A Variant of Herpes Simplex Virus Type 2 Strain HG52 with a 1.5 kb Deletion in R₀ between 0 to 0.02 and 0.81 to 0.83 Map Units Is Non-neurovirulent for Mice

By MAHMOUD Y. TAHA, GEOFFREY B. CLEMENTS and S. MOIRA BROWN

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

(Accepted 15 November 1988)

SUMMARY

The virulence of a deletion variant of herpes simplex virus type 2 (HSV-2) strain HG52 has been determined by intracranial inoculation of 3-week-old BALB/c mice. The variant JH2604 has a 1.5 kb deletion within each copy of the long repeat region (R₀) of the genome between 0 to 0.02 and 0.81 to 0.83 map units. JH2604 is avirulent for mice compared to the parental wild-type virus, and fails to replicate in mouse brain in vivo. Correction of the deletion by marker rescue resulted in the isolation of recombinants which gave LD₅₀ values comparable to those of individual plaque stocks of the parental HG52. Introduction of the deletion into wild-type virus resulted in recombinants which on intracranial inoculation of mice were avirulent. The results imply that sequences within the 3 kb terminal portion of R₀ are required for virulence of HSV-2 strain HG52.

INTRODUCTION

The identification of genes involved in viral pathogenicity and the elucidation of their precise functions is of fundamental importance to the understanding of the biology of herpes simplex virus (HSV). Many host factors have been shown to influence virulence including animal age (Kohl & Loo, 1980), route of inoculation (Caspary et al., 1980) and strain of experimental animal (Lopez, 1975). Virus strain (Dix et al., 1983) and the consequences of serial passage of virus in vivo (Kaerner et al., 1983) and in vitro (Goodman & Stevens, 1986) have also been implicated.

Mutation in or deletion of the viral thymidine kinase (TK) gene has been shown to impair virulence (Field & Wildy, 1978) and viral sequences between 0.7 and 0.83 map units (m.u.) have been shown to be involved in determining the virulence phenotype (Thompson & Stevens, 1983; Thompson et al., 1985; Javier et al., 1986; Rosen et al., 1986). Sequences between 0.25 and 0.53 m.u. have also been implicated (Thompson et al., 1986) as has the viral DNA polymerase (Field & Coen, 1986).

We have isolated and characterized a number of variants of both HSV type 1 (HSV-1) (strain 17) and HSV-2 (strain HG52) with defined deletions in the unique and repeat sequences of both the long and short regions of the viral genome (Brown et al., 1984; Harland & Brown, 1985; Brown & Harland, 1987; MacLean & Brown, 1987a, b). Availability of variants with deletions in specific regions of the viral genome affords a unique opportunity to determine the possible role of specific sequences in both latency and virulence.

Heterogeneity in the neurovirulence of plaque-purified stocks of the parental HSV-2 strain HG52 has recently been reported (Taha et al., 1988). A baseline was thus established from which to evaluate the neurovirulence of one variant, JH2604 (HG52XD192) (Harland & Brown, 1985), which has a deletion of approx. 1.5 kb within each copy of BamHI v (0 to 0.02 and 0.81 to 0.83 m.u.). Restriction endonuclease analysis and the lack of sequencing data for this region of the HG52 genome has not enabled determination of whether totally identical sequences have been removed from both the terminal repeat (TR₀) and internal inverted repeat (IR₀) or
whether the deleted sequences are contiguous. Intracranial inoculation of the variant JH2604 has shown it to be avirulent for mice, which implies that sequences within the 3 kb terminal portion of R\textsubscript{L} are required for virulence of HSV-2 strain HG52.

**METHODS**

*Cells.* Baby hamster kidney clone 13 cells (BHK-21 C-13) (Macpherson & Stoker, 1962) were propagated in Eagle's medium containing twice the normal concentration of vitamins and amino acids, 5% (v/v) tryptose phosphate broth and 10% (v/v) calf serum (ETC 10). BALB/c mouse embryo fibroblasts (3T3) cells (Flow Laboratories) were grown in Dulbecco's modified Eagle's medium containing 10% newborn bovine serum and 2% (v/v) glutamine.

*Viruses.* Virus stocks were grown and titrated in BHK-21 C-13 cells as previously described (Brown et al., 1973). The parental virus strain was HSV-2 HG52 (Timbury, 1971) and the HSV-2 deletion variant used was JH2604 (Harland & Brown, 1988), characterized (Harland & Brown, 1985) under its previous designation of HG52XD192.

*Restriction enzyme analysis of virus genomes.* Restriction enzyme analysis was carried out by a modification of the technique of Lonsdale (1979). BHK-21 C-13 cells (5 × 10\textsuperscript{6}) were infected at an m.o.i. of 10 p.f.u./cell in the presence of \textsuperscript{32}P in phosphate-free Eagle's medium containing 1% (v/v) calf serum and incubated at 31 °C for 48 h. Viral DNA was extracted with SDS and phenol, and ethanol-precipitated. The DNA was treated with various restriction enzymes using the manufacturers' recommended conditions. Digests were analysed by electrophoresis on agarose gels of the appropriate concentrations (0.5 to 0.8%) in TBE buffer (89 mM-Tris, 89 mM-boric acid, 2 mM-EDTA). Gels were air-dried and exposed to Kodak X1S film. Three rounds of plaque purification were carried out prior to further restriction enzyme analysis.

*Animal inoculation.* Three-week-old BALB/c mice (Bantin & Kingman) were inoculated intracranially with individual virus stocks. Mice were anaesthetized with ether and 0.025 ml of the appropriate virus dilution in phosphate buffered saline (PBS)/5% calf serum was inoculated into the central region of the left cerebral hemisphere. Four to eight mice were inoculated with each virus at a dose of between 10\textsuperscript{2} and 10\textsuperscript{7} p.f.u./animal. The virus stocks were always retitrated on BHK-21 C-13 cells on the day of inoculation to determine the precise titre inoculated. Mice were observed daily for 21 days after inoculation; the clinical state of the mice was recorded and the LD\textsubscript{50} calculated according to the formula of Reed & Muench (1938) on the basis of deaths between day 3 and day 21.

*Virus growth properties in vitro.* One-step growth experiments were carried out as described by Brown & Harland (1987). Confluent BALB/c mouse embryo fibroblast monolayers (3T3) (2 × 10\textsuperscript{6} cells) were infected at an m.o.i. of 5 p.f.u./cell and incubated at 37 °C or 38.5 °C. Samples were harvested at specific times post-infection and titrated for virus yield on BHK-21 C-13 cells at 37 °C.

*Virus growth properties in vivo.* Virus stocks (10\textsuperscript{6} p.f.u.) were inoculated into the left cerebral hemisphere of 3-week-old BALB/c mice (both sexes). At 0, 12, 24, 72, 96, 120 and 144 h, post-infection, two surviving mice from each time point were sacrificed and their brains were removed and frozen at −70 °C. After thawing, the brains were homogenized in 1 ml PBS/5% calf serum using a Dounce homogenizer, and 0.2 ml of the resulting suspension was titrated for infectious virus on BHK-21 C-13 cells at 37 °C.

*Infected cell polypeptide analysis.* The method used was essentially that described by Marsden et al. (1976). Confluent BHK-21 C-13 cells were infected at an m.o.i. of 20 p.f.u./cell. After adsorption for 1 h at 37 °C, the monolayers were washed twice with Eagle's medium containing 20% normal concentration of methionine plus 2% calf serum, and the same medium was used to overlay the monolayers. After incubation at 37 °C for 3 to 4 h, 100 μCi/plate of \textsuperscript{35}S methionine was added and the incubation was continued at 37 °C for 24 h. Samples were harvested into 300 μl sample buffer (150 mM-Tris-HCl pH 6.7, 6.28% w/v SDS, 0.15% v/v 2-mercaptoethanol, 0.31% v/v glycerol, 0.1% bromophenol blue) and after boiling for 5 to 10 min, they were electrophoresed on single concentration polyacrylamide gels.

*Correction of genome deletion in variant JH2604.* The variant JH2604 has an approx. 1.5 kb deletion in both copies of R\textsubscript{L} within the BantHI \textsubscript{e} fragment (0 to 0.02 and 0.81 to 0.83 m.u.) (Fig. 4). In an attempt to correct the deletion by marker rescue, three wild-type Xba\textsubscript{I} fragments were used in cotransfection experiments with intact JH2604 genomes. The Xba\textsubscript{I} fragments used were g (0-7 to 0.83), e (g + h) (0-83 to 0.91) and f (g + i) (0-94 to 1-0). The method used was a modification of that of Stow & Wilkie (1976) and the fragments were used at a five-, 10- and 20-fold molar excess over the intact JH2604 genome. The wild-type HG52 DNA was extracted from plaque-purified stock, designated plaque 17, which has been shown to be of high neurovirulence for BALB/c mice (Taha et al., 1988). The digested DNA was run in a 0.5% agarose gel from which the required fragments were excised; the DNA was electroeluted, purified with phenol and precipitated with ethanol using the standard procedures. Resulting individual plaques from cotransfections were isolated and their DNA was analysed by the method of Lonsdale (1979). Three rounds of plaque purification were carried out before growing a virus stock.

*Introduction of the JH2604 deletion into HG52 plaque 17.* To introduce the deletion in JH2604 into HG52 plaque 17 DNA, three Xba\textsubscript{I} fragments (g, e and f) from JH2604 were independently transfected at a 10- and 20-fold molar
HSV-2 \( R_L \) sequences are required for virulence

excess with intact DNA from HG52 plaque 17. Resulting individual plaques from cotransfections were isolated and their DNA was analysed by the method of Lonsdale (1979). Virus which appeared to have acquired the deletion was plaque-purified three times before growing a virus stock.

**Thymidine kinase assay.** The method used was a modification of that of Jamieson & Subak-Sharpe (1974). BHK-21 C-13 cells (5 \( \times \) 10⁵) were mock-infected or infected with wild-type or mutant virus at an m.o.i. of 5 p.f.u./cell. After absorption for 1 h and incubation for 6 h at 37 °C, the cells were scraped into 1 ml cold PBS and pelleted. The pellet was resuspended in 150 μl lysis buffer (20 mM-Tris-Cl pH 7.5, 2 mM-MgCl₂, 10 mM-NaCl, 0.5% v/v Nonidet P40, 6.5 mM-2-mercaptoethanol), maintained on ice for 5 min, mixed briefly and replaced on ice for a further 5 min. The samples were then centrifuged and the supernatant was retained. Five μl of extract was mixed with the reaction buffer in a total volume of 50 μl (0.5 mM-Na₃PO₄ pH 6, 100 mM-MgCl₂, 2 mM-dTTP, 100 mM-ATP, 5 μM aqueous [Me⁻³H]thymidine 1 mCi/ml). After incubating for 1 h the reaction was stopped by the addition of 10 μl of 100 mM-EDTA and 1 mM-thymidine. The samples were heated for 3 min at 100 °C and placed on ice for 3 min. After centrifugation, 40 μl of the supernatant was spotted onto DE81 discs which were washed three times (10 min each at 37 °C) with 4 mM-ammonium formate pH 6.0 and 10 μM-thymidine. After a further two washes with ethanol, the discs were dried and radioactivity due to [³H]thymidine was determined.

**RESULTS**

**Neurovirulence of the deletion variant JH2604 (HG52XD192) for BALB/c mice**

The DNA structure of the deletion variant JH2604 has been described in detail by Harland & Brown (1985). Essentially the genome bears a deletion of approx. 1.5 kb in both copies of \( \text{BamHI} \), located between 0 to 0.02 and 0.81 to 0.83 m.u. in \( \text{TRL} \) and \( \text{IRL} \) respectively. To determine the neurovirulence of this variant compared to the parental HG52 virus, experiments were carried out to estimate the LD₅₀ values of BALB/c mice. Twenty-five μl aliquots of different doses of HG52 and JH2604 were inoculated into the left cerebral hemisphere of 3-week-old BALB/c mice of each sex. Deaths from encephalitis were scored between day 3 and day 21 post-inoculation and the LD₅₀ values are shown in Table 1.

The elite parental stock of HG52 without further plaque purification showed an LD₅₀ of < 10⁶ p.f.u.; at doses of 10⁷ p.f.u./animal only one animal out of nine died and no others showed signs of encephalitis or any other symptoms of illness or distress. Also, two mice infected with 10⁸ p.f.u. of JH2604 remained healthy after inoculation. Thus the deletion variant JH2604 was at least 10⁶-fold less neurovirulent than the parental HG52 virus stock. The particle : p.f.u. ratios of 54 : 1 for HG52 and 71 : 1 for JH2604 are comparable and fall within the normal range of values for HSV-2.

**Growth of JH2604 in vivo**

To determine whether the low neurovirulence of JH2604 was due to failure of replication in mouse brain, the growth of the virus in vivo was tested. Samples of HG52 and JH2604 (10⁵ p.f.u./mouse) were inoculated into the left cerebral hemisphere of 3-week-old BALB/c mice of each sex. At various times post-infection, two mice were killed and their brains frozen at ~ 70 °C. The brain tissue was homogenized, and the resulting suspension was sonicated and assayed by plaque titration on BHK-21 C-13 cells at 37 °C. The results plotted in Fig. 1 show that

**Table 1. Neurovirulence of HG52 and JH2604 in BALB/c mice**

<table>
<thead>
<tr>
<th>Virus</th>
<th>10²</th>
<th>10³</th>
<th>10⁴</th>
<th>10⁵</th>
<th>10⁶</th>
<th>10⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG52*</td>
<td>5/12</td>
<td>8/12</td>
<td>12/12</td>
<td>6/6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>JH2604*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0/4</td>
<td>0/4</td>
<td>1/9</td>
</tr>
</tbody>
</table>

* Particle : p.f.u., 54 : 1.
† Number of deaths/number of animals inoculated.
‡ Particle : p.f.u., 71 : 1.
§ ND, Not determined.
Fig. 1. *In vitro* growth kinetics of HG52 and JH2604. HG52 and JH2604 were inoculated into the left cerebral hemisphere of 3-week-old BALB/c mice of either sex (10⁵ p.f.u./mouse). At 0, 12, 24, 72, 96, 120 and 144 h post-infection, two surviving mice for each time point were sacrificed. The brain tissue was homogenized, the resulting cell suspension sonicated and released infectious virus titrated on BHK-21 C-13 cells at 37 °C. O, HG52; J, JH2604.

Fig. 2. Single cycle growth kinetics of HG52 (O) and JH2604 (●). Mouse embryo fibroblasts (3T3 cells) were infected at an m.o.i. of 5 p.f.u./cell with HG52 and JH2604. At various times post-infection at 38.5 °C the infected cells were harvested, and virus was released by sonication and titrated on BHK-21 C-13 cells at 37 °C.

The parental HG52 grew well in mouse brain with the titre continuing to rise at 120 h post-inoculation; by 144 h all the mice had died from encephalitis. In contrast, the variant JH2604 failed to show significant replication and the titre diminished progressively with time.

**Growth of JH2604 in vitro**

The deletion variant JH2604 grows like wild-type virus in a single-cycle growth experiment in BHK-21 C-13 cells at 37 °C (Harland & Brown, 1985); the parental elite stock of HG52 is somewhat temperature-restricted at 38-5 °C (Brown & Harland, 1987) in that, though displaying exponential growth, the highest titre of virus reached by 12 h post-infection is not appreciably higher than the starting titre immediately post-absorption. The variant JH2604 is not temperature-sensitive in the classical sense but is impaired in single-cycle growth experiments at 38.5 °C to the same extent as the parental HG52. The titre of the JH2604 stock used for all experiments was 4 × 10⁷ p.f.u./ml at 31 °C and 3 × 10⁷ p.f.u./ml at 38.5 °C.

To determine whether JH2604 was temperature-sensitive in mouse cells at the accepted ambient body temperature of mice, i.e. 38.5 °C, comparative one-step growth experiments were carried out in BALB/c mouse embryo fibroblasts (3T3 cells) at 38.5 °C, and virus yield was titrated on BHK-21 C-13 cells at 37 °C. Results are given in Fig. 2 and show that the parental HG52 initially replicated poorly, reaching a titre comparable to the input titre by 8 h post-infection and then diminishing. The mutant JH2604 showed little evidence of replication and
HSV-2 $R_l$ sequences are required for virulence

Table 2. TK assays on HG52, JH2604 and TK$^{-7}$

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-infected</td>
<td>2573</td>
</tr>
<tr>
<td>TK$^{-7}$ (17)</td>
<td>2216</td>
</tr>
<tr>
<td>HSV-2 (HG52)</td>
<td>8713</td>
</tr>
<tr>
<td>JH2604</td>
<td>9265</td>
</tr>
</tbody>
</table>

the virus titre progressively fell with time. In a multi-step growth experiment performed at 37 °C in 3T3 cells, again the parental HG52 demonstrated some replication and reached a low peak titre by 24 h post-infection. The variant JH2604 failed to replicate and the infective titre dropped with time (data not shown).

It is concluded that BALB/c 3T3 cells are poor hosts for replication of HSV-2 (HG52) although some replication of wild-type virus does occur; these cells appear to be non-permissive for the deletion variant JH2604 both at 37 °C and 38.5 °C.

Infected cell polypeptide synthesis

It has been shown previously that JH2604 synthesizes normal amounts of the HSV-induced immediate early polypeptides (Harland & Brown, 1985). To determine whether the deletion in JH2604 affected any other polypeptide synthesis, the infected cell polypeptides of HG52 and JH2604 were labelled with $[^{35}S]$methionine and analysed by SDS-PAGE. There were no detectable differences between the infected cell polypeptides synthesized by HG52 and by JH2604 (data not shown).

TK assay

To determine whether the lack of neurovirulence of JH2604 was due to a mutation in TK synthesis, TK assays were performed on HG52, JH2604 and a known TK-negative mutant (TK$^{-7}$). The results are shown on Table 2. With the TK$^{-7}$ mutant the $^3$H c.p.m. were marginally below the background (mock-infected) levels in both experiments, while the wild-type HG52 gave counts of 3.4 and 2.4 times background levels and variant JH2604 gave counts of 3.6 and 3.0 times the mock-infected levels. Thus JH2604 is at least as efficient as HG52 in synthesizing TK.

Correction of the deletion in JH2604

Purified DNA from the neurovirulent plaque 17 of HG52 ($LD_{50} < 10^5$ p.f.u.) (Taha et al., 1988) was digested with XbaI, the $g$, $e$ and $f$ fragments were excised and after purification by electroelution were used independently in cotransfection experiments with intact JH2604 DNA in order to correct the deletion in JH2604 by marker rescue. The $M_{r}$ of fragment XbaI $g$ (0.7 to 0.83 m.u.) was $13 \times 10^6$, that of XbaI $e$ ($g + h$ 0.83 to 0.91) was $21 \times 10^6$ and of $f$ ($g + i$ 0.94 to 1.0) was $19 \times 10^6$. In the cotransfection experiments the fragments were used at a five-, 10- and 20-fold molar excess to the intact JH2604 DNA. Although the deletion in JH2604 is only $1 \times 10^6$ $M_{r}$ (1.5 kb) in both copies of BamHI $v$, these large XbaI fragments were used to facilitate recombination between the fragment and the intact genome (the larger the fragment, the greater the chance of recombination occurring). No selection system was available to isolate the sought recombinants between the intact JH2604 genomes and the wild-type fragments.

Two-hundred plaques were picked, and the DNA of 46 of these was digested with XbaI; four of these digests showed profiles indicating that the deletion in XbaI $g$ had been corrected, as the $g$, $e$ and $f$ bands ran in the normal wild-type position. Three were from cotransfections using a 20-fold molar excess of XbaI $e$ and the fourth from a plate in which XbaI $f$ had been cotransfected at a 20-fold molar excess with JH2604 DNA.

After three rounds of plaque purification, XbaI digestion confirmed that recombination had occurred and that the deletion in JH2604 had been corrected. The recombinants were designated R17(192)E pl. 8, R17(192)E pl. 14, R17(192)E pl. 15 and R17(192)F pl. 10. The XbaI
Fig. 3. Autoradiographs of restriction digests of viral DNA 32P-labelled in vivo. XbaI digestion of HG52 (lane 1), R17(192)E pl. 8 (lane 2), R17(192)E pl. 14 (lane 3), R17(192)E pl. 15 (lane 4), R17(192)F pl. 10 (lane 5) and JH2604 (lane 6). Letters refer to specific fragments, arrowheads indicate the positions where fragments are missing and e', f', g' indicate novel fragments.

Fig. 4. Restriction endonuclease maps for the DNA of HSV-2 strain HG52, from Cortini & Wilkie (1978).

profiles of the four recombinants are shown compared to HG52 and JH2604 in Fig. 3. The relevant restriction maps for HG52 are also shown in Fig. 4 (Cortini & Wilkie, 1978).

XbaI digestion demonstrated that the deletion had been corrected only in IRb, i.e. within XbaI g. The size of the other XbaI fragment containing BamHI v (i.e. c, 45 × 10⁶ Mv) is such that an
HSV-2 R<sub>L</sub> sequences are required for virulence

Fig. 5. Autoradiographs of restriction digests of viral DNA <sup>32</sup>P-labelled in vivo. HpaI (lanes 1 to 6), HindIII (lanes 7 to 12), HG52 (lanes 1 and 7), JH2604 (lanes 6 and 12), R17(192)E pl. 8 (lanes 2 and 8), R17(192)E pl. 14 (lanes 3 and 9), R17(192)E pl. 15 (lanes 4 and 10), R17(192)F pl. 10 (lanes 5 and 11). Letters refer to specific fragments, arrowheads indicate the positions where fragments are missing and f', g' etc. indicate novel fragments.

alteration in size of Mr 1 x 10<sup>6</sup> would not be detectable. Therefore HindIII and HpaI digestion was carried out. The results are shown in Fig. 5. BamHI v in TR<sub>L</sub> is located within HpaI g, and BamHI v in IR<sub>L</sub> is located within HpaI f. The variant JH2604 shows f and g deleted by approx. 1 x 10<sup>6</sup> (Fig. 5, lane 6) compared to f and g of HG52 (lane 1). The recombinants show that HpaI f and g have returned to their wild-type positions (lanes 2 to 5). BamHI v in TR<sub>L</sub> is located within HindIII i, and in IR<sub>L</sub> within HindIII j. Therefore with the variant JH2604, HindIII i and j and the i- and j-containing joint fragments i.e. c, d, f and g (lane 12) are deleted compared to the HG52 fragments (lane 7). In the recombinants, i, j, c, d, f and g have all returned to their wild-type position (lanes 8 to 11). The HpaI and HindIII digests demonstrate that the deletion has been corrected in both copies of BamHI v i.e. in TR<sub>L</sub> and IR<sub>L</sub>.

Neurovirulence of the R17(192) recombinants compared to HG52

The three-times plaque-purified recombinants were inoculated intracranially into 3-week-old BALB/c mice at titres ranging from 10<sup>2</sup> to 10<sup>5</sup> p.f.u. as described previously. As controls, the highly neurovirulent plaque 17 stock of HG52 and the JH2604 variant were used at titres ranging from 10<sup>2</sup> to 10<sup>5</sup> and 10<sup>5</sup> to 10<sup>7</sup> p.f.u. respectively. The results given in Table 3 show that all four recombinants have regained neurovirulence with somewhat variable levels ranging from 10<sup>3</sup> to 3 x 10<sup>5</sup> p.f.u. These virulence levels are within the range of the LD<sub>50</sub> levels of the individual plaque stocks of HG52 (10<sup>3</sup> to 3 x 10<sup>5</sup>) (Taha et al., 1988) and suggest the occurrence of genetic variation outside the deletion region which modifies virulence. The avirulent phenotype of JH2604 has been confirmed with an LD<sub>50</sub> of > 10<sup>7</sup> p.f.u. The avirulent phenotype has therefore been corrected by insertion of wild-type sequences replacing the deletion in both copies of BamHI v.

Insertion of the deletion in JH2604 into wild-type HG52 and neurovirulence of the resulting deletion variants

Purified DNA from JH2604 was digested with XbaI, and the three fragments g, e and f containing the deletion within BamHI v were excised and purified from the gel. The three
fragments were independently used in cotransfection experiments with intact DNA from HG52 plaque 17 (high neurovirulence). Again, no selection system was used to isolate recombinants when 200 plaques were picked. The DNA of 40 of these was digested with XbaI (Fig. 6), and two showed XbaI profiles indicating that the fragments containing the deletion had recombined into the wild-type genome (lanes 2 and 3) in that the g, e and f' bands were now running at the position of the equivalent bands in JH2604 (lane 4) and not in their normal wild-type position (lane 1). HpaI and HindIII digestion (Fig. 7) confirmed that the deletion was in both copies of RL in that HpaI g and f (lanes 2 and 3) and HindIII i, j, c, d, f and g (lanes 6 and 7) were running below their normal wild-type positions (lanes 1 and 5). The recombinants were plaque-purified.
three times, their restriction profiles confirmed and virus stock was grown. The recombinants were designated R192(17)E pl.2 and R192(17)E pl.5.

The two recombinants R192(17)E were injected intracranially into 3-week-old BALB/c mice. Control infections with HG52 plaque 17 and JH2604 were performed at the same time. The results are shown in Table 3. It can be seen that each of the recombinants had LD50 values of > 10^7 p.f.u., i.e. the same as that of the non-neurovirulent deletion variant JH2604.

**DISCUSSION**

The nervous system is a critical target organ for HSV infection. The relationship between HSV and the peripheral nervous system is usually benign, in that the virus remains latent in the neurons of dorsal root ganglia from which it reactivates intermittently. However, when HSV invades the central nervous system the likely outcome is a fatal encephalitis. It is therefore important to determine those genes of HSV which predispose it to invade and destroy nervous tissue. Viral genes controlling neurovirulence were only identified recently, and various laboratories have predominantly implicated the viral sequences between 0.7 to 0.83 m.u. in the control of virulence (Thompson & Stevens, 1983; Thompson et al., 1985; Javier et al., 1987; Rosen et al., 1986). This region contains the whole of IRa and part of UL. The genes encoded therein are one copy of IE1 and UL50 to UL56 (Clements et al., 1979; Perry et al., 1986; Whitton et al., 1983; McGeoch et al., 1988). The mapping of virulence genes to this region of the genome has for the most part relied on the construction of recombinants between low and high virulence strains of HSV using specific cloned viral fragments (Thompson & Stevens, 1983; Thompson et al., 1985). Deletion mutants in the HSV genome allow defined regions to be studied for their neurovirulence properties. A mutant of HSV-1 strain HFEM (Halliburton et al., 1980) with a 4 kb deletion between 0.76 and 0.79 m.u. (Rosen & Darai, 1985) has been shown to be non-pathogenic for tree shrews and mice, and its pathogenicity can be restored by the insertion of sequences spanning 0.76 to 0.79 m.u. from the pathogenic strain F (Rosen et al., 1986).

Before studying biological properties such as latency and virulence of defined deletion variants in animal model systems, it is important to determine the phenotype of the parental wild-type virus. We have recently shown that the elite stock of HSV-2, HG52, used in our institute has an LD50 of < 10^2 p.f.u. on intracranial inoculation of 3-week-old BALB/c mice. However, when 10 individual plaques were isolated at random from this stock, plaque-purified three times and a virus stock was grown up from each, the LD50 values of these 10 re-plaqued stocks exhibited individual values ranging from < 10^3 to 3 x 10^5 p.f.u. (Taha et al., 1988), demonstrating a marked heterogeneity in virulence within the original unselected virus stock.

The deletion variant JH2604 has a 1.5 kb deletion in both copies of *BamHI v*. As no sequencing data for this region of HG52 are available, the location of the deletion relied solely on restriction endonuclease analysis. The same sequences could be deleted from each copy but the size of the deletion and the size of *BamHI v* (3 kb) does not preclude the deleted sequences being at least in part contiguous (Harland & Brown, 1985). The results presented in this paper have demonstrated that JH2604 is avirulent on intracranial inoculation of 3-week-old BALB/c mice (LC50 > 10^7 p.f.u.) compared with the plaque-purified plaque 17 stock of HG52 (< 10^3). JH2604 and its parental HG52 grew equally well in BHK-21 C-13 cells at 37 °C and JH2604 is no more temperature-restricted at 38.5 °C than HG52. The variant failed to grow in BALB/c mice-derived 3T3 cells at both 37 °C and 38.5 °C, but as the wild-type showed poor replication in these cells the significance of apparent lack of growth of JH2604 is unclear. Of more importance is the finding that JH2604 does not replicate *in vivo* in mouse brain. The wild-type virus grows well within the brain at the accepted ambient mouse temperature of 38.5 °C, reaching peak titres by 120 h post-infection with all the experimentally infected animals dying by 144 h post-infection. JH2604 showed some replication by 12 and 24 h, but by 48 h the amount of infectious virus recoverable from infected brains was over 1 log_{10} unit lower than the input virus measurable immediately post-absorption. Viral functions required for neurovirulence may modify the process in a variety of ways, e.g. by affecting replication at the peripheral site, at neural entry, spread within the nervous system and by facilitating escape from immunological surveillance.
The intracranial route of inoculation, although artificial, has the advantage of bypassing most of these steps. The brain pathology induced by HG52 and the JH2604 variant has been studied and is being reported in a separate communication; suffice to say that JH2604-infected brains demonstrate no gross pathology.

The TK phenotype of the variant JH2604 when compared to that of the parental HG52 was found to be equally efficient. The low neurovirulence and lack of growth in mouse brain is therefore not a result of the virus being TK-. Support for this conclusion comes from the finding that JH2604 is capable of establishing latency in the dorsal root ganglia of mice (G. B. Clements, unpublished data).

Cotransfection of intact DNA of JH2604 and XbaI fragments of HG52 plaque 17 containing sequences deleted from JH2604 resulted in the isolation of recombinants in which the deleted sequences had been replaced by equivalent sequences from the XbaI fragment. The isolation of the recombinants enabled us to determine whether the replaced sequences had a direct effect on neurovirulence. From 28 mice infected with R17(192)E pl. 8, the LD₅₀ value of the recombinant was calculated to be 2 × 10⁵ p.f.u.; from 40 mice infected with R17(192)E pl. 14, 1 × 10⁵ p.f.u.; from 28 infected with R17(192)E pl. 15, 3 × 10⁵ p.f.u.; and from 46 infected with R17(192)F pl. 10, 3 × 10⁵ p.f.u. These values are within the range (10⁴ to 3 × 10⁵) of the individual plaque stocks of HG52 (Taha et al., 1988). The deletion in JH2604 was corrected by the use of fragments from the neurovirulent plaque 17 of HG52 (LD₅₀ < 10³). The range of LD₅₀ values obtained with the recombinants in which the deletion has been corrected suggest either that (i) there are sequences apart from those deleted in JH2604 which act either in conjunction with or independently of those between 0 to 0.02 and 0.81 to 0.83 m.u. to determine neurovirulence or (ii) the DNA stock of HG52 plaque 17 is heterogeneous, in that viruses arising from individual genomes have different virulence potentials. The latter would mean that the virus population of HG52 plaque 17 despite its recent purification contains a genome mixture with virulence LD₅₀ values similar to those of the individual plaque stocks of the elite HG52 laboratory stock (< 10³ to 3 × 10⁵). To determine whether this was the case, a DNA stock of HG52 pl. 17 was transfected and five well separated progeny plaques were isolated. Virus stocks from the five plaques were inoculated into mice as before and the LD₅₀ values were determined for each; these values were < 10², < 10³, 10⁴ and > 10⁴ p.f.u., confirming the virulence heterogeneity within the stock of HG52 pl. 17. However, the deaths of 10/33 animals infected with the R17(192) recombinants at a dose of 10⁵ p.f.u. and 10/15 with 10⁶ p.f.u. compared to 0/12 with 10⁵ p.f.u., 0/12 with 10⁶ p.f.u. and 2/18 with 10⁷ p.f.u. of JH2604 point to the avirulent phenotype having been corrected by the replacement of the deleted sequences within both copies of BamHI v, or the exchange of sequences with the remainder of XbaI e. It would have been more critical to correct the deletion by cotransfecting with the BamHI g joint fragment (v + u), but because this fragment is so small (4 × 10⁶ Mr) and no selection system was available, we chose to facilitate recombination by using XbaI fragments. It could be argued that as well as replacing the deleted sequences the recombinant could also have other sequences from XbaI e of HG52 plaque 17. This could of course imply that apart from the deletion the sequences within XbaI e of JH2604 and XbaI e of HG52 plaque 17 are not equivalent and affect neuropathogenicity.

The isolation of two recombinants in which the deletion in JH2604 has been introduced into the HG52 plaque 17 stock allowed the determination of whether the reciprocal recombinants to those isolated previously behaved differently upon inoculation into mice. Intracranial inoculation of 18 mice with each recombinant produced an LD₅₀ value of > 10⁷ p.f.u., in each case. This is identical to the LD₅₀ value determined from 42 animals infected at the same time with JH2604. These results provide substantive evidence that the sequences deleted in JH2604 are involved in the determination of neurovirulence of HSV-2 HG52.

BamHI v of HG52 co-maps with BamHI s of HSV-1. The nucleotide sequence of v is not known, but in HSV-1 strain 17 no complete genes have been identified in BamHI s. The S' end and 500 bp from that end of the immediate early gene IE1 are located within BamHI s, the BamHI s/b site being located within the first intron of the gene (McGeoch et al., 1988). We have shown previously that JH2604 makes VₘₐIE118 of normal size and in normal amounts
compared to the parental HG52 (Harland & Brown, 1985) indicating that the deletion within BamHI v in both copies of R∞ does not extend into the reading frame for IE1. BamHI v is 3 kb in size; the size of the deletion in each copy of BamHI v is 1.5 kb. Taking into account the extent of the transcript for IE1 into BamHI v (assuming that they are collinear in HSV-1 and HSV-2 and the type 1 BamHI s/b site is coincident with the type 2 p/v site) and the normal synthesis of Vmw IE118 by JH2604, then if the deletions in each copy of BamHI v are not the same, they must overlap by at least 500 bp.

General infected cell polypeptide synthesis by JH2604 is not identifiably different from that of HG52. In the HSV-1 strain F Chou & Roizman (1986) have reported a gene in R∞ coding for an M, 43.5 K protein, ICP 34.5, whose promoter is in the terminal 'a' sequence. The gene has not been identified in the sequencing of HSV-1 strain 17 (McGeoch et al., 1988) and it is not known whether its equivalent is present in HG52.

In vivo analysis of the HG52 deletion variant JH2604 has demonstrated that at least 500 bp and at most 1500 bp of the terminal sequences of R∞ are required to confer neurovirulence. As no genes have as yet been identified in this region it must be assumed that the deleted sequences have a control function. Sequence analysis of R∞ in HG52 and JH2604 should help to clarify their precise role.

Our thanks are due to John H. Subak-Sharpe for his interest and critical reading of the manuscript. M.Y.T. is supported by an Iraqi Government scholarship.

REFERENCES


(Received 14 September 1988)