Generation of a Herpes Simplex Virus Type 2 Variant Devoid of XbaI Sites: Removal of the 0.91 Map Coordinate Site Results in Impaired Synthesis of Glycoprotein G-2

By JUNE HARLAND AND S. MOIRA BROWN*

MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR, U.K.

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SUMMARY

Herpes simplex virus (HSV) type 2 (strain HG52) has four XbaI sites at map coordinates (m.c.) 0.45, 0.7, 0.91 and 0.94, i.e. two in the unique long and two in the unique short regions of the genome. Previously, we had isolated a genome containing only the 0.45 and 0.94 XbaI sites. Here we report the isolation of a mutant (JH2611) in which all four XbaI sites have been removed using an enrichment selection procedure, without any loss of viability. Removal of each site has been shown to be due to a base change or small undetectable deletion/insertion of less than 100 bp. In HSV-1, the XbaI site at 0.45 m.c. is in an open reading frame coding for a polypeptide of 14K. Both the 0.7 and 0.94 m.c. HSV-2 XbaI sites are in intergenic positions. The 0.91 m.c. XbaI site has been shown to be within the coding sequence of the glycoprotein gG-2. Synthesis of gG-2 by JH2611 and two other mutants, JH2610 (formerly HG52X163X3) and JH2609 (formerly HG52X163X21), in which the 0.91 m.c. site has been deleted was analysed by immunoprecipitation using the gG-2-specific monoclonal antibodies AP1 and LP5 and the anti-peptide serum 14 713. In the mutants JH2610 and JH2611 neither gG-2 nor its precursor were detected but the monoclonal antibodies detected two polypeptides migrating above the normal positions of gG-2 and the gG-2 precursor; these were not precipitated by the anti-peptide serum. With the mutant JH2609 neither gG-2 nor its precursors could be detected by either the monoclonal antibodies or the anti-peptide serum. The results strongly suggest that gG-2 is non-essential for the growth of HSV-2 in vitro.

INTRODUCTION

Recombination in herpes simplex virus (HSV) is poorly understood and has for the most part been studied by using temperature-sensitive (ts) mutants (Brown et al., 1973; Brown & Ritchie, 1975; Schaffer et al., 1974). However, following our suggestion of using restriction endonuclease sites as unselected markers for the study of recombination in HSV (Brown et al., 1984), Umene (1985) used two HSV type 1 (HSV-1) strains with eight restriction enzyme site differences to evaluate recombination frequency. In order to study recombination within a single strain and to use genomes which are totally homologous in genetic content apart from restriction endonuclease site differences, we have removed XbaI sites from HSV-1 strain 17 and HSV type 2 (HSV-2) strain HG52 (Brown et al., 1984; Harland & Brown, 1985; Brown & Harland, 1987; MacLean & Brown, 1987a). In conjunction with ts markers these special genomes will enable us to study the role of sequence homology in recombination by determining the distribution of restriction endonuclease sites in intra- and intertypic crosses. In addition, by studying the time course of recombination the contribution of parental and progeny molecules in multiple rounds of mating will become apparent. The introduction of ts mutations of different genes into restriction endonuclease site-negative genomes should allow determination of the role of specific genes in recombination. The present paper reports the isolation of an HSV-2 (strain HG52)
The glycoprotein gG-2 (formerly g92K) has been shown to map in the unique short (Us) region (Marsden et al., 1978, 1984; Roizman et al., 1984; Olofsson et al., 1986). Recent work by McGeoch et al. (1987) has determined the sequence of the gG-2 gene and shown that the XbaI site at map coordinate (m.c.) 0-91 lies within the coding region for the gG-2 polypeptide at residue 4785 near the carboxy terminus. Balachandran & Hutt-Fletcher (1985) have demonstrated the processing steps involved in the synthesis of gG-2 through a 120K precursor to the final processed form of 108K. The 108K polypeptide was shown to be equivalent to gG-2. The construction of mutants with changes in the XbaI site in the gene coding for gG-2 has made it possible to study the effect of these specific mutations on the production of the gG-2 glycoprotein.

**METHODS**

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

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

**Restriction endonuclease digestion of viral DNA.** Digestion of DNA at 50 to 100 μg/ml was carried out at 37-5 °C in 0·006 M-Tris–HCl pH 7·5, 0·006 M-MgCl₂, 0·006 M-²-mercaptoethanol, 0·02 M-KCl, 1 mg/ml bovine serum albumin using 1 unit of XbaI per μg of viral DNA. Incubation was for 1, 3 or 4·5 h.

**Transfection of virus DNA.** Intact and XbaI-treated DNA was transfected at 1 to 2 μg/plate onto BHK21/C13 monolayers (4 × 10⁶ cells) using the calcium phosphate infectivity assay technique (Stow & Wilkie, 1976). Single plaques obtained from transfection of XbaI-treated DNA were isolated, plaque-purified three times, grown into individual stocks and titrated.

**Restriction enzyme analysis of virus genomes.** Restriction enzyme analysis was carried out using the Linbro well technique (Lonsdale, 1979). Cells were infected in the presence of 32P at an m.o.i. of 10 p.f.u./cell of titrated virus stocks obtained from single plaques; the cells were incubated at 31 °C for 24 to 48 h. 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 electrophoresis on agarose gels of appropriate concentrations (0-5% to 1-2%). Gels were air-dried and exposed to Kodak XSI film for 24 to 48 h.

**Immunoprecipitation.** The monoclonal antibodies used were a mixture of API and LP5 (Marsden et al., 1984) which have been shown to precipitate specifically the M92K glycoprotein gG-2; for the experiments, API and LP5 were used in a 1:1 ratio. Cells (4 × 10⁶) were infected with mutant or wild-type virus at an m.o.i. of 20 p.f.u./cell and maintained in Eagle's MEM containing one-fifth of the normal methionine concentration plus 2% calf serum. Labelling was carried out from 3 to 24 h post-infection with [35S]methionine (50 μCi/ml), 100 μCi having been added to each plate. The cells were then washed twice with phosphate-buffered saline. Two ml of extraction buffer (0·05 M-Tris–HCl pH 7·2, 0·15 M-NaCl, 1% sodium deoxycholate, 0·1% SDS, 1% Triton X-100, 0·5 mM-PMSF) was added to each plate and the cells were scraped off. The suspension was sonicated, and ultracentrifuged in a T150 rotor at 35000 r.p.m. at 4 °C for 1 h. The supernatant was the antigen extract. Ten μl of the monoclonal antibody mixture was mixed with 500 μl antigen and incubated at room temperature for 30 min. Sixty μl of Protein A-Sepharose diluted 1:1 in extraction buffer was added and the mixture incubated for 2 h at 4 °C. The pellet was washed four times in extraction buffer and proteins were eluted by boiling in 100 μl sample buffer. Fifty μl of the sample was run on either a 7-5% or a 5% to 12·5% polyacrylamide gel (Marsden et al., 1978).

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

In addition, immunoprecipitation was carried out using antiserum 14713 raised against amino acids 687 to 698 of Us gene 4 (USA); these form a near C-terminal sequence but exclude the C-terminal residue itself (McGeoch et al., 1987). Confluent monolayers of BHK cells were infected with 20 p.f.u./cell of virus and labelled with 100 μCi/ml [3H]mannosone from 5 to 12 h post-infection. Immunoprecipitation was carried out as described by Frame et al. (1986) and precipitated proteins were analysed by electrophoresis in 5% to 12·5% polyacrylamide gels.

Reciprocal labelling experiments were carried out using [3H]mannosone-labelled extracts with a mixture of API and LP5 and [35S]methionine-labelled extracts with 14713.
XbaI site deletion variant of HSV-2

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; EcoRI, b = f + k, c = h + k, d = f + m, e = h + m; BamHI, g = u + v.

Nomenclature. As the number of HG52 XbaI site deletion mutants and mutants with large genomic deletions has increased to a point where the current nomenclature is too cumbersome, the mutants will from now on be designated as follows.

- HG52XD94 = JH2601
- HG52X163 = JH2602
- HG52XD86 = JH2603
- HG52XD94 = JH2604
- HG52X163/5 = JH2605
- HG52XD85/4 = JH2606
- HG52X163/5 = JH2607
- HG52X163X14 = JH2608
- HG52XD85/21 = JH2609
- HG52X163X3 = JH2610
- HG52X163X3X53 = JH2611
- HG52X163X3X45 = JH2612
- HG52X163X3X27 = JH2613

RESULTS

Removal of the 0.45 and 0.94 m.c. XbaI sites

Wild-type HG52 contains four XbaI sites at 0.45, 0.7, 0.91 and 0.94 m.c. (Fig. 1; Cortini & Wilkie, 1978) i.e. two in the long unique and two in the short unique region of the genome. Using the enrichment selection procedure described previously (Brown et al., 1984), we isolated a mutant, JH2602, lacking the XbaI site at 0.7 m.c.; the site loss was due to an approximately 150 base pair (bp) insertion as demonstrated by the altered mobility of the BamHI k fragment in which the XbaI site is situated (Harland & Brown, 1985). JH2602 was used as the parental virus from which a variant lacking the 0.91 m.c. site in addition to the 0.7 m.c. site was isolated (Brown & Harland, 1987). The variant with the two deleted sites, JH2610, had lost the 0.91 m.c. site by a base change or small undetectable deletion or insertion. The BamHI l fragment containing the 0.91 m.c. site had an unaltered mobility. A JH2610 DNA preparation was digested with XbaI (1 unit/µg DNA) for 1, 3 or 4.5 h at 37 °C. The digested DNA was transfected onto BHK21/C13 monolayers and 32 plaques were picked from two plates for each time point. The DNA from each plaque was subjected to restriction endonuclease digestion. In order to identify mutants lacking the 0.45 m.c. site it was necessary to carry out XbaI/EcoRI double digestions. XbaI alone would not clearly identify such mutants as the fragments generated would comigrate at the top of the gel. The XbaI site at 0.45 m.c. is within the EcoRI o fragment and the XbaI site at 0.94 m.c. is within the EcoRI a fragment. Thus EcoRI o, a
Fig. 2. Autoradiographs of Xbal/EcoRI digests of viral DNA $^{32}$P-labelled in vivo. Lane 1, JH2613; lane 2, JH2610; lane 3, JH2612. Letters refer to specific fragments and ▶ indicates missing fragments.

Fig. 3. Autoradiographs of restriction digests of viral DNA $^{32}$P-labelled in vivo. Lanes 1, 2 and 3, XbaI; lanes 4, 5 and 6, EcoRI; lanes 7, 8 and 9, XbaI/EcoRI. Lanes 1, 4 and 7, JH2611; lanes 2, 5 and 8, JH2610; lanes 3, 6 and 9, HG52. Letters refer to specific fragments and ▶ indicates missing fragments.

Fig. 4. Autoradiograph of a BamHI digest of viral DNA $^{32}$P-labelled in vivo: lane 1, JH2610; lane 2, JH2611.
20 × 10^6 M_r fragment, is cleaved by XbaI to two a' fragments of M_r approximately 18 × 10^6 and 2 × 10^6. The EcoRI fragment (0.8 × 10^6 M_r) is cleaved by XbaI to two o' fragments of M_r approximately 0.6 × 10^6 and 0.2 × 10^6.

Of the 96 plaques analysed, two appeared to have lost the XbaI site at 0.45 m.c. An XbaI/EcoRI digest of one of these, JH2613, is shown in Fig. 2. When the parental JH2610 was cleaved with XbaI/EcoRI it could be seen that the EcoRI a band disappeared and the two resulting a' bands comigrated with c and n (lane 2). When JH2613 DNA was cut with XbaI/EcoRI, the EcoRI a band ran in its normal position at the top of the gel (lane 1). Hence this mutant had lost the 0.45 m.c. XbaI site in addition to the 0.7 and 0.91 m.c. sites. This mutant also had a deletion in the short region of the genome such that EcoRI m comigrated with n and the two joint fragments containing m, i.e. d and e, were not in their normal position; e comigrated with f and d ran above f. The deletion was similar to that described in JH2607 (Brown & Harland, 1987). The XbaI/EcoRI analysis also revealed that 20 of the 96 plaques appeared to have lost the XbaI site at 0.94 m.c. An XbaI/EcoRI digest of one of these, JH2612, is shown in Fig. 2 (lane 3). When the parental JH2610 DNA was cut with XbaI/EcoRI, it could be seen that the EcoRI o band was missing and one o' band ran at the bottom of the gel. The other o' band of M_r 0.2 × 10^6 had migrated off the gel (lane 2). In the mutant JH2612 the EcoRI o band migrated in its normal position (lane 3). The XbaI site at 0.94 m.c. had therefore been deleted.

Restriction endonuclease analysis revealed that five of the 96 plaques had lost both the 0.45 and 0.94 m.c. sites. XbaI, EcoRI and XbaI/EcoRI digests of one of these mutants (JH2611) are shown in Fig. 3. XbaI digestion of JH2610 (lane 2) showed missing g, h, j, d, e and f bands; the i band was present and the new band running above the g position was made up of h and j. The mutant had lost the 0.7 m.c. d/g site and the 0.91 m.c. h/j site. XbaI analysis of JH2611 showed only a large band at the top of the gel (lane 1). EcoRI digestion of both JH2610 (lane 5) and JH2611 (lane 4) gave an identical pattern to that of the wild-type HG52 (lane 6). All the fragments were present and their mobilities were normal. When HG52 was cut with XbaI and EcoRI (lane 9) the EcoRI a band was cut to give two a' fragments as described for Fig. 2, the EcoRI l(baI/EcoRI) band was cut to give two l' fragments, the EcoRI n fragment was cut to give a slightly smaller n' fragment and an n' fragment which was too small to be seen on the gel, and the EcoRI o fragment was cut to give two o' fragments as also described for Fig. 2. In JH2610 (lane 8) the 0.7 m.c. XbaI site was absent, therefore the EcoRI l band was not cut and migrated normally; the 0.91 m.c. XbaI site was also absent, therefore EcoRI n migrated in its normal position. The 0.45 and 0.94 m.c. sites were present giving rise to the two EcoRI a' and two o' bands (lane 8). XbaI/EcoRI digestion of JH2611 showed the EcoRI a, l, n and o bands in their normal positions (lane 7). In other words the XbaI/EcoRI double digest was indistinguishable from the EcoRI digest, demonstrating that no XbaI site was present in the mutant. The 0.94 m.c. site loss must have been due to a base change or small undetectable deletion or insertion. Any deletion or insertion > 100 b.p. would have been detected in the mobility of the EcoRI o fragment (lane 4). The 0.45 m.c. XbaI site lies within BamHI o. It can be seen in Fig. 4 (lane 2) that the mobility of BamHI o of JH2611 was unaltered compared to the parental JH2610 (lane 1). The site loss at 0.45 m.c. was therefore due to a base change or small undetectable deletion or insertion. The mutants JH2611, JH2612 and JH2613 were all viable in tissue culture and grew normally to give titres of at least 10^8 p.f.u./ml.

Effect of removal of the 0.91 m.c. XbaI site on the US4 gene product, gG-2

The monoclonal antibodies AP1 and LP5 have been shown to immunoprecipitate specifically the polypeptide gG-2 (g92K; Marsden et al., 1984) which has been shown to be encoded by US4 of HSV-2 (McGeoch et al., 1987). The XbaI site at 0.91 m.c. lies within the coding region for gG-2 starting at residue 4785. Our analysis has led to the identification of three mutants in which the XbaI site at 0.91 m.c. has been lost. These are JH2610, JH2609 (Brown & Harland, 1987) and JH2611. The 0.91 m.c. site loss in the mutant JH2610 was shown to be due to either a base change or a small undetectable deletion or insertion in that the BamHI l fragment in which the site resides was shown to have an unchanged mobility compared to wild-type virus on restriction endonuclease analysis (JH2610 retains the XbaI sites at 0.45 and 0.94). The mutant JH2609 on
Fig. 5. Immunoprecipitation of HSV-2 glycoprotein G by the monoclonal antibodies AP1 and LP5 (Marsden *et al.*, 1984). Immunoprecipitations were carried out on extracts of [35S]methionine-labelled HSV-2-infected BHK cells and precipitated species were resolved by gel electrophoresis and detected by autoradiography. Lane 1, an extract from HSV-2-infected cells; lanes 14, 15 and 16, extracts from mock-infected cells; lanes 3, 5, 7, 9, 11 and 13, controls using normal ascitic fluid; lane 2, gG-2 from HG52-infected cells as precipitated by AP1 and LP5; lane 4, gG-2 from JH2602; lane 10, gG-2 from JH2608; lane 8, no specific precipitation from JH2609; lane 6, precipitation of two species from JH2610; lane 12, precipitation of two species from JH2611. The location of gG-2 is marked □. The absence of gG-2 is marked ◊. The two aberrant species precipitated are marked ○ and ●.

*XbaI* digestion showed a fused *XbaI* *h* and *j* fragment indicating loss of the 0·91 m.c. *XbaI* site; the mobility of the *BamHI* *l* fragment was unchanged. In addition, the information in *XbaI* *i* was replaced by the information within *XbaI* *h*. A detailed structure of this mutant genome which again retains the *XbaI* sites at 0·45 and 0·94 is shown by Brown & Harland (1987). The third mutant JH2611 was derived from JH2610 and had lost all four *XbaI* sites.

We have previously shown the one-step growth kinetics of JH2610 and JH2609 (Brown & Harland, 1987). JH2610 showed normal growth curve characteristics but JH2609 demonstrated poor single cycle growth kinetics. However, as this mutant in addition to the deleted 0·91 *XbaI* site has lost the genes US10, −11, −12, one copy of immediate early (IE) gene 3 and one copy of an origin of replication (ORI), the growth restriction cannot be attributed to a specific gene. The mutant JH2611 derived from JH2610 grew to high titre (> 10⁸ p.f.u./ml) under normal growth conditions.

To determine whether the removal of the 0·91 m.c. *XbaI* site from these mutants had any effect on the synthesis of gG-2, immunoprecipitation experiments were carried out using a 1:1 mixture of the monoclonal antibodies AP1 and LP5, both of which specifically precipitate gG-2.
Viruses used as controls were HG52, JH2602 which has the XbaI site at 0.7 m.c. deleted, and JH2608 which has a structure similar to JH2609 except that the 0.91 m.c. XbaI site has been retained. Ascites fluid was used as a further control with each mutant. The results are shown in Fig. 5. It can be seen that as expected AP1 and LP5 specifically precipitated gG-2 of HG52 (Fig. 5, lane 2) and of mutants JH2602 (lane 4) and JH2608 (lane 10). In contrast, with the mutants JH2610 (lane 6) and JH2611 (lane 12) the monoclonal antibodies did not precipitate a band in the normal gG-2 position but precipitated two novel bands of apparent higher Mr, running just above gG-2. In the mutant JH2609 (lane 8) there was no detectable specific precipitation.

To confirm these findings, immunoprecipitation experiments were carried out using the antiserum 14713 directed against the dodecapeptide of the near C-terminal portion of US4 (McGeoch et al., 1987). The label used was [3H]mannose whereas in the AP1 and LP5 experiments that used [35S]methionine. Competition controls were set up by addition of the specific peptide. Fig. 6 shows that the oligopeptide antiserum specifically precipitated gG-2 of HG52 (lane 2) and of the mutants JH2602 (lane 4) and JH2608 (lane 8). No specific precipitation was seen with the mutants JH2609 (lane 10), JH2610 (lane 6) and JH2611 (lane 12). Reciprocal labelling experiments using [35S]methionine with 14713 and [3H]mannose with AP1 and LP5 gave confirmatory results. Fig. 7 shows an immunoprecipitation of [3H]mannose-labelled extracts with AP1 and LP5. With HG52 (lane 2), JH2602 (lane 4) and JH2608 (lane 8) the monoclonal antibodies precipitated both the precursor and the processed forms of gG-2. With the mutant JH2609 (lane 10) no precipitate was detectable. With the mutants JH2610 (lane 6)
Fig. 7. Immunoprecipitation of HSV-2 glycoprotein G by the monoclonal antibodies AP1 and LP5 (Marsden et al., 1984). Immunoprecipitations were carried out on extracts of [3H]mannose-labelled, HSV-2-infected BHK cells, and the precipitated species were resolved by gel electrophoresis and detected by autoradiography. Lane 1, an extract from HG52-infected cells; lanes 14, 15 and 16, extracts from mock-infected cells; lane 2, gG-2 and its precursor from HG52-infected cells as precipitated by AP1 and LP5. Similarly, lanes 4 and 8 show gG-2 and its precursor from JH2602- and JH2608-infected cells, respectively. Lanes 6 and 12, the aberrant gG-2 precursor and a small amount of the aberrant processed form of the precursor from JH2610- and JH2611-infected cells, respectively. Lane 10, no specific precipitation from JH2609-infected cells. Lanes 3, 5, 7, 9, 11 and 13 are controls using normal ascitic fluid. Location of gG-2 is marked □ and its precursor □. The location of the aberrant precursor is marked ○ and the aberrant processed form of gG-2 is marked ●.

and JH2611 (lane 12) there was precipitation of aberrantly migrating precursor polypeptides with little detectable precipitation of the processed form.

To determine whether the apparent higher M, species precipitated from extracts of the mutants JH2610 and JH2611 were precursors of gG-2, pulse-chase experiments were carried out. The wild-type HG52 was labelled with [35S]methionine for 10 min at 5 h post-infection and samples were harvested immediately. In addition, after labelling for 10 min at 5 h post-infection, a further 5 h chase was allowed before harvesting of the sample. As controls, wild-type and mutant were labelled from 3 to 20 h with [35S]methionine. The results with HG52 using the monoclonal antibodies AP1 and LP5 are shown in Fig. 8. When a pulse was given for 10 min at 5 h post-infection with HG52, a single labelled species was specifically precipitated (lane 6). This would be equivalent to the 120K polypeptide described by Balachandran & Hurt-Fletcher (1985) under similar conditions. When the 10 min pulse was followed by a 5 h chase (lane 8), the only species specifically precipitated was the processed form of gG-2 equivalent to the 108K polypeptide seen by Balachandran & Hutt-Fletcher (1985). This migrated to the same position as gG-2 seen in a 3 to 20 h labelled infection (lane 2). As already seen in Fig. 5 when JH2611 was labelled from 3 to 20 h, an aberrant precursor polypeptide was specifically precipitated and a small amount of an aberrant processed form was also precipitated (Fig. 5, lane 4). The aberrant precursor migrated above the precursor seen in (Fig. 8, lane 6) and therefore was not the 120K polypeptide described as the normal precursor of gG-2.
XbaI site deletion variant of HSV-2

Fig. 8. Immunoprecipitation of HSV-2 glycoprotein G by the monoclonal antibodies AP1 and LP5 (Marsden et al., 1984) in a pulse–chase experiment. Immunoprecipitations were carried out on extracts of [35S]methionine-labelled, HSV-2-infected BHK cells, and the precipitated species were resolved by gel electrophoresis and detected by autoradiography. Lane 1, extract from HG52-infected cells; lanes 10, 11 and 12, extracts from mock-infected cells. Lane 2, gG-2 from HG52-infected cells labelled from 3 to 20 h post-infection; lane 4, the aberrant precursor and aberrant gG-2 from JH2611-infected cells labelled from 3 to 20 h post-infection; lane 6, the gG-2 precursor from HG52-infected cells labelled for 10 min at 3 h post-infection; lane 8, gG-2 from HG52-infected cells labelled for 10 min at 5 h post-infection and chased for 5 h in medium containing 100 times the normal concentration of unlabelled methionine plus cycloheximide (50 μg/ml). Lanes 3, 5, 7 and 9 are controls using normal ascitic fluid. Location of gG-2 is marked □ and its precursor □. The location of the aberrant precursor is marked ○ and the aberrant processed form of gG-2 is marked ●.

DISCUSSION

The 0.45 m.c. XbaI site in HSV-1 has been shown to be within an open reading frame coding for a polypeptide of 14K (D. J. McGeoch, personal communication). As the 0.45 m.c. site is coincident in HSV-1 and HSV-2 and has been lost without affecting the viability of the virus it must be assumed that either the DNA alteration causing the site loss is conservative or the gene function is non-essential. The 0.7 m.c. site lies very close to the coding region for 65K but in HSV-2 strain 333 it has been shown that the XbaI site is not within BglII i which contains the coding sequence for 65K. No sequence data are available for the 0.7 m.c. site of HSV-2 strain HG52 but as for the 0.45 m.c. site, it must be assumed that the DNA insertion causing the site loss at 0.7 m.c. is conservative, intergenic or, less likely, in the coding sequence for a non-essential polypeptide. The 0.94 m.c. XbaI site is in an intergenic position (Whitton, 1984) and therefore any DNA alteration should be of no consequence.

The XbaI site at 0.91 m.c. lies near the 3' end of US4 starting from residue 4785 of HSV-2 (McGeoch et al., 1987). Immunoprecipitation experiments using a mixture of the monoclonal antibodies AP1 and LP5 showed that in HG52 infections, gG-2 was specifically precipitated.
The mutants JH2602 and JH2608 which retained the 0.91 m.c. XbaI site also synthesized a normal gG-2. The mutants JH2610 and JH2611 which were derived from JH2602 and in which the XbaI site has been removed by a base change or a deletion/insertion of less than 100 bp, showed no specific precipitation of gG-2 but specific precipitation of two bands of higher apparent Mr. Balachandran & Hutt-Fletcher (1985) have shown that the processing of gG-2 is complex. They proposed that an unglycosylated precursor of 110K is glycosylated to give a high mannose peptide of 120K which undergoes peptide cleavage to give a product of 108K; this is equivalent to the 92K gG-2. In the pulse–chase experiment with HG52, when the virus was pulse-labelled with \(^{35}\)S methionine for 10 min at 5 h post-infection only a single species equivalent to the main 120K precursor was precipitated by the AP1 and LP5 monoclonal antibody mixture, but when the 10 min pulse was followed by a 5 h chase the single 92K polypeptide was precipitated. With the mutant JH2611 the pulse–chase experiment showed that the higher Mr species was an aberrant precursor which was inefficiently processed to the smaller polypeptide of the doublet seen in the long label infection (data not shown).

The data suggest that JH2610 and JH2611 fail to make normal gG-2 but synthesize a polypeptide either of higher Mr or of different conformation, which is inefficiently processed to give a form corresponding to gG-2 which again is either of higher Mr than normal or has a different tertiary structure. The data would be consistent with the sequence alteration removing the XbaI site at 4785, altering the reading frame such that the stop codon at residue 4939 (McGeoch et al., 1987) is not read but another stop codon downstream and in another frame is used. The only out-of-frame stop codon in one of the two alternative reading frames in the coding region is actually included in the XbaI site. There are no other out-of-frame stop codons until residues 5007 (alternative frame 1) and 5024 (alternative frame 2) which would result in either an extra 23 or 29 amino acids. Due to the frameshift, the amino acids encoded will all be changed between the destroyed XbaI site and the new stop codons. The fact that the antipeptide serum directed against the carboxy terminus of gG-2 fails to detect either the aberrant precursor or its processed form is what would be expected from this hypothesis. Upstream of the carboxy terminus of gG-2 are the sequences thought to confer the transmembrane properties of gG-2. If the normal reading frame is not used the transmembrane ability of the polypeptide may be impaired. Experiments are currently under way to determine whether the aberrant gG-2 is incorporated into virions. Until we have sequenced the US4 gene of the mutants, the exact nature of the deletion and hence its specific consequences cannot be determined.

With the mutant JH2609 neither gG-2 nor its precursors were precipitated by either the monoclonal antibody mixture or the anti-peptide serum 14713. The results obtained by the use of both sets of reagent make it unlikely that the explanation is non-recognition by all three reagents. 14713 is known to recognize the carboxy terminus of gG-2, although the precise epitopes recognized by AP1 and LP5 are unknown. The mutants JH2608 and JH2609 have essentially the same structure except for the missing XbaI site in JH2609, and thus absence of any gG-2-related polypeptide must be a direct consequence of the genomic alteration removing the XbaI site of JH2609. The unidentified sequence rearrangement which removes the XbaI site could have introduced a stop signal such that a rapidly degraded truncated form of gG-2 is made. The XbaI recognition signal is TCTAGA. Removal of one nucleotide from between T-CT or insertion there of two nucleotides bring the TAG stop codon into the reading frame. Similarly insertion of a T between A and G would produce an in-frame stop codon in the XbaI site. Other deletions or insertions which take out the site and introduce an in-frame stop codon would have the same effect. Until the sequencing data are available for JH2609, the reason for lack of detectable polypeptide can only be conjecture.

The data demonstrate that (i) a fully processed form of gG-2 is not necessary for viable growth in tissue culture and (ii) gG-2 is either dispensable or needed only in undetectable amounts in the lytic cycle of the virus. It has recently been shown that US4 of HSV-1 is non-essential in vitro (Longnecker & Roizman, 1987). Varicella-zoster virus has been shown to lack the glycoprotein gene equivalent to HSV US4 (McGeoch, 1984; Davison & McGeoch, 1986). Perhaps the lack of gG-2 in the HSV mutants could be compensated by the evolutionarily related gD (McGeoch et al., 1987).
The HG52 mutant with all four XbaI sites deleted arose at a frequency of five plaques out of 96 isolated after transfection of XbaI-digested JH2610 DNA. As the five could have been clonally related, this isolation frequency is approximately 1%. This high frequency of isolation has been maintained throughout the study on the removal of XbaI sites from HG52 (Harland & Brown, 1985; Brown & Harland, 1987). In addition, deletions in the long repeat regions of HG52 occur at a frequency of 24% and in the short repeat/unique region at a frequency of 7% (Harland & Brown, 1985; Brown & Harland, 1987).

In HSV-1 strain 17, genomes with deleted XbaI sites at 0·07 and 0·63 m.c. were isolated at a frequency of 3·6% (Brown et al., 1984). However, genomes lacking the 0·29 m.c. XbaI site (which lies within the gH gene) were not detected despite over 2500 plaques being screened after enrichment selection. The 0·29 m.c. XbaI site was eventually disrupted by insertion of a synthetic oligonucleotide (MacLean & Brown, 1987a). The XbaI site at 0·45 m.c. in strain 17 was shown to be deleted in 14 out of 196 plaques screened after a single transfection of XbaI-treated DNA. Again as these plaques could have been clonally related this represents an isolation frequency of approximately 0·5%. However, this mutant was detected only after screening over 2500 plaques for the loss of the 0·45 m.c. site. Similarly, in screening HSV-1 strain 17 plaques for genomic deletions, only four genomes with deletions have been isolated from over 5000 plaques tested. These mutations occurred around the Us/IRs junction (MacLean & Brown, 1987b,c). It is concluded that genomic alterations, i.e. base changes, deletions and insertions, occur at a much higher frequency in HSV-2 strain HG52 than in HSV-1 strain 17.

The isolation of JH2611 will allow its use in intratypic and intertypic recombination studies and as a eukaryotic vector or recipient for viral plasmids cloned into desired positions, similar to adenovirus (Jones & Shenk, 1978; Stow, 1981). The role of restriction endonuclease site-negative mutants in superinfection experiments has already been exploited (Cook & Brown, 1987) and they are currently being used to study recombination in vivo.

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REFERENCES


BROWN, S. M. & HARRLAND, J. (1987). Three mutants of herpes simplex virus type 2: one lacking the genes US10, US11 and US12 and two in which Rs has been extended by 6 kb to 0·91 map units with loss of Us sequences between 0·94 and the Us/IRs junction. Journal of General Virology 68, 1–18.


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