Functional analysis of the herpes simplex virus type 2 strain HG52 RL1 gene: the intron plays no role in virulence

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Sequence analysis predicts that herpes simplex virus type 2 (HSV-2) strain HG52 contains an open reading frame, RL1, encoding a polypeptide equivalent to ICP34.5 of HSV-1. Similarly to HSV-1, deletion of the region spanning RL1 abolishes the virulence of HSV-2 strain HG52 and its ability to grow in stationary 3T6 cells. In contrast to HSV-1, the HSV-2 strain HG52 RL1 gene is predicted to contain a 154 bp intron. Previously, we have demonstrated that this intron is spliced from RL1 poly(A) mRNA at the predicted splice donor/acceptor sites. To determine if the intron affects the function of ICP34.5 of HSV-2 strain HG52, we have constructed a virus, 2624, in which the RL1 intron is deleted: 2624 retains wild-type growth both in vitro and in 3T6 cells, indicating that the presence of an intron does not affect the function of RL1 in HSV-2 strain HG52. 2624 has wild-type growth kinetics in BHK21/C13 cells.

Herpes simplex virus types 1 and 2 (HSV-1/2) are important human pathogens which cause a variety of diseases ranging from benign superficial cutaneous lesions to life threatening encephalitis (Whitely, 1985). Identification and characterization of the genes that control virus pathogenicity are of fundamental importance in understanding the mechanisms of herpesvirus disease.

Any alteration in a virus gene that impairs replication in vitro will also affect the in vivo replication of HSV and can therefore be loosely considered a ‘pathogenicity gene’ (Larder et al., 1986). Such an example is UL10 (MacLean et al., 1993). In addition, a number of virus encoded enzymes involved in DNA metabolism, which are not essential in dividing cells but play a role in replication in stationary cells, are also involved in HSV neurovirulence. These include thymidine kinase (Efstathiou et al., 1989), ribonucleotide reductase (Cameron et al., 1988) and dUTPase (Pyles et al., 1992).

We and others have previously demonstrated that the RL1 gene of HSV-1 encodes a polypeptide, ICP34.5, which is absolutely required for HSV-1 virulence (Thompson et al., 1983, 1989; Chou et al., 1990; MacLean et al., 1991; McKie et al., 1994). RL1 is a diploid gene situated in the long repeat element of the genome between IE1 and the ‘a’ sequence (Fig. 1; Chou & Roizman, 1986, 1990; Dolan et al., 1992). We have demonstrated that, similarly to HSV-1, deletion of sequences in the equivalent region of HSV-2 strain HG52 also abolishes virulence (Taha et al., 1989a, b). Although both HSV-1 and -2 RL1 negative mutants replicate with wild-type characteristics in BHK21/C13 cells, they fail to replicate in stationary 3T6 mouse embryo fibroblasts due to a defect in virus maturation (Brown et al., 1994a, b), illustrating that RL1 negative virus replicates in a cell type and state dependent manner.

Sequence analysis of the region between IE1 and the ‘a’ sequence in HSV-2 strain HG52 by McGeoch et al. (1991) demonstrated the presence of an open reading frame (ORF) predicted to encode an ICP34.5 homologue; however, none of our antisera against the HSV-1 protein cross-react with the HSV-2 strain HG52 gene product which has yet to be identified (McKay et al., 1993; Brown et al., 1997). The organization of the HSV-2 strain HG52 RL1 gene differs from that of HSV-1: although both initiation codons are in equivalent positions, the HSV-2 strain HG52 coding sequence lacks the central (PAT)10 repeat element and is disrupted by a 154 bp intron, containing six complete copies and one partial copy of a 19 bp repeat which encodes a stop codon (McGeoch et al., 1991). Downstream of the intron there is a 63 amino acid sequence which is highly conserved between HSV-1 and -2 and in non-herpesvirus homologues in African swine fever virus (Sussman et al., 1992) and cellular genes involved in differentiation (MyD116; McGeoch & Barnett, 1991) and growth arrest (GADD34; He et al., 1996): this 63 amino acid sequence has been shown to be essential but not necessarily adequate for ICP34.5 function in a non-permissive neuro-
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Fig. 1. (a) HSV-2 strain HG52 genome. (b) An expansion of the BamHI g fragment encoding RL1. (c) Schematic diagram of the SphI fragment cloned in pSB1 containing the RL1 gene of HSV-2 strain HG52. The locations of the primers used for the deletion of the intron are illustrated: this consists of nt 12505–12530 and 12685–12700. The positions of the restriction enzyme sites Smal (S), and RsaI (R) are marked. Illustrated are (1) the sequenced 263 bp RsaI–Smal fragment spanning the intron deletion and (2) the Smal bands (960 bp and 804 bp in HG52 and 2624 respectively) used to map the deletion. All nucleotide numbers are from McGeoch et al. (1991).

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As (1) introns are not a normal feature of the HSV genetic arrangement, being present in only four other HSV genes, and (2) this is the only gene predicted to have an intron in HSV-2 but not HSV-1, we wished to determine if the intron plays a role in the function of ICP34.5. To do this, we have constructed a mutant (2624) with the intron sequences precisely deleted and analysed its phenotype in vivo and in vitro. 2624 was generated from a 1-kb SphI fragment spanning the RL1 gene (pSB1; nt 11632–13252; Fig. 1; McGeoch et al., 1991) cloned into pGEM-3Zf(®) (Promega). Site directed mutagenesis was carried out using a 52-mer oligonucleotide consisting of 26 bp from the ends of each exon (nt 12505–12530 and 12685–12700; Fig. 1, and Harland et al., 1996), to precisely delete the intron. Briefly E. coli strain BW313 (dut− ung−) (Promega) was transformed with the SphI fragment containing recombinant plasmids. The resulting recombinant phagamid was superinfected with M13K07 to prepare uracil enriched single-stranded DNA as a template for site directed mutagenesis. The primer was hybridized and extended by T4 DNA polymerase to generate double-stranded phagemids. Following transformation into dut+ ung+ E. coli to select against the input wild-type DNA, single colonies were analysed to isolate recombinant phagemids which had taken up the oligonucleotide. Reombinat phagemids were identified by a 154 bp deletion on restriction enzyme digestion (data not shown). Once a recombinant phagemid (pSB2) was constructed and prior to the generation of recombinant virus, a 263 bp RsaI–Smal fragment spanning the inserted oligonucleotide was cloned into the Smal site of M13 and sequenced (Fig. 1): the intron was precisely deleted and no other alterations from the wild-type exon sequences were found (data not shown). The mutated plasmid was cotransfected with HSV-2 strain HG52 DNA by calcium phosphate precipitation onto BHK21/C13 cells (Stow & Wilkie, 1976). When CPE was complete, the virus was harvested, sonicated, titrated and single plaques were isolated. To detect recombinant virus, DNA was prepared from the single plaque isolates and its profile analysed by restriction enzyme digestion and Southern blotting. A virus (2624) with a 154 bp deletion in the appropriate region of both copies of RL1 was isolated. On Southern blotting using pSB1 as a probe.
Role of HSV-2 RL1 gene intron in virulence

Fig. 2. Confluent monolayers (2 x 10^6 cells) of (a and c) BHK21/C13 (MacPherson & Stoker, 1962) and (b and d) 3T6 (Brown et al., 1994a) cells were infected at a multiplicity of 10 p.f.u. per cell (a and b) for a one-cycle and 0.01 p.f.u. per cell (c and d) for multi-cycle growth experiments. After adsorption at 37 °C for 60 min, the monolayers were washed, overlaid with the appropriate medium and incubated at 37 °C. At various times post-infection (a and b): 0, 2, 4, 6, 8, 12 and 24 h; c and d: 0, 12, 24, 36, 48, 60, 72 h) samples were harvested, sonicated and stored at −70 °C until titration on BHK21/C13 cells. Yields are expressed as p.f.u. per 10^6 cells.

Table 1. Intracerebral LD_{50} of HSV-2 recombinants

<table>
<thead>
<tr>
<th>HSV-2 Strain</th>
<th>Dose:</th>
<th>LD_{50}</th>
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<tbody>
<tr>
<td>HG52</td>
<td>10^1</td>
<td>2/4</td>
</tr>
<tr>
<td>2604</td>
<td>10^2</td>
<td>4/4</td>
</tr>
<tr>
<td>2624</td>
<td>10^3</td>
<td>ND</td>
</tr>
<tr>
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<td>10^4</td>
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<td>0/4</td>
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<td></td>
<td>10^6</td>
<td>0/4</td>
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<tr>
<td></td>
<td></td>
<td>3-16 x 10^6</td>
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Figures were previously observed, HSV-2 strain HG52 has an LD_{50} of 10 p.f.u. per mouse and 2604 an LD_{50} of > 3-16 x 10^6 p.f.u. per mouse. 2624 has an LD_{50} of 31, comparable to that of wild-type virus, indicating that the virus is fully virulent and the intron plays no detectable role in RL1 function in vivo. Consistent results were obtained on two other occasions, confirming no apparent difference between HG52 and 2624.

a 960 bp SmaI band spanning the intron is detected in wild-type DNA (Fig. 1 and data not shown): in 2624 this band disappears and is replaced by an 806 bp band (Fig. 1 and data not shown). 2624 was plaque purified a further three times before preparing a virus stock.

The growth characteristics of 2624 were analysed, in comparison to wild-type and the RL1 negative virus, 2604 (Harland & Brown, 1985; Taha et al., 1989a, b), in BHK21/C13 cells and stationary 3T6 mouse embryo fibroblasts during both low (0.01 p.f.u. per cell) and high (10 p.f.u. per cell) multiplicity infections. As anticipated, in BHK21/C13 cells all three viruses grew equally well following both high and low m.o.i. infection (Fig. 2a, c). In 3T6 cells at low m.o.i. (Fig. 2d) HG52 and 2624 increased in titre by 10^4-fold between 12 and 72 h p.i.: in contrast 2604 increased in titre by less than 10-fold giving RL1 positive virus a 10^3 growth differential in 3T6 cells as observed previously (Brown et al., 1994a). On high m.o.i. infection in 3T6 cells (Fig. 2b) HG52 and 2624 gave a final yield of approximately 5 x 10^6 p.f.u. per 10^6 cells whereas 2604 only gave a yield of less than 10^5 p.f.u. per 10^6 cells. The wild-type growth characteristics exhibited by 2624 demonstrated that the intron sequences play no role in HSV-2 strain HG52 growth in 3T6 cells.

To determine if the intron sequences in the RL1 gene play a role in in vivo growth and virulence, the virulence of 2624 was assessed. Three-week-old female BALB/C mice (Harlan) were injected in the left cerebral hemisphere with 25 µl of appropriate 10-fold serial dilutions of HG52, 2604 and 2624 (MacLean et al., 1991). At the time of inoculation the virus was titrated on BHK21/C13 cells to ensure that the correct dose had been administered. Animals were monitored on a daily basis and deaths up to day 21 scored; the LD_{50} was calculated as described by Reed & Muench (1938) and the results are shown in Table 1. As previously observed, HSV-2 strain HG52 has an LD_{50} of 10 p.f.u. per mouse and 2604 an LD_{50} of > 3-16 x 10^6 p.f.u. per mouse. 2624 has an LD_{50} of 31, comparable to that of wild-type virus, indicating that the virus is fully virulent and the intron plays no detectable role in RL1 function in vivo. Consistent results were obtained on two other occasions, confirming no apparent difference between HG52 and 2624.
The question remains as to whether the presence of an intron affects the level of ICP34.5 expression. Although preliminary Northern blot analysis (Liz McKie, personal communication) suggests that a similar amount of poly(A)+ mRNA is made by HG52 and 2624, we are unable to fully address this question in the absence of an antiserum which recognizes the HSV-2 protein. However, if the presence of the intron sequences does affect the level of expression there is no effect on the growth of virus in our assays.

In HSV-1 strain F Langunoff & Roizman (1994) identified an ORF encoding a polypeptide named ORF P running in the antisense direction to ICP34.5. An equivalent ORF is also present in HSV-1 strain 17 (L. McKie and others, unpublished). However, there is only homology in HSV-2 strain HG52 at the 5’ end of ORF P: this homology disappears when ORF P encounters the RL1 intron in HSV-2 (Langunoff & Roizman, 1994). The presence and extent of an ORF P homologue in HSV-2 strain HG52 are therefore unclear at present. Removing the RL1 intron is liable to affect the coding sequence and frame of the 3’ part of an ORF P homologue in HSV-2 strain HG52, confirming that the observed phenotype of 2604 is due solely to lack of ICP34.5.

Thus in conclusion we can state that the structure proposed for the HSV-2 HG52 RL1 gene is correct but like the situation in IE1 (Everett, 1991) deletion of the intron has no apparent effect on the phenotype of the virus. The implication of the RL1 gene being spliced in HSV-2 but not -1 is unclear especially with regards to the presence of an ORF P homologue in HSV-2 strain HG52.

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