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Generation of a Herpes Simplex Virus Type 1 Variant Devoid of XbaI Sites

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SUMMARY

Using both selection enrichment and site-directed mutagenesis, a herpes simplex virus type 1 (HSV-1) strain 17 genome lacking all four XbaI sites has been generated. The site at 0.45 map units which lies within the gene coding for a polypeptide of 28,000 molecular weight was removed by selection enrichment, while the site at 0.29 map units which lies within the gene coding for glycoprotein H was removed by site-directed mutagenesis. The parental virus from which these two XbaI sites were deleted had previously had the sites at 0.07 and 0.63 map units removed through selection enrichment. The variant devoid of XbaI sites (X4) showed normal growth characteristics; its phenotype was normal apart from the absence of the thymidine kinase protein, which is believed to be unrelated to the loss of XbaI sites.

Recombination in herpes simplex virus (HSV) is poorly understood, both in terms of the role of viral and host functions (DasGupta & Summers, 1980) and of the possible effect of particular base sequences, e.g. hot spots, on recombination frequency. The contribution of different genome isomers to the recombination process is unclear, although Honess et al. (1980) suggested a circular linkage map indicating the involvement of all four isomers. Linkage maps of several temperature-sensitive (ts) mutants of HSV-1 have been constructed (Brown et al., 1973; Brown & Ritchie, 1975; Schaffer et al., 1974) and although the maps were shown to be correct in ordering these ts lesions, the distances between markers did not accord with their physical map locations, possibly due to multiple crossovers between distant markers (Stow et al., 1978). Honess et al. (1980), investigating recombination between selected and unselected pairs of markers, showed two-factor recombination frequencies of 2 to 40%. Umene (1985), following our suggestion of using restriction enzyme sites as unselected markers (Brown et al., 1984), used two HSV-1 strains differing in eight restriction enzyme sites. He demonstrated that no region of the genome had an excess of recombination and calculated an overall recombination frequency of 0.007 per kbp which concords with the values previously found in both HSV (Roizman, 1979) and adenovirus (Young & Silverstein, 1980). Whether progeny as well as parental genomes take part in recombination is controversial. Ritchie et al. (1977), on the strength of triparental crosses and time course studies, suggested that in HSV-1, progeny virus contributed to recombination whereas Ben-Porat et al. (1982) concluded from density labelling experiments that in pseudorabies virus only parental molecules were involved.

To determine the role of parental and progeny molecules, the contribution of specific genes and the role of sequence homology in recombination, we are constructing HSV-1 and HSV-2 genomes with multiple unselected markers. To this end we have been selecting genomes with deleted restriction endonuclease sites (Brown et al., 1984; Harland & Brown, 1985) and these will be used in conjunction with selected ts markers to study both inter- and intratypic recombination in HSV. This paper reports the isolation of an HSV-1 genome devoid of XbaI sites.

Wild-type HSV-1 strain 17 contains four XbaI sites at map positions 0.07, 0.29, 0.45 and 0.63 (Fig. 1a and Wilkie, 1976). By using a modification of the selection enrichment technique described by Jones & Shenk (1978) we have previously isolated the mutant X2, which lacks two of the XbaI sites, at map positions 0.07 and 0.63 (Fig. 1a and Brown et al., 1984). In order to
remove the remaining XbaI sites two approaches have been employed. First, plasmids containing HSV genomic segments spanning the two remaining XbaI sites in X2 at 0.29 and 0.45 map units, KpnI m and KpnI c respectively (Fig. 1a), were mutated by several means to remove the XbaI sites and recombined with X2 DNA by cotransfection using calcium phosphate precipitation (Stow & Wilkie, 1976). Single plaques were isolated and virus stocks were generated in BHK21/C13 cells (50 mm Petri dishes). These isolates were analysed by restriction enzyme analysis of 32P-labelled DNA (Lonsdale, 1979). Second, selection enrichment was carried out as described by Brown et al. (1984) with one modification: digestion with XbaI was from 1 to 16 h and plaques were picked from the virus-positive plate that had received DNA cleaved for the longest period of time. As well as single plaques being picked and analysed, total plate harvests were prepared from the transfected Petri dishes. DNA was isolated and subjected to a further round of XbaI digestion and transfection. In this way it was hoped to enrich for minor populations which lacked one or both XbaI sites within the total plate harvests.

In order to identify mutants lacking either the 0.29 or 0.45 XbaI sites, XbaI–BglII double digestions were carried out. XbaI alone would not discriminate between mutants lacking one or other of the sites (Brown et al., 1984). The 0.29 and 0.45 XbaI sites are contained within the BglII m and d fragments respectively (Fig. 1b). Thus BglII m, a 4 x 106 mol. wt. band is cleaved by XbaI to two smaller m' bands of around 2.1 x 106 mol. wt. and 1.9 x 106 mol. wt. which comigrate with BglII f and n respectively (Fig. 2a, lanes 1 and 2).

Initial attempts at cotransfection experiments with plasmids lacking the XbaI sites and X2 DNA failed to isolate any variants lacking either XbaI site despite analysis of several thousand plaques. Control marker-rescue experiments of ts mutants rescued these ts lesions at the expected frequency (2 to 15%), suggesting that the failure to select variants lacking these sites was not due to technical reasons. After these experiments, it became apparent that the 0.29 XbaI site was contained within the coding region of a newly recognized glycoprotein, gH (Sharp et al., 1983; Buckmaster et al., 1984; McGeoch & Davison, 1986) in which a ts lesion has been mapped (Weller et al., 1983), thus indicating an essential role for the polypeptide. One reason for our failure could be that lethal mutations were introduced into essential viral polypeptides. Recent
work in our laboratory, using recombinants between HSV-1 strain McKrae and HSV-2 strain HG52, has mapped the major HSV-1 28K protein (Marsden et al., 1976) to the region spanning the 0.45 XbaI site (S. K. Batra, personal communication).

Using the serial selection enrichment procedure, 14 plaques were isolated that had identical gel profiles differing from that of X2. They were assumed to be clonally related and one (X3) was selected as the prototype. A BgII–XbaI digestion of X3 is shown in Fig. 2(a), lane 3. The two BgII m' bands were present, demonstrating retention of the 0.29 XbaI site. However, the two BgII d' bands were absent and the BgII d band was present, demonstrating the absence of the 0.45 XbaI site. To determine the nature of the alteration leading to the removal of the 0.45 XbaI site, an EcoRI digestion was carried out. The 0.45 XbaI site is within EcoRI α, a 10^6 mol. wt. band (Fig. 1c). Cleavage of X2 and X3 by EcoRI (Fig. 2b, lanes 1 and 2) showed the mobility of the o band to be unaltered, indicating that the site loss had arisen with no detectable deletion or
Amino acid sequence  R F O t t\~ • S
Wild-type DNA sequence  CGC TTT GAT CT6 GAC GAG AGC
Mutated DNA sequence  CGC TTT GAT CT6 GAC GAG AGC
\[\text{No XbaI}\]

Fig. 3. Amino acid and DNA sequence of gH around the 0-29 XbaI site. Underneath is written the sequence of the 18-mer synthetic oligonucleotide used to mutate the XbaI site. The left hand C and right hand GC are not part of the oligonucleotide and are included to show the amino acids around the XbaI site. The A (underlined) in the XbaI site has been mutated to a T (underlined) to destroy the XbaI site. This is in the third base position of the codon and does not alter the amino acid, thus having no effect on gH.

Fig. 4. General polypeptide extracts run on 5 to 12-5% gradient polyacrylamide gels. Infected cell extracts were prepared by the method of Marsden et al. (1976, 1978). 2 × 10⁶ BHK21/C13 cells were infected at an m.o.i. of 20 p.f.u./cell in methionine-reduced Eagle's medium. At 4 h post-infection [³⁵S]methionine was added at 25 μCi/ml. Twenty-four h post-infection the cells were harvested into 0-75 ml sample buffer (50 mM-Tris-HCl pH 6-7, 2% SDS, 700 mM-2-mercaptoethanol and 10% glycerol) and run on 5 to 12-5% SDS–polyacrylamide gels. Lane 1, 17; lane 2, X2; lane 3, X3; lane 4, X4 and lane 5, mock-infected. The absent 43K polypeptide is marked ▲.

Attempts to remove the 0-29 XbaI site by selection enrichment were unsuccessful despite analysis of over 2000 plaques. At this time the coding sequence of gH containing the 0-29 XbaI site became available and was kindly supplied by Drs A. C. Minson and D. J. McGeoch. This allowed the synthesis on an Applied Biosystems synthesizer of an 18-mer oligonucleotide with a one base alteration which destroyed the XbaI site but did not alter the amino acid sequence (Fig. 3). The oligonucleotide was inserted into KpnI m (method of Oostra et al., 1983; Liang et al., 1986) which was then cotransfected with X3 and single plaques were analysed as before. Eight plaques giving identical DNA profiles were isolated. Of these one (X4) was designated as the prototype. It differed from X3 in that the two BglII m' bands were absent and the BglII m band was present (Fig. 2a, lane 4). Thus, X4 had lost the 0-29 XbaI site and contained no site for this enzyme. A BglII–XbaI digestion produced an identical profile to a BglII digestion of X2 (Fig. 2a, lanes 1 and 4) and wild-type strain 17 (not shown). That the mobility of the BglII m band in X4 was normal showed that the 0-29 XbaI site had not been lost by a detectable deletion or insertion (less than 150 bp). The difference in mobility of the EcoRI k bands of X2 and X3 (Fig. 1c and 2b) is due to variation in the number of 'a' sequences between isolates as previously described (Davison & Wilkie, 1981).
Short communication

Fig. 5. (a) One-step growth curves of HSV-1 strains 17 (△), X2 (○), X3 (□) and X4 (●) 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 containing twice the normal concentration of vitamins and amino acids, 5% (v/v) tryptose phosphate broth and 10% (v/v) calf serum and incubated at 37 °C. Cultures were harvested at 0, 2, 4, 6, 8, 12 and 24 h post-infection and the virus titre was measured by plaque assay on BHK21/C13 cells (Dargan & Subak-Sharpe, 1985). (b) Long term growth curves of HSV-1 strains 17 (△), X2 (○), X3 (□) and X4 (●) in BHK21/C13 cells. The method was as above except that an infecting multiplicity of 0.001 p.f.u./cell was used and samples were harvested at 0, 2, 4, 12, 24, 48 and 72 h and titrated on BHK21/C13 cells.

The growth characteristics of X3 and X4 are very similar to those of strains 17 and X2. A one-step growth curve in BHK21/C13 cells over 24 h (Dargan & Subak-Sharpe, 1985) showed no marked difference in growth properties between strains 17, X2, X3 and X4 (Fig. 5a). All four viruses also showed almost identical growth characteristics over several rounds of replication (Fig. 5b) and high titre stocks (10⁹ p.f.u./ml) of X3 and X4 have been obtained.
The HSV-1 variant totally lacking XbaI sites will now be used for various recombination studies, and construction of genomes lacking other more frequent restriction enzyme sites (e.g. BamHI in U5 and HindIII in L and S) is underway. This will allow the study of recombination over smaller distances. As most of the HSV-1 genome has now been sequenced (D. J. McGeoch, personal communication), site-directed mutagenesis will be preferentially used.

HSV-1 genomes lacking XbaI restriction enzyme sites have also been useful for superinfection experiments rescuing viral DNA from latent or transformed cultures (Cook & Brown, 1987). A virus lacking XbaI restriction sites allows the possibility of introducing new XbaI sites at desired positions for use as a eukaryotic vector or as a recipient for mutagenized cloned viral fragments at desired positions using adenovirus mutant dl309 as a model (Jones & Shenk, 1979; Stow, 1981).

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REFERENCES


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