R 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 XbaI 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 XbaI 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 XbaI 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 XbaI 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 XbaI 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 XbaI restriction endonuclease site. The genome structures of two independently isolated mutants lacking the XbaI site at map coordinate 0.7 are described. In addition, genome analysis of the survivors from XbaI 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 (ts1) (Timbury, 1971), and a freshly re-cloned 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 TRl and IRL 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), ts1 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-Cl 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 fivefold excess of XbaI (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 XbaI-digested DNA (1 to 2 μg/plate) was transfected onto BHK21/C13 monolayers (4 × 10⁶ 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 BamHI digests of HG52 were transferred from agarose gels to nitrocellulose sheets (BA-85, Schleicher & Schull) 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 BamHI 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-Na2HPO4, 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, ts1 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 (PBS/Ca). The single plaques were picked into 0.5 ml PBS/Ca, sonicated and 0.2 ml was used to infect monolayers of BHK21/C13 cells on 50 mm Petri dishes (4 × 10⁶ 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 IRL. This re-cloned stock was also treated as above.

Preparation and analysis of immediate early polypeptides. Confluent human foetal lung (HFL) cells (2 × 10⁶ 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. [35S]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-MgCl2, 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 XbaI 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 x 10⁶ 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 XbaI at 5 units/μg DNA for 2 h at 37 °C failed to give any plaques. Reduction of the XbaI 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 XbaI/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 XbaI site loss. One-hundred plaques each were picked from plates transfected with DNA which had been digested with 1 unit XbaI/μg DNA for either 1 or 2 h at 37 °C. Only two of these 200 plaques were found to contain genomes lacking XbaI 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 XbaI RE mutants (following treatment with 5 units XbaI/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 XbaI 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 x 10⁶), fragment d (m.c. 0.45 to 0.7, mol. wt. 25 x 10⁶), fragment g (m.c. 0.7 to 0.82, mol. wt. 12 x 10⁶), fragment h (m.c. 0.82 to 0.9, mol. wt. 8 x 10⁶), fragment j (m.c. 0.9 to 0.944, mol. wt. 5.6 x 10⁶), and fragment i (m.c. 0.944 to 1, mol. wt. 5.6 x 10⁶) plus four fusion fragments [a=(c + h), mol. wt. 53 x 10⁶; b=(c + i), mol. wt. 50.6 x 10⁶; e=(g + h), mol. wt. 20 x 10⁶; f=(g + i), mol. wt. 17.6 x 10⁶]. The XbaI, HpaI, HindIII, EcoRI and BamHI maps of HSV-2 (strain HG52) are shown in Fig. 1 (Cortini & Wilkie, 1978).

One of the isolated variants, HG52X94, gave an XbaI 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 XbaI site at m.c. 0.7, giving rise to a new fused terminal fragment (d + g) of mol. wt. 36 x 10⁶ and two new joint fragments, d + g + h of mol. wt. 44 x 10⁶ and d + g + i of mol. wt. 41.6 x 10⁶. 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 x 10⁶). HindIII and HpaI digests of this mutant detected no changes in the DNA profile compared to the parental HG52, suggesting that the XbaI site loss at 0.7 was not due to an extensive deletion which would have resulted in alterations in mobility of the large HindIII a and HpaI e fragments (data not shown). However, a BamHI digest of HG52X94 showed that the site loss at m.c. 0.7 was due to a deletion of about 150 base pairs. The BamHI k fragment which contains the XbaI site at m.c. 0.7 had an altered mobility equivalent to a 0.1 x 10⁶ change in mol. wt. compared to the parental HG52 k fragment (Fig. 3, lanes 1 and 2).

Another variant, HG52X163, had an XbaI profile indistinguishable from HG52X94 (Fig. 2, lane 3) although the method of isolation precluded the two mutants from being clonally related. Again, the XbaI profile suggested that HG52X163 had lost the XbaI site at m.c. 0.7 and the HindIII and HpaI profiles were indistinguishable from the parental HG52 pattern. The BamHI 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. XbaI: \( a = c + h, b = c + i, e = g + h, f = g + i \). HindIII: \( c = i + k, d = j + k, f = i + m, g = j + m \). EcoRI: \( b = f + k, c = h + k, d = f + m, e = h + m \). HpaI: \( b = f + [S], c = g + [S] \). BamHI: \( g = v + u \).
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 4.4 × 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 BamHI f/p site (Fig. 4, lane 2). A normal f + p joint fragment would have a mol. wt. of 6.9 × 10^6. We conclude that the deletion in HG52XD86 is 2.5 × 10^6 mol. wt. (3.75 kb) in size and includes the BamHI 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_m,JEll8 protein (m.c. 0.785 to 0.81).

The XbaI 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 HpaI 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 HpaI g fragment (m.c. 0 to 0.064) (data not shown). This was confirmed by a HindIII analysis which showed a deletion in both HindIII i (0 to 0.07) and j (0.75 to 0.82) with new i and j bands running between the f 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 BamHI digest and a Southern blot hybridization of a BamHI digest with nick-translated HG52 DNA and the plasmid pGZ1 (BamHI v + u) were carried out.

The BamHI digest (Fig. 5, lanes 5 and 6) showed that the BamHI p fragment was present in normal 2-molar amounts and the BamHI e and f fragments were normal in size. The BamHI g joint fragment (v + u) was missing and it appeared that it was running in two places, just above the f 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 BamHI 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 (BamHI 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 BamHI g fragment, there was no hybridization but positive hybridization was detected to two smaller fragments running at 2 × 10^6 and 2.9 × 10^6 mol. wt. band was due to an additional 'a' sequence. The deletion in TRL is thought to be of approx. 1.5 kb deleted in each g 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 BamHI v fragment.

Another isolate, HG52XD85, on initial XbaI digestion also seemed to lack XbaI 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.

XbaI 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 HpaI 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 HindIII digest which showed a missing f fragment and a new joint fragment running midway between n and m. Two new
Fig. 2. Autoradiographs of XbaI restriction digests of viral DNA (12P-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 XbaI 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 BamHI (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 BamHI 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 BamHI 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 XbaI digestion (Fig. 7, lane 1) a deletion of 9 to 9.3 kb in that the XbaI g fragment was running marginally above the i fragment (mol. wt. 5.6 × 10^6). The HpaI 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 HindIII 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 *BamHl* restriction digests of viral DNA (*³²P*-labelled *in vitro*) of the RE site mutant HG52X94 (lane 1), strain HG52 (lane 2) and the RE site mutant HG52X163 (lane 3). Letters refer to specific *BamHl* fragments; △, new fragments.
HG52XD85/4 practically the whole of HindIII j (internal repeat region) had been deleted. The HindIII o fragment appeared as normal. The BamHI 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 BamHI f/p site, the total BamHI p and v fragments excluding 'a' sequences [the molecule still inverts, e.g. HindIII 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 x 10^6. The deletion therefore extends from approximately 0.76 to 0.82 map units, including the total coding region for V_mwIE118 and the U_L/IR_L junction.
**HSV-2 deletion mutants**

Fig. 5. Autoradiographs of XbaI, HindIII and BamHI restriction digests of viral DNA $^{32}$P-labelled in vivo. Lanes 1 and 2, XbaI digests of HG52 (lane 1) and HG52XD192 (lane 2); lanes 3 and 4, HindIII digests of HG52 (lane 3) and HG52XD192 (lane 4); lanes 5 and 6, BamHI 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 XbaI treatment**

As the frequency of genome deletions isolated from XbaI-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 XbaI 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 BamHI 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 κ and γ and the joint fragment g. Lane 2 shows normal hybridization to the κ terminus from the short repeat and hybridization to the new long terminal fragments v1 and v2 and the new joint g fragment. Hybridization to bands thought to contain larger numbers of 'α' 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 32P 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.)
The DNA of the 50 HG52P4 clone stocks was digested with XbaI and EcoRI to facilitate identification of deletions in both IR1 and TR1. Twelve of the 50 clones showed alterations in both the XbaI and EcoRI 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 XbaI profile with an increased mobility of the g, e and f bands and an EcoRI profile which indicated a deletion in the EcoRI h fragment. From the change in mobility in the XbaI g fragment and the EcoRI c and e fragments (joint fragments) the size of the deletion was calculated to be between $2 \times 10^6$ and $3 \times 10^6$ mol. wt. This was more accurately determined by BamHI digestion which showed a missing f fragment, only one copy of the p fragment and a new band of mol. wt. $44 \times 10^6$ running above f. The variant HG52/5 is therefore deleted by $2.5 \times 10^6$ mol. wt. or 3.75 kb in the BamHI f and p fragments; the deletion spans the BamHI f/p site and may include the U1/IR1 junction and/or part of the coding region for VmwIE118. The size of the deletion in HG52/5 suggests that it was similar to that in HG52XD86, isolated after XbaI digestion.
Analysis of HG52/10 gave the following results. The XbaI g, e and f bands had mobilities consistent with a deletion in XbaI g but the EcoRI 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 EcoRI 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 1.5 × 10^6. The BamHI digest results were consistent with a deletion in the BamHI v fragment in both TR1 and IR1: 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 XbaI treatment of the DNA, which suggests that these variants were not generated during the XbaI 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 1.5 × 10^6 in both IR1 and TR1 has been isolated, although it is not known whether completely identical sequences have been lost from IR1 and TR1. 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 XbaI and EcoRI, one showed a deletion of 1 × 10^6 in XbaI 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 XbaI and EcoRI, no alterations were seen in any of the bands except for additional 'a' sequences on the terminal EcoRI (m and k) and XbaI (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 XbaI 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 32P. In both the XbaI and EcoRI profiles, all 50 clones were shown to be identical without detectable deletions in the genome. Therefore, deletions in TR1 or IR1 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 (ts1) of HG52. Treatment was as before (50 single plaque stocks) and DNA was restricted with XbaI and EcoRI. No deletions were detected with either enzyme. As ts1 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 XbaI and HpaI 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 BamHI f/p site, showed a reduction in the amount of VmwIE118 synthesized both in whole cells (lane 2) and nuclei (lane 8). This would support the conclusion reached from the DNA analysis: one copy of
HSV-2 deletion mutants

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 \( V_{\text{mw}} \text{IE118} \) (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 \( \text{BamHI v} \) appeared to synthesize wild-type amounts of \( V_{\text{mw}} \text{IE118} \). 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 \( V_{\text{mw}} \text{IE118} \).
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\textsubscript{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 BamHI \(v\), 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 X\_Bai 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 X\_Bai 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 X\_Bai 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 BamHI k fragment. The latter had a consistently smaller BamHI u fragment but Southern blot experiments showed that the insertion into BamHI 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 X\_Bai 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\_mIE65.

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 X\_Bai 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 U1/IR1 junction, one in the middle and one within BamHI s near the IR1/IRs 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 TR1/IR1. 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 BamHI v fragment in both TR1 and IR1 must have lost at least 150 base pairs of equivalent sequences from both copies of the repeat. When a Southern blot of a BamHI 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 $0.1 \times 10^6$ and $0.2 \times 10^6$ from wild-type, showing that a minimum of 150 base pairs within the BamHI 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 joins reflect nothing more than additional 'a' sequences, then a 1.5 kb sequence within BamHI v can be deleted and the viability of HSV retained. Sequence analysis of the BamHI s fragment from HSV-1 (equivalent to HSV-2 BamHI v) has not identified a gene coding, but it could contain control sequences similar to those upstream of the 5' end of the VmwlE110 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 VmwlE118 gene in that the amount of polypeptide synthesized was reduced compared to wild-type HG52. In contrast, the amount of VmwlE118 made by HG52XD192 was the same as wild-type, confirming that the deletion in this variant, although confined to BamHI v, is outside the VmwlE118 coding region. Interestingly, in HG52XD86 the amounts of the other IE polypeptides, Vmwl182, 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 VmwlE110 or VmwlE118 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 EcoRI 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 VmwlE110 in conjunction with VmwlE175 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 TR1/IR1 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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REFERENCES


HSV-2 deletion mutants


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