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Genetic and Biological Analyses of a Herpes Simplex Virus Intertypic Recombinant Reduced Specifically for Neurovirulence

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RS6 is a herpes simplex virus intertypic recombinant derived from type 1 strain 17 syn$^+$ and type 2 strain HG_{52}. With a 50% lethal dose of about 10^5 PFU after intracerebral inoculation of mice, RS6 was approximately 100,000 times less neurovirulent than either of its wild-type parental viruses were. When compared with strains 17 syn$^+$ and HG_{52}, RS6 replicated intermediately in primary mouse embryo fibroblasts in vitro at 38.5°C (mouse temperature) and to wild-type peak titers in mouse feet in vivo. In contrast, following intracranial inoculation of mice, RS6 replicated significantly less well than did either of its parental viruses in brains. The genetic defect(s) responsible for the reduced neurovirulence of RS6 (i) was stable after in vitro and in vivo serial passage, (ii) was not manifested as temperature-sensitive plaquing in vitro, and (iii) did not affect thymidine kinase expression. These data indicate that RS6 has a genetic defect(s) specifically affecting its ability to replicate in the mouse brain. Using marker rescue technologies, we increased the neurovirulence of RS6 and localized one genetic determinant(s) involved with the reduced neurovirulence of this agent to 0.72 to 0.87 map units (and, tentatively, to 0.79 to 0.83 map units) of the herpes simplex virus genome. When coupled with the work suggesting that thymidine kinase expression is essential for efficient replication in nerve tissues and earlier reports from this laboratory and others, the results presented in this study indicate that more than one herpes simplex virus gene is involved with neurovirulence.

The mouse is commonly used as a model system for studying acute herpes simplex virus (HSV)-induced encephalitis. In this system, intracerebral inoculation of a very low dose (1 to 10 PFU) of a standard HSV type 1 (HSV-1) or type 2 (HSV-2) leads to acute fatal encephalitis (14). HSV variants that require significantly larger doses to elicit fatal encephalitis are potentially useful for identifying viral genes and gene products associated with this disease. Two classes of such agents have been distinguished (25). The first and most common class displays generalized replication defects in most cell types in vitro and in vivo. The second class of virus, on the other hand, replicates efficiently in most cell types in vitro and in vivo but exhibits a reduced ability to replicate in brain cells in vivo. This result indicates that there are genes which enable HSV to replicate efficiently in the central nervous system. Thus, with the use of specific-neurovirulence mutants, HSV genes and gene products associated with acute central nervous system disease can be identified and mechanisms can be established.

In this paper, we biologically characterize the HSV-1 × HSV-2 intertypic recombinant RS6, a low-neurovirulence mutant which was found to display replication defects specific for the mouse brain in vivo. Then, using the cloned HSV-1 *Eco*RI e+k fragment in marker rescue experiments, we localized a genetic lesion(s) of RS6 to 0.72 to 0.87 map units (m.u.) of the HSV genome. Additional results suggest that 0.79 to 0.83 m.u. is the region involved.

MATERIALS AND METHODS

Cells, viruses, and mouse inoculation. The techniques of tissue culture of rabbit skin cells, production of primary mouse embryo fibroblasts (MEF), and infection, propagation, titration, and plaque purification of viruses have been described previously (25, 26). The standard laboratory viruses HSV-1 17 syn$^+$ and HSV-2 HG_{52} (21), TK^{-7} (a thymidine kinase (tk)-negative mutant derived from 17 syn$^+$), and the intertypic recombinant RS6 were kindly provided by colleagues at the Medical Research Council Virology Unit, Glasgow, Scotland. RS6 was generated by cotransfection of unit-length *ts*S (a temperature-sensitive [*ts*] mutant of 17 syn$^+$) DNA and *Xba*I-generated DNA fragments derived from HG_{52} (20), and its genomic structure has been described previously (6, 13, 20). Examination of comparative viral replication kinetics and the production of tk by the Ara-T test (22) were as described earlier (25). The tk assay was that used by Cheresh and Haines (4), except that cellular cytoplasmic extracts were derived from LM(TK^-) cells (10) lysed by two cycles of freeze-thaw at 14 h postinfection. Except where noted, 4- to 5-week-old outbred male Swiss Webster mice were used in all experiments, and standard procedures for intracranial inoculation (25, 26) and quantitation of neurovirulence (15) were used.

Restriction enzyme analysis and transfections. Isolation of DNA used for restriction endonuclease and Southern blot analyses were performed as described earlier (26), except that viral DNA was layered onto sodium iodide gradients rather than onto cesium chloride for isopycnic centrifugation. The restriction endonucleases *Asp*718 (*Kpn*I isoschizomer), *Bam*HI, *Hin*dIII, and *Hpa*I were used to analyze the genomic structures of putative recombinant viruses. Methods for transfection and isolation of unit-length RS6 DNA for transfection have been previously detailed (8, 19, 26). In this study, however, transfections were performed without the use of carrier DNA, a modification which greatly increased the number of plaques normally obtained during transfection of unit-length RS6 DNA. Electroelution of *Xba*I fragments b, c, and f derived from HSV-1 17 syn$^+$ was performed by standard methods (12).

Production, selection, and isolation of neurovirulence-enhanced recombinants. The technique of marker rescue

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TABLE 1. Production of HSV-specific tk: phosphorylation of [^{3}H]thymidine by infected-cell cytoplasmic extracts[a]

Virus	Phosphorylation of thymidine (cpm)[b]
17 syn$^+$	73,170
RS6	74,520
TK^{-7}	2,910
Mock infected	3,930

[a] Cytoplasmic extracts were derived from LM(TK$^-$) cells 14 h postinfection.
[b] Counts per minute of [^{3}H]thymidine phosphorylated per 30 min per milligram of protein.

used in combination with in vivo selection has been outlined in a previous study by this laboratory (26). Briefly, unit-length RS6 DNA and a DNA fragment(s) (approximately 0.1 and 0.4 μg, respectively) derived from the highly neurovirulent HSV-1 17 syn$^+$ were cotransfected. The resulting viruses (~10^6 PFU/0.1 ml) were then harvested and collectively inoculated (10-fold diluted and undiluted) intracerebrally into mice. From mice that succumbed to encephalitis, brains were removed and homogenized, and the homogenate was cleared by centrifugation (3,000 × g for 5 min in a Sorvall SS-34 rotor). Cleared brain homogenate (30 to 50 μl) was then injected intracerebrally into two mice. Mice invariably survived the injection of brain homogenate when RS6 DNA was transfected alone, so when at least one of the two mice died from encephalitis after this injection, the transfection plate was scored as positive (it contained a virus with increased neurovirulence), and a single virus was plaque purified from the brain of one of the dead mice.

Southern blot analysis. Standard methods for agarose gel electrophoresis and transfer of DNA to nitrocellulose were used (12, 16). The *Hpa*I t fragment was isolated from the cloned *Eco*RI e+k fragment of 17 syn$^+$ by *Hpa*I digestion and electroelution (12), nick translated in the presence of biotinylated-11-dUTP, and used in hybridization experiments as suggested by the manufacturer (DNA Detection System Instructional Manual, catalog no. 8239SA; Bethesda Research Laboratories, Inc., Gaithersburg, Md. [11]).

Isolation and purification of cloned HSV DNA. The 17 syn$^+$ *Eco*RI e+k fragment cloned into pUC12 was isolated by standard methods from 250-ml cultures of *Escherichia coli* HB101 in log-phase growth in the presence of ampicillin (12). After purification on cesium chloride gradients, the plasmid DNA was dialyzed extensively against TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA), and before use in transfection experiments, the *Eco*RI e+k plasmid DNA was digested with *Eco*RI, phenol and chloroform extracted, ethanol precipitated, and dissolved in TE buffer.

RESULTS

Comparative LD_{50}s after intracerebral inoculation into mice. (i) RS6 versus 17 syn$^+$ and HG_{52}. To assess the neurovirulence of RS6, we first performed a comparative 50% lethal dose (LD_{50}) experiment on a clone of RS6 that was plaque purified three times and the wild-type parental viruses HSV-1 17 syn$^+$ and HSV-2 HG_{52}. Approximately 30 μl of serial 10-fold dilutions of RS6, 17 syn$^+$, and HG_{52} were inoculated into the left cerebral hemisphere of 4- to 5-week-old outbred male Swiss Webster mice (five mice per dilution); deaths from encephalitis were scored, and LD_{50}s were determined. Both parental viruses displayed LD_{50}s of 1 PFU or lower, while the LD_{50} of RS6 was greater than 10^5 PFU. Thus, RS6 was approximately 100,000 times less neurovirulent than the parental viruses.

(ii) Plaquing efficiency of RS6 at 31 versus 38.5°C. RS6 is derived from a *ts* mutant of 17 syn$^+$ (*ts*S) whose mutation was rescued with *Xba*I fragments of HG_{52} in marker rescue experiments (20). To eliminate the possibility that RS6 exhibited low neurovirulence because of a *ts* mutation affecting general viral replication, a comparison of its plaquing efficiencies at 31 and 38.5°C was performed. For this purpose, approximately 100 PFU of RS6 was plated onto cultured rabbit skin cells or primary MEF, and parallel flasks of each were incubated, with an anti-HSV antibody overlay (18), at either 31 or 38.5°C (mouse temperature). At 72 h postinfection, plaques were counted, and RS6 demonstrated 38.5°C/31°C plaquing ratios of 1 on both rabbit skin cells and primary MEF, a result indicating that *ts* mutations for replication were not responsible for the reduced neurovirulence of RS6.

Expression of the enzyme tk. Since production of the enzyme tk by HSV has been implicated for complete expression of neurovirulence (7), tk enzyme assays (4) and Ara-T tests (22) were performed to test whether RS6 efficiently expressed this enzyme.

In the tk enzyme assay, RS6, 17 syn$^+$ (a highly neurovirulent and tk-positive HSV-1 strain) and TK^{-7} (a weakly neurovirulent and tk-negative mutant) were used. LM(TK$^-$) cells in monolayer were mock infected or infected with RS6, 17 syn$^+$, or TK^{-7} at a multiplicity of infection of 1 PFU per cell, and following adsorption for 1 h, the infection proceeded for 14 h. Cells were then harvested and lysed, and a tk assay was performed on the cytoplasmic extracts. The results of this assay (Table 1) indicate that RS6 expressed wild-type levels of HSV-specific tk. These results were confirmed in an Ara-T test, in which replication of RS6 and 17 syn$^+$ was decreased more than 10,000-fold in the presence of the drug, while the replication of TK^{-7} was unaffected (data not shown).

RS6, 17 syn$^+$, and HG_{52} replication in mouse tissues in vivo and in vitro. We next tested the likely possibility that the cause of the low neurovirulence of RS6 was inefficient replication in the mouse brain. Samples (10^5 PFU) of RS6, 17 syn$^+$, or HG_{52} were inoculated into the left cerebral hemispheres of 4- to 5-week-old outbred male Swiss Webster mice. At 0, 12, 24, 48, 72, and 96 h postinfection, surviving mice were sacrificed, and their brains were removed and frozen at −70°C until assayed for virus. The results of this experiment show that RS6 replicated significantly less well than either of its parental viruses did in the mouse brain in vivo (Fig. 1A).

To ascertain whether the brain cell replication deficiency of RS6 was specific or generalized to all mouse cells at the temperature of a mouse (38.5°C), a comparative multistep replication study was performed in primary MEF in vitro at 38.5°C. MEF monolayers 1 day old were infected at a multiplicity of infection of 0.001 PFU per cell with RS6, 17 syn$^+$, or HG_{52}, and at 0, 24, 48, 72, and 96 h postinfection, cells were harvested and frozen at −70°C until assayed for virus. RS6 displayed intermediate replication kinetics in MEF (Fig. 1B), a result which likely reflects the hybrid nature of RS6. These results strongly suggest that RS6 is not replication defective for mouse cells in general at the temperature of a mouse.

In an extension of these studies, we also examined the replication of RS6, 17 syn$^+$, and HG_{52} in nonneural mouse tissues in vivo. RS6 and its parental viruses (10^6 PFU) were

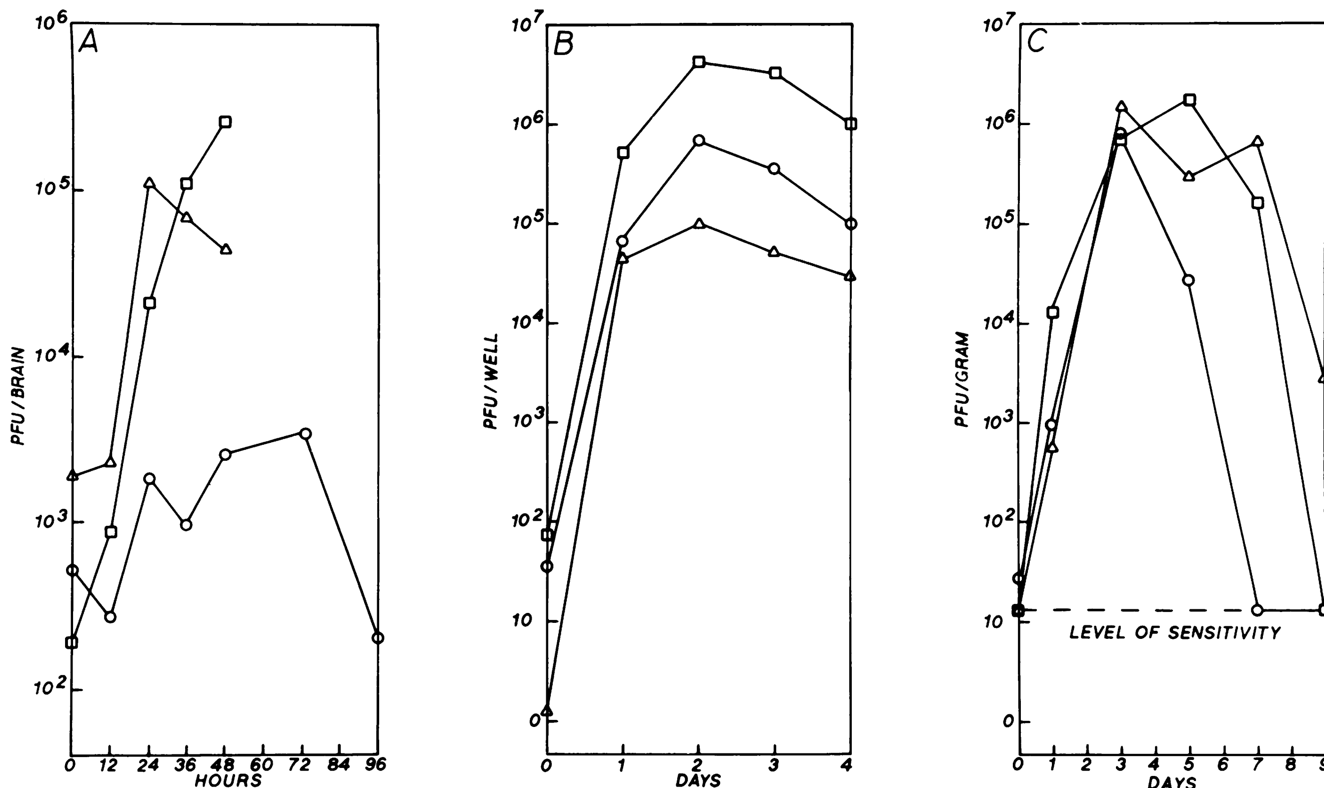

FIG. 1. Comparative viral replication of RS6, 17 syn$^+$, and HG_{52}. (A) Mouse brains in vivo. Mice were inoculated intracranially with approximately 0.03 ml containing 10^5 PFU of virus, and at the indicated times postinfection, surviving mice were sacrificed (three mice per datum point), and their brains were removed and stored at −70°C. The brains were then homogenized as a 10% (wt/vol) solution and centrifuged for 5 min at 3,000 × g in a Sorvall SS-34 rotor, and the resulting supernatant was titrated for virus. (B) MEF in vitro. Primary MEF (10^5) in 24-well tissue culture plates were infected with 100 PFU of virus, and at the indicated times postinfection, cells were harvested (two wells per datum point) by scraping with a pipette and were stored at −70°C. The cells were then frozen and thawed three times before titration for virus. (C) Mouse feet in vivo. The abraded rear footpads of mice were infected with 10^6 PFU of virus, and at the indicated times postinfection, mice were sacrificed (two mice per datum point), and their feet were removed and stored at −70°C. The feet were then ground in a mortar and pestle with coarse sea sand; the homogenate was centrifuged for 5 min at 3,000 × g in a Sorvall SS-34 rotor, and the resulting supernatant was titrated for virus. ○, RS6; □, 17 syn$^+$; △, HG_{52}.

inoculated onto both rear footpads of mice as described previously (4, 21). At 0, 1, 3, 5, 7, and 9 days postinfection, mice were sacrificed, and their feet were removed and stored at −70°C until assayed for virus. The results indicate that RS6 replicated and peaked to titers comparable to its wild-type counterparts (Fig. 1C); however, beyond 3 days postinfection, RS6 did not sustain viral titers like those of 17 syn$^+$ and HG_{52}. This result has been observed previously (23) and may represent the lack of virus passing centrifugally from acutely infected nerve tissue of the ganglia to the foot (17).

Phenotypic stability of RS6 after brain passage and transfection. The stability of the low-neurovirulence phenotype of RS6 was next examined (9). A lethal dose of RS6 (~10^6 PFU) was inoculated into the brain of a mouse, and when the mouse died (24 to 48 h postinfection), its brain was removed and homogenized, and the recovered virus was passed to rabbit skin cells in culture to boost the titer. This procedure was repeated eight times, and when the neurovirulence of passage 8 virus was tested, its LD_{50} (3.0 × 10^5 PFU) after intracerebral inoculation of mice was similar to that of the parental RS6, i.e., 1.2 × 10^5 PFU.

Finally, since transfection of DNA into mammalian cells is known to be mutagenic (2), we transfected unit-length RS6 DNA alone onto five independent plates, harvested the viruses (~10^6 PFU/0.1 ml), and inoculated these viruses intracerebrally into mice. The brain homogenates (cleared by centrifugation at 3,000 × g for 5 min in a Sorvall SS-34 rotor) of mice killed by encephalitis were then inoculated into another group of mice. All mice invariably survived this second brain injection. Nevertheless, an RS6 isolate, one for each of the five independent transfection plates, was plaque purified from the brains of the mice at 4 days postinfection. When LD_{50}s following intracerebral inoculation were determined for each of the five independent isolates, they ranged from 1.0 × 10^5 to 3.0 × 10^5 PFU. Also, when the DNAs of these five viruses were analyzed by restriction enzyme analysis (*Asp*718 and *Bam*HI), their profiles were identical to that of RS6 (data not shown). These experiments indicate that the low-neurovirulence phenotype and the restriction enzyme profile of RS6 were stable after both brain passage and transfection.

Physical localization of the genetic lesion of RS6. (i) RS6 DNA cotransfected with total genomic *Xba*I fragments of 17 syn$^+$. Since RS6 was shown to possess a stable genetic lesion(s) specifically associated with a reduced capacity to replicate in the mouse brain, we next attempted to localize its defect(s) physically on the HSV genome. The technique of marker rescue in combination with in vivo selection (26) was used for this purpose. Initially, we cotransfected unit-

TABLE 2. LD_{50}s after intracranial inoculation of RS6, 17 syn$^+$, and the six neurovirulence-enhanced viruses derived from the cotransfection of unit-length RS6 DNA and total genomic *Xba*I DNA fragments of 17 syn$^+$ [a]

Virus	LD_{50} (PFU)
RS6	1.2×10^5
17 syn$^+$	6
X3-1	260
X3-9	1.5
X3-14	15
X10-13	7
X3-13	4.6
X3-20	3.7

[a] Mice were inoculated with serial 10-fold dilutions of each virus, and deaths from encephalitis were scored for 21 days postinfection. LD_{50}s were determined by using the method of Reed and Muench (15).

length RS6 DNA and total genomic *Xba*I fragments derived from the highly neurovirulent HSV-1 17 syn$^+$. Our rationale was that if a genetic lesion(s) of RS6 resided within or near one of its type 2-encoded regions, all resulting viruses with increased neurovirulence would display a total or partial replacement of this type 2 region with type 1 DNA of 17 syn$^+$. If, on the other hand, a genetic lesion(s) of RS6 resided solely within type 1-encoded regions, this would be indicated by the isolation of neurovirulence-enhanced viruses resembling RS6 in structure.

From the cotransfection of unit-length RS6 DNA with 17 syn$^+$ total genomic *Xba*I fragments, 6 of 20 transfection plates contained neurovirulence-enhanced viruses. When these viruses were plaque purified, one from each infected brain, they were found to exhibit wild-type or near-wild-type neurovirulence (Table 2). As determined by restriction endonuclease analysis using four different enzymes (*Asp*718, *Bam*HI, *Hin*dIII, and *Hpa*I), four of these six viruses (X3-1, X3-9, X3-13, and X3-14) were shown to be recombinants, and except for minor differences, the restriction enzyme profiles of the other two (X3-20 and X10-13) resembled the DNA donor virus 17 syn$^+$ (data not shown). Although each of the six neurovirulence-enhanced viruses had different genomic structures, they all shared two genomic changes (Fig. 2), i.e., replacement of RS6 type 2 DNA with type 1 DNA from approximately 0.11 to 0.14 m.u. (data not shown) and approximately 0.79 to 0.83 m.u.. The latter change (Fig. 3) was manifested as all six viruses gaining an *Asp*718 band in the region of the strain 17 syn$^+$ e (0.80 to 0.83 and 0.95 to 1.0 m.u.), f (0.12 to 0.19 m.u.), and g (0.73 to 0.80 m.u.) triplet, fragments which RS6 lacks. By analyzing the four different restriction enzyme profiles of each of the six recombinant viruses, we determined that of the three possible fragments (*Asp*718 e, f, or g), it was the gain of the 17

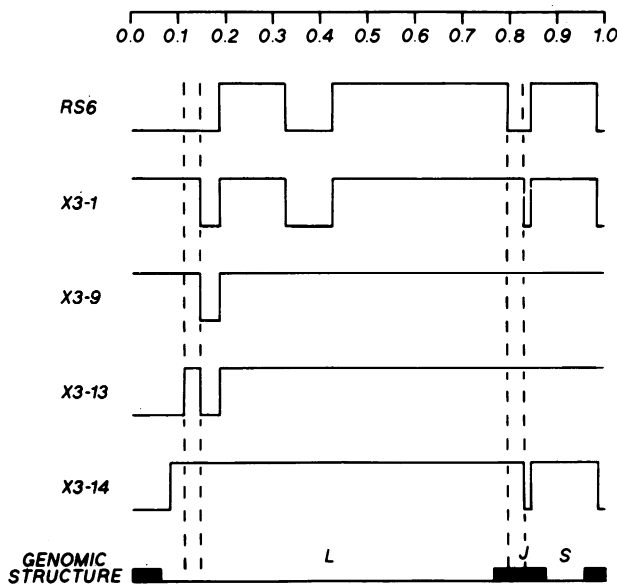

FIG. 2. Genomic structures of RS6 and four of the neurovirulence-enhanced recombinants derived from the cotransfection of unit-length RS6 DNA and 17 syn$^+$ total genomic *Xba*I DNA fragments. The upper line of each genomic structure indicates regions encoded by type 1 DNA (17 syn$^+$), and the lower line indicates regions encoded by type 2 DNA (HG_{52}). The two areas demarcated by the dashed lines (~0.11 to 0.14 m.u. and ~0.79 to 0.83 m.u.) indicate regions where RS6 type 2 DNA was replaced with type 1 DNA in all of the neurovirulence-enhanced viruses.

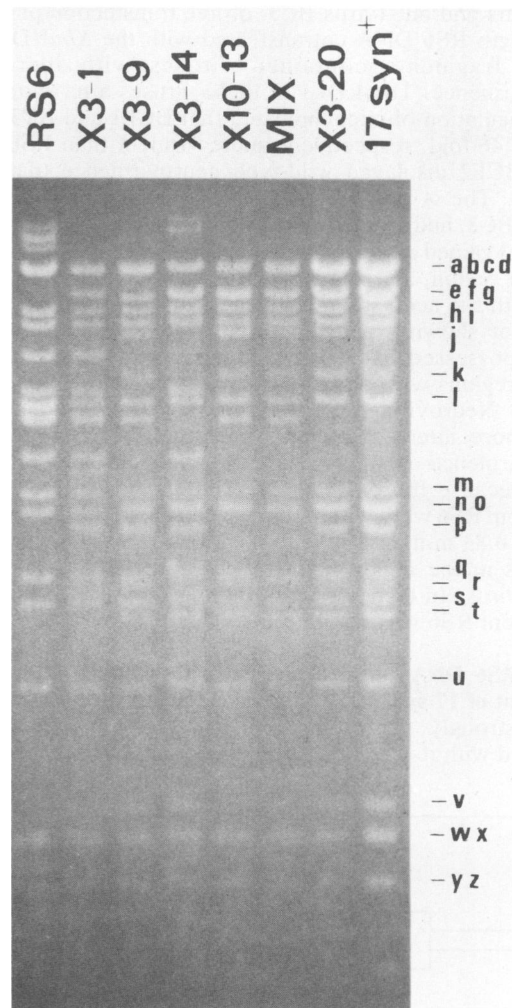

FIG. 3. Agarose gel electropherogram of *Asp*718-generated DNA fragments of RS6, 17 syn$^+$, and the neurovirulence-enhanced viruses derived from the cotransfection of unit-length RS6 DNA and 17 syn$^+$ total genomic *Xba*I DNA fragments. All of the neurovirulence-enhanced viruses acquired a novel band, which RS6 lacks, in the 17 syn$^+$ e, f, and g triplet region. The lane labeled MIX shows the DNA of a virus isolated from the same brain as virus X10-13, and virus X3-13 is not shown.

syn$^+$ *Asp*718 g fragment that was common to all of the neurovirulence-enhanced recombinant viruses (data not shown). The gain of the type 1 *Asp*718 g fragment by these six viruses arose by the acquisition of a type 1 *Asp*718 site at approximately 0.8 m.u., which RS6 lacks because of a type 2 region extending from 0.79 to 0.83 m.u. (Fig. 4). Therefore, the regions located from approximately 0.11 to 0.14 m.u. and 0.79 to 0.83 m.u. were tentatively associated with the low-neurovirulence characteristics of RS6.

(ii) RS6 DNA cotransfected with electroeluted HSV-1 *Xba*I fragments. To confirm and extend the observation that all recombinant viruses with increased neurovirulence displayed replacement of type 2 DNA with type 1 DNA at approximately 0.11 to 0.14 and 0.79 to 0.83 m.u., we electrophoretically purified *Xba*I fragments of 17 syn$^+$ spanning these regions and used them in cotransfection experiments with unit-length RS6 DNA. Two (viruses BCF1 and BCF2) of five transfection plates of unit-length RS6 DNA cotransfected with the *Xba*I DNA b (0 to 0.07 and 0.77 to 1.0 m.u.), c (0.07 to 0.29 m.u.), and f (0.29 to 0.45 m.u.) fragments and one (virus BC3) of five transfection plates of unit-length RS6 DNA cotransfected with the *Xba*I DNA b and c fragments contained viruses with increased neurovirulence. The LD_{50}s of these viruses after intracerebral inoculation of mice indicated that BCF1 and BC3 were 81- and 46-fold, respectively, more virulent than RS6 was, while BCF2 displayed wild-type neurovirulence (data not shown). The *Asp*718 restriction enzyme profiles of RS6, BCF1, BC3, and 17 syn$^+$ (Fig. 5) indicate that viruses BCF1 and BC3 gained a novel band in the 17 syn$^+$ *Asp*718 e, f, and g triplet region, and as before, this was found to be associated with the acquisition of the 17 syn$^+$ *Asp*718 g fragment (data not shown); highly neurovirulent recombinant virus BCF2 possessed extensive genomic replacements of RS6 type 2 regions with type 1 DNA and was not characterized further. Neurovirulence-enhanced viruses BCF1 and BC3 were more interesting than were any of the original six neurovirulence-enhanced viruses discussed above, however, because the only genomic differences of BCF1 and BC3 from RS6 were within the two adjacent type 2 regions at 0.79 to 0.83 m.u. and 0.83 to 0.85 and 0.99 to 1.0 m.u.; no changes in the region 0.11 to 0.14 m.u. were evident for either virus. In fact, for virus BC3 the only evident difference from RS6 was within the type 2 region at 0.79 to 0.83 m.u..

(iii) RS6 DNA cotransfected with the cloned *Eco*RI e+k fragment of 17 syn$^+$. The marker rescue experiments in this study strongly suggest that RS6 has a genetic lesion(s) involved with its low neurovirulence within or near the type 2 region at 0.79 to 0.83 m.u. To lend support to this hypothesis, we next used a cloned fragment, 17 syn$^+$ *Eco*RI e+k (0.72 to 0.87 m.u.), which spans the region of interest. In this experiment, unit-length RS6 DNA cotransfected with the cloned *Eco*RI e+k fragment (five plates) and unit-length RS6 DNA transfected alone (five plates) were screened for the presence of viruses with increased neurovirulence. The cotransfection of unit-length RS6 DNA with the cloned *Eco*RI e+k fragment resulted in three of the five plates yielding viruses with enhanced neurovirulence (RB1, RB3, and RB4). On the other hand, no viruses with enhanced neurovirulence were obtained from plates of RS6 DNA transfected alone. Viruses RB1, RB3, and RB4 were then inoculated intracerebrally into mice to determine their LD_{50}s; they were all more neurovirulent than RS6 was (Table 3). Restriction enzyme analysis with *Asp*718 revealed that RB1, RB3, and RB4 were recombinants which also displayed a novel band in the *Asp*718 e, f, and g triplet region of 17 syn$^+$ (Fig. 6A). A Southern blot analysis of this gel by using 17 syn$^+$ *Hpa*I fragment t (0.74 to 0.76 m.u.) as a probe (Fig. 6B) proved that the novel *Asp*718 band present for RB1, RB3, and RB4 in the e, f, and g triplet region of 17 syn$^+$ was fragment g. In addition, since none of these three viruses lost the adjacent type 2 *Asp*718 sites at approximately 0.84 and 0.99 m.u., there is a strong suggestion that the type 2 region from 0.79 to 0.83 m.u. is involved with the low neurovirulence of RS6.

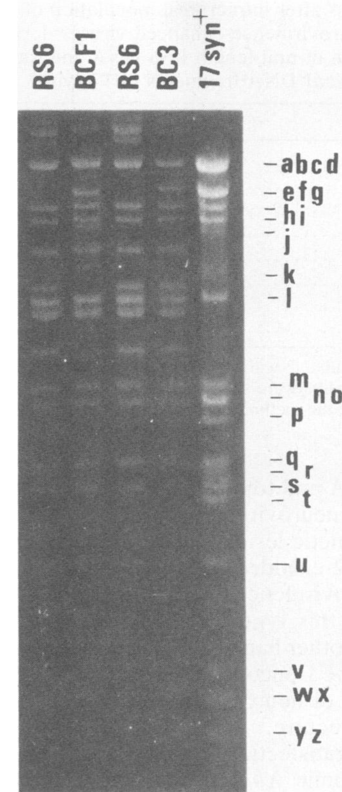

FIG. 5. Agarose gel electropherogram of *Asp*718-generated DNA fragments of RS6, 17 syn$^+$, and two of the neurovirulence-enhanced viruses derived from the cotransfection of unit-length RS6 DNA with electrophoretically purified 17 syn$^+$ *Xba*I DNA fragments b, c, and f (virus BCF1) or b and c (virus BC3). Both BCF1 and BC3 acquired a novel band, which RS6 lacks, in the 17 syn$^+$ e, f, and g triplet region.

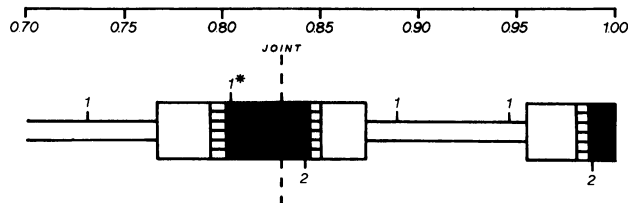

FIG. 4. HSV-1 and HSV-2 *Asp*718 (*Kpn*I isoschizomer) sites encoded within the region 0.73 to 1.0 m.u. of the RS6 genome. 1 and 2, HSV-1- and HSV-2-encoded *Asp*718 sites, respectively. ■, regions encoded by HSV-2 DNA; □, regions encoded by HSV-1 DNA; □, regions of crossover uncertainty. The wide areas signify inverted repeat sequences, and the thin areas are unique sequences. RS6 lacks the HSV-1 *Asp*718 site at ~0.8 m.u. (*), and this is why RS6 does not possess an *Asp*718 g fragment (0.73 to 0.80 m.u.).

TABLE 3. LD_{50}s after intracranial inoculation of RS6 and the three neurovirulence-enhanced viruses derived from the cotransfection of unit-length RS6 DNA and the 17 syn$^+$ *Eco*RI e+k fragment clone[a]

Expt and virus	LD_{50} (PFU)
1	
RS6	6.0×10^5
RB3	1.9×10^4
2	
RS6	1.5×10^5
RB4	4.7×10^3
3	
RS6	3.0×10^5
RB1	1.9×10^4
RB3	2.4×10^4
RB4	2.0×10^4

[a] No neurovirulence-enhanced recombinants were isolated by using the cloned 17 syn$^+$ *Eco*RI d (~0.08 to 0.18 m.u.) or f (~0.32 to 0.43 m.u.) fragments. *Eco*RI fragments e+k, d, and f are cloned into pUC12. Mice were inoculated with serial 10-fold dilutions of each virus, and deaths from encephalitis were scored for 21 days postinfection. LD_{50}s were determined by using the method of Reed and Muench (15).

DISCUSSION

The results show that the HSV intertypic recombinant RS6 is a specific-neurovirulence mutant and identify one genomic region involved with this property. Thus, we have shown that the region 0.72 to 0.87 m.u. contains at least one gene involved with HSV neurovirulence, since the 17 syn$^+$ *Eco*RI e+k fragment conferred increased neurovirulence to RS6. This general region has previously been implicated as contributing to HSV stromal disease in rabbits (3), neurovirulence in mice (24), and virulence following intraperitoneal injection of mice (1). Candidate genes encoded within the region 0.72 to 0.87 m.u. include immediate-early genes ICP0, ICP4, and ICP27, genetic loci involved in syncytial plaque morphology, and the late gene ICP34.5. Additionally, we tentatively conclude that the region 0.79 to 0.83 m.u. contains the gene(s) of interest, since all neurovirulence-enhanced recombinant viruses displayed replacement of type 2 DNA with type 1 DNA in this region. The known genes encoded within the region 0.79 to 0.83 m.u. are ICP0 and ICP34.5.

Since repair of the genetic lesion(s) within the region encoded by the *Eco*RI e+k fragment (0.72 to 0.87 m.u.) alone does not result in recombinant viruses with wild-type neurovirulence, it seems likely that at least one other gene is involved with HSV neurovirulence. As suggested by the structures of recombinant viruses described in this study, a candidate region for the site of another gene(s) is the area from 0.11 to 0.14 m.u. since, as with the region 0.79 to 0.83 m.u., all highly neurovirulent recombinants had type 2 DNA replaced with type 1 DNA in this region. This result suggests that repair of genetic lesions within these two regions may result in highly neurovirulent recombinant viruses. Marker rescue experiments to test this possibility by using cloned fragments spanning both of these regions are in progress.

With respect to related studies, this laboratory has previously reported the characterization of another intertypic recombinant, RE6, which is also a specific-neurovirulence mutant (25). The gene associated with the nonneurovirulence of RE6 was mapped to 0.71 to 0.83 m.u. of the HSV genome (24, 26), and it could be suggested that RS6 and RE6 have a genetic lesion in common. In an experiment to test this possibility, RS6 and RE6 were mixedly infected in tissue culture in attempts to produce a recombinant virus more neurovirulent than RS6 or RE6 is. So far, we have not found such a recombinant. This result suggests either that RS6 and RE6 share the same genetic lesion or that these viruses have two different lesions very near one another. Additional marker rescue experiments should permit us to distinguish between these possibilities.

Finally, it is important to consider the reason that RS6 is deficient in replicating and producing fatal encephalitis following intracranial inoculation of mice (i.e., the nature of the block to replication in the brain). In initial attempts to study this, we used immunohistochemical techniques to locate HSV antigens in the brains of mice acutely infected with RS6, 17 syn$^+$, and HG_{52}. Preliminary results indicate that RS6 antigens are not found at distant locations from the initial site of inoculation at times when antigens of its wild-type parental strains are found (unpublished results). A likely interpretation of these results is that RS6 is defective for replication in initially infected cells, and future experi-

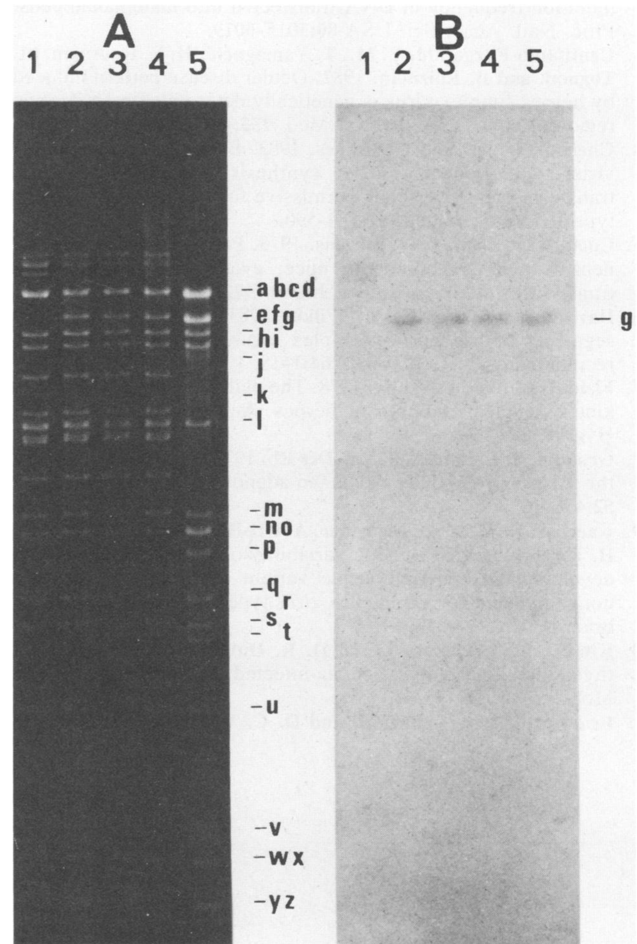

FIG. 6. (A) Agarose gel electropherogram of *Asp*718-generated DNA fragments of RS6, 17 syn$^+$, and the three neurovirulence-enhanced viruses derived from the cotransfection of unit-length RS6 DNA and the cloned *Eco*RI e+k fragment of 17 syn$^+$. Note that all three neurovirulence-enhanced viruses display a novel band, which RS6 lacks, in the 17 syn$^+$ e, f, and g triplet region. (B) Southern blot of the gel in panel A probed with a biotinylated *Hpa*I t fragment (0.74 to 0.76 m.u.) of 17 syn$^+$. The novel *Asp*718 band acquired by the three neurovirulence-enhanced recombinants is fragment g (0.73 to 0.80 m.u.). 1, RS6; 2, RB1; 3, RB3; 4, RB4; 5, 17 syn$^+$.

ments in this area will be designed with this possibility in mind.

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