The Herpes Simplex Virus Type 2 (HG52) Variant JH2604 Has a 1488 bp Deletion which Eliminates Neurovirulence in Mice

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SUMMARY

The herpes simplex virus type 2 (HG52) deletion variant JH2604 is avirulent (LD50 > 107 p.f.u./mouse) for mice compared to the parental wild-type virus (LD50 < 102 p.f.u./mouse) and fails to replicate in vivo. JH2604 has a 1488 bp deletion within the 3 kb BamHI v fragment (0 to 0.02 and 0.81 to 0.83 map units) which removes one copy of the 17 bp direct repeat DR1 element of the 'a' sequence and terminates 522 bp upstream of the 5' end of the immediate early gene 1. In vivo selection after transfection of intact JH2604 DNA with the BamHI g (v + u) joint fragment of HG52 results in the isolation of wild-type virus with an LD50 of < 102 p.f.u./mouse. These results show that a 1488 bp sequence within the terminal portion of the genome long repeat region confers neurovirulence on strain HG52.

Sound understanding of the biology of herpes simplex virus (HSV) requires identification of genes involved in pathogenicity and latency and determination of their precise functions in vivo. To this end specific viral genes and regions of the HSV genome as well as many host factors have been implicated in the control of virulence (Kohl & Loo, 1980; Caspary et al., 1980; Field & Wildy, 1978; Thompson & Stevens, 1983; Thompson et al., 1985, 1986; Javier et al., 1986; Rosen et al., 1986; Field & Coen, 1986). Moreover heterogeneity of virulence within a single virus stock of HSV-2 strain HG52 has recently been demonstrated. Thus the LD50 values of 10 individual plaque stocks isolated from the elite laboratory stock ranged from < 103 to > 105 p.f.u./mouse, with plaque isolate 17 (pl. 17; the most neurovirulent) possessing an LD50 of < 102 p.f.u./mouse (Taha et al., 1988).

Using HG52 as a standard, the virulence of deletion variants of HG52 (Harland & Brown, 1985; Brown & Harland, 1987) is under study. One of the variants, JH2604, with a 1.5 kb deletion within each copy of the long repeat region (RL) from 0 to 0.02 and 0.81 to 0.83 map units (m.u.) has been shown to be avirulent on intracranial inoculation of mice, with an LD50 value of > 107 p.f.u./mouse (Taha et al., 1989). We have previously demonstrated that recombinants isolated after cotransfection of JH2604 DNA with XbaI fragments e or f from the highly neurovirulent pl. 17 regained the neurovirulence levels of HG52. Recombinants isolated from the reciprocal cotransfection of HG52 pl. 17 DNA with XbaI e or f of JH2604 as expected had lost their neurovirulence, exhibiting LD50 values of > 107 p.f.u./mouse, equivalent to that of JH2604 (Taha et al., 1989). The XbaI e fragment is composed of XbaI g (0-7 to 0-83 m.u.) plus XbaI h (0-83 to 0-91 m.u.) and XbaI f is composed of g plus i (0-94 to 1-0 m.u.). These results implied that sequences within the 3 kb terminal portion of RL probably within BamHI v are required for virulence of HSV-2 strain HG52. The deletion in JH2604 had been shown to be within BamHI v (0-81 to 0-83 m.u.) (Harland & Brown, 1985) but we chose to correct or insert the deletion using large XbaI fragments because of the small size of the relevant BamHI fragment and the lack of a selection system. (The positions of the relevant BamHI, XbaI, HindIII and HpaI fragments are shown in Fig. 1.) This approach is subject to the criticism that apart from

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having the deleted sequences replaced, the recombinants could also have gained other sequences from the cotransf lecting Xbali e or f fragments. The implication is that besides the deleted sequences, the sequences within XbaI e and f were not equivalent in HG52 pl. 17 and JH2604 and that the observed effect on neurovirulence was not due to the deletion but to some other mutation. To resolve this question we have corrected the deletion in JH2604 by in vivo selection.

This paper demonstrates that the variant JH2604 has a 1488 bp deletion within the 3 kb BamHI v fragment (0 to 0-02 and 0.81 to 0.83 m.u.) upstream of the coding region of immediate early (IE) gene 1, and in vivo selection after transfection of intact JH2604 DNA with the BamHI g fragment of HG52 (0.81 to 0.85 m.u.) resulted in the isolation of wild-type virus with an LD50 of < 102 p.f.u./mouse. These results show that a 1488 bp sequence within the terminal portion of Rv confers neurovirulence on HSV-2 strain HG52.

Cotransfection experiments were carried out using intact JH2604 genomes and different molar ratios (1 x, 5 x, 10 x) of the BamHI g fragment (0.81 to 0.85 m.u.) of pl. 17. When extensive c.p.e. was apparent the cells were harvested, sonicated and the resulting virus suspension, serially diluted, was inoculated intracranially into the left cerebral hemisphere of 3 week old BALB/c mice (two/dilution) as described previously (Taha et al., 1988). By 3 days post-infection, two mice had died, one inoculated with the virus from a transfection plate infected with a tenfold molar excess of BamHI g and the other from a transfection with a fivefold molar excess of BamHI g. At post mortem the brain of the mouse inoculated with the products of the tenfold BamHI g transfection plate was putrefied and could not be used. The brain of the other one was homogenized and the resulting suspension titrated on BHK21/C13 cells. One plate of infected cells was harvested, the virus released by sonication and used to reinfect mice intracranially to determine its LD50. This stock gave an LD50 of < 103 p.f.u./mouse. Single virus plaques from the original infected brain suspension were picked and their DNA was subjected to restriction enzyme analysis [with HindIII and HpaI by the method of Lonsdale (1979)] which demonstrated that a mixture of JH2604 and HG52 virus had been recovered (data not shown). Two plaques showing these mixed profiles were each subjected to three rounds of stringent plaque purification after which a stock of virus was prepared from each. The two virus stocks were inoculated intracerebrally at 102, 103 or 104 p.f.u./mouse into groups of five 3 week old BALB/c mice and their LD50 values determined. Both isolates killed all the infected mice at all three doses, giving an LD50 of < 102 p.f.u./mouse. The HpaI and HindIII profiles of the two viruses had DNA structures equivalent to wild-type HG52 and not the deletion variant JH2604 (Fig. 2). These results demonstrate unequivocally that sequences within BamHI g correct the deletion in JH2604 and restore its neurovirulence to the level of wild-type virus.

It had been shown that in JH2604 the deletion of approximately 1.5 kb was in each copy of BamHI v (0 to 0-02 and 0.81 to 0.83 m.u.), and that the deleted v fragments caused the formation of joint fragments of different sizes indicating a difference in the size or position of the deletion in each copy of v (Harland & Brown, 1985). A BamHI digestion of JH2604 DNA showed that the g fragment (i.e. v + u) was missing from its normal location and ran in two novel positions, just above the f fragment and just above the m fragment (Fig. 3). These two fragments were purified and cloned into the BamHI site of pAT153 using standard procedures (Twigg & Sherratt, 1980).
Fig. 2. Autoradiographs of restriction digests of viral DNA $^{32}$P-labelled in vivo. *HpaI* (lanes 1 to 4); *HindIII* (lanes 5 to 8). Recombinant viruses (lanes 1, 2, 5 and 6); JH2604 (lanes 3 and 7); HG52 (lanes 4 and 8). Letters refer to specific fragments; * indicates novel fragment.

Fig. 3. Autoradiographs of *BamHI* restriction digests of viral DNA $^{32}$P-labelled in vivo. JH2604 (lane 1): HG52 (lane 2). Letters refer to specific fragments; ▶ indicates missing fragment; * indicates novel fragment.

Fig. 4. Autoradiographs of Southern blots containing *BamHI* restriction fragments of clone 28 (lanes 1 and 5), clone 22 (lanes 2 and 6), JH2604 (lanes 3 and 7) and HG52 (lanes 4 and 8) to which nick-translated probes have been hybridized. The probes were the recombinant plasmid clone 22 (lanes 1 to 4) and HG52 DNA (lanes 5 to 8). Letters refer to specific fragments. Hybridizations were carried out according to the method of Southern (1975) and the probes were nick-translated by the method of Rigby et al. (1977).
Fig. 5. Structure of the HG52 genome (top line) showing UL and Us flanked by TRL/IRL and IRs/TRs respectively. The second line shows an expansion of the BamHI g fragment (0.81 to 0.85 m.u.) which is 5815 bp. The position of the ‘a’ sequence and the transcript for Vmw118 is indicated. The bottom line shows the positions of the HincII and XhoI sites within BamHI g.

Fig. 6. Portion of an autoradiograph of a sequencing gel showing the cloning site (3053) within the ‘a’ sequence and the start (3083) and end (4571) of the deletion in JH2604. Sequencing products were separated on denaturing 6% polyacrylamide gels (Sanger et al., 1980) containing 9 M-urea (McGeoch et al., 1986). Gels were covalently bonded to one glass plate (Garoff & Ansorge, 1981), fixed, dried and exposed to X-ray film. Sequences were read from the autoradiograph and assembled using a DEC VAX computer running the GCG software (Devereux et al., 1984) and analysed using the BESTFIT, GAP and FROMSTADEN programs.

One positive clone from each, designated 22 and 28, was isolated. Southern blot analysis of a BamHI digest using nick-translated intact HG52 DNA as the probe showed hybridization to one band in clone 22 running just above the BamHI I fragment and to one clone 28 band running just above the BamHI m fragment (Fig. 4). When clone 22 or clone 28 DNA was nick-translated and used as a probe in Southern blots with BamHI-digested DNA of HG52, JH2604, clone 22 and clone 28, the following results were obtained. With HG52 there was positive hybridization to BamHI v, u and g; with JH2604 there was positive hybridization to u and to the two bands running close to BamHI l and m. With clones 22 and 28 there was hybridization to two bands, the lower one representing the pAT153 DNA and the upper in each case the deletion-affected BamHI g fragment running coincidentally with BamHI l and m respectively. It is assumed that the small size of the v' fragment precluded its identification. The variation in the mobility of the fragments from clones 22 and 28 could be due either to the deletion in each case being a different size or to variability in the copy number of reiterated sets of sequences. Such variation in the HSV-2 HG52 joint fragment was observed previously by Davison & Wilkie (1981) and in HSV-1 strain KOS by Wagner & Summers (1978).

The wild-type HG52 BamHI g fragment has been shown to be 5815 bp in length and to extend from 1Rs to 1RI (D. J. McGeoch, unpublished results). There is a HincII site at nucleotide
position 3053 within the 'a' sequence and a XhoI site at nucleotide position 5069 just upstream of the 5' end of IE gene 1 (Vmw118) (Fig. 5). Plasmids containing the JH2604 BamHI g (v + u) inserts were cut with XhoI/HincII and in both cases the expected 2016 bp fragment had been replaced by a 516 bp fragment; the remaining BamHI g fragments were normal. This indicated that the deletion in JH2604 was contained completely within the HincII/XhoI fragment, that the deletion was approximately 1500 bp in size as previously predicted and that both copies of BamHI v had a deletion with identical endpoints and size.

These 516 bp fragments were ligated into the Smal–SalI sites of M13 mp18 and mp19 phage (Sanger et al., 1980; Norrander et al., 1983) and the nucleotide sequence was determined from both ends of the clone by the dideoxynucleotide chain termination method (Sanger et al., 1977). Sequencing of clones 22 and 28 showed that homology to the wild-type sequence started at the HincII site (3053) and continued for 30 bp to nucleotide position 3083 (Fig. 6). There is then a deletion of 1488 bp before homology starts again at nucleotide position 4571 and continues with no alterations, to the XhoI site at 5069 (Fig. 5). The deletion includes one complete copy of the 17 bp direct repeat DR1 element of the 'a' sequence, AGTCCCCGTCCTGCCGC, and terminates 522 bases upstream of the 5' end of the IE1 gene. Immediate early polypeptide synthesis in JH2604 has been analysed and the amount of Vmw118 made under such conditions appeared to be normal although it was not precisely quantified (Harland & Brown, 1985). It seems unlikely that the promoter sequences of IE1 could be within the deletion more than 522 bp from the 5' end of the gene but as the control sequences for IE1 in HSV-2 have not yet been defined, the possibility cannot be ruled out.

Sequence analysis showed that the nucleotide sequences deleted, and hence the size of the deletion in both clones 22 and clone 28, were identical indicating that the difference in mobility of the remnant of BamHI g in each clone was due to variable copy numbers of the reiterated sequences outside the deleted region. Both HincII and XhoI also cut at nucleotide positions 1237 and 1769 respectively within the u fragment of BamHI g. This 532 bp sequence in JH2604 was shown to be identical to that of HG52 with no point mutations, deletions or insertions. In total 2548 bp of the JH2604 BamHI g fragment have been sequenced and shown to be identical to HG52 except for the deletion. However, a point mutation within the remaining 3267 bp cannot be excluded.

Chou & Roizman (1986) presented sequencing data and proposed that the region between the IE1 gene and the 'a' sequence in HSV-1 strain F codes for a gene in the same orientation as IE1. An anti-peptide serum based on this predicted open reading frame identified a protein, ICP34.5, in extracts of infected cells (Ackermann et al., 1986). When comparisons were made between the published sequence for this region in strain F and the equivalent region in HSV-1 strain 17, Perry & McGeoch (1988) concluded that it was unlikely that the entire open reading frame assigned by Chou & Roizman (1986) was genuinely protein-coding. The data presented by Perry & McGeoch (1988) were thought to be consistent with there being protein-coding sequences in this locality but they did not consider the analysis satisfactory enough to propose any definite gene layout.

It is concluded from the nucleotide sequence analysis and the in vivo studies presented here and previously (Taha et al., 1988, 1989) that sequences within a 1488 bp fragment located between 0 and 0.02 m.u. in TR2 and from 0.81 to 0.83 m.u. in IR2 of HSV-2 strain HG52 confer neurovirulence for BALB/c mice. The variant JH2604 in which these sequences are deleted fails to grow in mouse brain and causes no discernible pathology (M. Y. Taha et al., unpublished). The analysis of the sequence of the whole R2 in HG52 is under study at present (D. J. McGeoch, personal communication) and is being compared to the R2 nucleotide sequence of HSV-1 strain 17. Our analysis clearly demonstrates a function in vivo for the sequences which may suggest that the region is protein-coding.

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Short communication

REFERENCES


BROWN, S. M. H., HARLAND, J. (1987). Three mutants of herpes simplex virus type 1: one lacking the genes US10, US11 and US12 and two in which R, has been extended by 6 kb to 0.91 map units with loss of Us sequences between 0.94 and the Us/Trs1 junction. *Journal of General Virology* 68, 1–18.


TAHA, M. Y., CLEMENTS, G. B. & BROWN, S. M. (1989). A variant of herpes simplex virus type 2 strain HG52 with a 1.3 kb deletion in R, between 0 to 0.02 and 0.81 to 0.83 map units is non-neurovirulent for mice. *Journal of General Virology* 70, 705–716.


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