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Localization of a Herpes Simplex Virus Neurovirulence Gene Dissociated from High-Titer Virus Replication in the Brain

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Previous studies with the herpes simplex virus type 1 × type 2 intertypic recombinant RS6 suggested that the genomic region from 0.11 to 0.14 map units is involved in neurovirulence (R. T. Javier, R. L. Thompson, and J. G. Stevens, J. Virol. 61:1978–1984, 1987). To study this further, we isolated an RS6-derived herpes simplex virus intertypic recombinant (R13-1) which has a genetic defect within this area. After inoculation into mouse brains, R13-1 was found to be approximately 10,000-fold less neurovirulent than either the wild-type type 1 or type 2 parental virus. However, R13-1 replicated in the mouse brain to titers resembling those of the wild-type parents. Further comparisons with wild-type counterparts indicated that R13-1 expressed equivalent levels of the enzyme thymidine kinase and replicated to intermediate levels in primary mouse embryo fibroblasts maintained at the normal body temperature for mice. Using marker rescue techniques combined with in vivo selection, we found that recombination between unit-length R13-1 DNA and a cloned type 1 DNA fragment spanning the region from 0.11 to 0.14 map units (*Eco*RI-d, 0.079 to 0.192 map units) generated viruses with a wild-type neurovirulence phenotype. To further refine the genomic region of interest, we performed marker rescue experiments using two *Eco*RI-d subclones, *Eco*RI/*Bam*HI dc (0.079 to 0.143 map units) and *Bam*HI/*Eco*RI ad (0.143 to 0.192 map units), representing the left and right halves of the *Eco*RI d fragment, respectively. In these experiments the *Eco*RI/*Bam*HI dc clone, but not the *Bam*HI/*Eco*RI ad clone, yielded recombinant viruses exhibiting wild-type neurovirulence. These results show that at least one herpes simplex virus gene function associated with neurovirulence is located within a 9.1-kilobase region at 0.079 to 0.143 map units of the viral genome. Perhaps more significantly, the results indicate that this neurovirulence property functions independently of high-titer virus replication in the brain.

Herpes simplex virus type 1 (HSV-1) induces a life-threatening encephalitis in humans (for a review, see reference 13), and there is a continuing interest in understanding the properties of the agent relating to this syndrome. To define various aspects of this disease, mice have served as an experimental model because after intracranial inoculation they succumb to very low doses of most HSV-1 or HSV-2 isolates. By exploiting this model to identify mutations in viral genes whose primary function is the enhancement of virulence within the central nervous system, it should ultimately be possible to establish molecular mechanisms underlying HSV-related neurovirulence.

Genetic analyses designed to identify specific neurovirulence-associated genes are limited by the availability of suitable mutant viruses, and to date, the viral thymidine kinase (TK) gene (4) and, more recently, the viral DNA polymerase gene (3) have been closely linked to HSV-induced central nervous system disease. To identify other viral genes associated with this property, we have been studying two HSV-1 × HSV-2 intertypic recombinants (RE6 and RS6) which exhibit phenotypes useful for the study of neurovirulence (6, 18). Although attenuated for virulence and replication after intracranial inoculation of mice, these viruses express wild-type (wt) levels of thymidine kinase and replicate efficiently in nonneural mouse tissues. Using cloned wt HSV-1 DNA in marker rescue experiments, we found that genetic defects contributing to the reduced neurovirulence of both RE6 and RS6 are located in an overlapping genomic region (0.72 to 0.83 map units [m.u.]) which does not include the viral-encoded TK or DNA polymerase gene (6, 17). Since increased neurovirulence of recombinant viruses derived from these two intertypic viruses is always associated with heterologous DNA replacements (HSV-2 DNA replaced with HSV-1 DNA) in specific genomic regions and since HSV-1 and HSV-2 DNA are easily distinguishable by restriction enzyme analysis, genetic studies using these intertypic viruses are greatly facilitated.

In a previous study with RS6 (6), it was apparent that at least one other genetic defect was responsible for the avirulent phenotype of this virus. Thus, in marker rescue experiments (using unit-length RS6 DNA and total-genomic HSV-1 DNA fragments), only recombinant viruses in which RS6 HSV-2 DNA was replaced with HSV-1 DNA in both the regions from 0.79 to 0.83 m.u. and 0.11 to 0.14 m.u. exhibited wt neurovirulence. This observation suggested that the region from 0.11 to 0.14 m.u. also participated in HSV neurovirulence. Despite this, attempts to increase the neurovirulence of RS6 by using only a cloned HSV-1 DNA fragment spanning the region from 0.11 to 0.14 m.u. were unsuccessful (6; unpublished results), suggesting that repair of a more dominant genetic defect was required before the function of this region could be revealed. We now report the isolation and characterization of such a recombinant which retains the low-neurovirulence phenotype of RS6. Unexpectedly, this virus was also found to replicate to wt levels in the mouse brain. Using marker rescue techniques, we mapped the defect(s) associated with these phenotypes to the region from 0.079 to 0.143 m.u. of the HSV genome.

MATERIALS AND METHODS

Cells, viruses, and mouse inoculations. The techniques for culture of rabbit skin cells, production of primary mouse

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embryo fibroblasts (MEFs), and infection, propagation, and titration of viruses have been described previously (18, 19). Plaque purification of virus was performed by overlaying cells with medium containing 0.3% agarose (SeaKem; FMC Corp., Marine Colloids Div.). After 3 days, well-isolated plaques were collected with a Pasteur pipette. The details of examining multistep replication in vitro and mouse brain replication in vivo were also described previously (18). Neurovirulence was measured by intracranial inoculation of mice because this route of infection is not influenced by the neuroinvasiveness of the virus (16). Six-week-old outbred male Swiss Webster mice (Simonsen Laboratories) were used in all experiments, and standard procedures for intracranial inoculation (18, 19) and quantitation of neurovirulence (14) were used.

TK enzyme assay. The TK enzyme assay was a modification of that used by Jamieson and Subak-Sharpe (5). LM(TK$^-$) cells (10^7) (kindly provided by S. Kit; 8) in tissue culture dishes (100 by 15 mm) were mock infected or infected with 5 PFU per cell (1 h adsorption at 37°C) and incubated at 38.5°C (normal body temperature for mice) for 5 h. The cells were then harvested by scraping with a rubber policeman, chilled on ice, and pelleted (500 × *g* for 10 min at 4°C) in an International Equipment Company CRU-5000 centrifuge. The cell pellet was washed with ice-cold 0.9% NaCl, repelleted as described above, resuspended, lysed by sonication at 0°C in 0.2 ml of storage buffer (50 mM Tris [pH 7.2], 5 mM mercaptoethanol, 0.005 mM thymidine), and centrifuged for 15 min at 4°C in an Eppendorf centrifuge (model 5414). A 0.1-ml portion of the resulting cellular cytoplasmic extract was then mixed with 0.08 ml of 2× reaction buffer (100 mM Tris [pH 7.2], 20 mM ATP, 20 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride) and 0.02 ml of aqueous [*methyl*-^{3}H]thymidine (52 Ci/mmol, 1.0 mCi/ml) and incubated at 38.5°C for 30 min. The solution was then boiled for 1 min and pipetted onto a 2.5-cm^2 Whatman DE-81 filter paper square. After 5 min, each filter square was washed four times with 15 ml of wash solution (1 mM ammonium formate [pH 6.0], 0.002 mM thymidine) and twice with 15 ml of 95% ethanol and then dried in a desiccator for 1 h. Radioactivity was measured by submersing the filter square in 5 ml of scintillation cocktail {0.5% 2,5-diphenyloxazole, 0.01% 1,4-bis[2-(5-phenyloxazolyl)] benzene in toluene} and counting in a scintillation counter. Protein was quantitated by the Lowry method.

Transfections and in vivo selections. Methods for the transfection and isolation of unit-length DNA used for transfection have been described (19). The marker rescue technique combined with in vivo selection was a modification of that developed by Thompson et al. (19) and can be described in four parts. (i) To generate recombinant viruses, unit-length R13-1 DNA was cotransfected with a cloned wt HSV-1 DNA fragment onto rabbit skin cells which were harvested when fully infected. (ii) As the initial step toward selecting highly neurovirulent recombinant viruses, 0.03 ml of the resulting virus mixture was inoculated undiluted into the brains of two mice. The two mice invariably died from encephalitis, at which time their brains were removed and homogenized together as a 10% (wt/vol) solution, and the homogenate was cleared by centrifugation (3,000 × *g* for 5 min at 4°C in a Sorvall SS-34 rotor). (iii) As a final selection for the most neurovirulent viruses, 0.03-ml portions of the cleared brain homogenate were inoculated intracranially into two more mice. (iv) If at least one of these mice died from encephalitis, virus was isolated from the brain and plaque purified (one virus per transfection plate) for further analysis. Since some viruses derived from R13-1 DNA transfected alone were able to progress to this step, transfection plates were scored as positive for neurovirulence-enhanced recombinant viruses only when viruses more neurovirulent than those derived from R13-1 DNA transfected alone and processed as described above were isolated.

Restriction enzyme and Southern blot analyses. Small viral DNA preparations used for restriction enzyme and Southern blot analyses were obtained by a rapid method in which 10^7 rabbit skin cells were infected with approximately 1 PFU per cell and after 24 h harvested by scraping with a rubber policeman. The infected cells were pelleted (500 × *g* for 10 min at 4°C) in an IEC CRU-5000 centrifuge, the supernatant was discarded, and the cell pellet was suspended by vigorous vortexing in 5 ml of ice-cold hypotonic lysis buffer (10 mM Tris [pH 8.0], 10 mM EDTA, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] deoxycholate). After sitting on ice for 20 min, the cells were again vortexed vigorously and centrifuged (3,000 × *g* for 5 min at 4°C) in a Sorvall SS-34 rotor to pellet nuclei. The resulting supernatant was extracted once with an equal volume of phenol and once with an equal volume of chloroform, collected, and adjusted to 0.1 M NaCl. Viral DNA was precipitated by adding 2 volumes of ethanol (−20°C) and centrifuging (30,000 × *g* for 1 h at 4°C) in a Beckman ultracentrifuge SW28.1 swinging bucket rotor. The resulting DNA pellet was dried in a desiccator and dissolved in 0.1 ml of TE (10 mM Tris [pH 8.0], 1 mM EDTA) containing 0.02 mg of RNase A per ml. A 0.02-ml portion of this solution was normally sufficient for analysis with one restriction enzyme.

Standard methods for agarose gel electrophoresis, DNA transfer to nylon membranes, and preparation of cloned HSV DNA were used (10). Cloned DNA was nick translated in the presence of biotin-11-dUTP (Bethesda Research Laboratories, Inc.) and used in hybridization experiments as described previously (6).

RESULTS

Reconfirming the significance of the region from 0.11 to 0.14 m.u. for neurovirulence, and isolation of virus R13-1. The importance of the genomic region from 0.11 to 0.14 m.u. for HSV neurovirulence was first suggested in a previous study (6) in which we found that one of two common genomic changes exhibited by recombinant viruses with wt neurovirulence characteristics was replacement of HSV-2 DNA with HSV-1 DNA between 0.11 and 0.14 m.u. One of these recombinant viruses (X3-13) exhibited a genomic structure in which a narrow region of HSV-2 DNA at 0.11 to 0.14 m.u. had been replaced with HSV-1 DNA (Fig. 1). To reconfirm that the region from 0.11 to 0.14 m.u. functioned in HSV neurovirulence, we used total genomic *Hin*dIII-digested X3-13 DNA in cotransfections with unit-length RS6 DNA. It was predicted that if the X3-13 HSV-1 region from 0.11 to 0.14 m.u. was essential for neurovirulence, then all resulting recombinant viruses with wt neurovirulence would have acquired this HSV-1 region from X3-13.

From 15 transfection plates, we were able to isolate 11 neurovirulence-enhanced recombinant viruses (each from a different plate), 7 of which displayed wt neurovirulence (data not shown). When examined by restriction enzyme analysis (data not shown), all seven of these viruses had gained the X3-13 HSV-1 region at 0.11 to 0.14 m.u., among other genomic changes, reconfirming the importance of this region for neurovirulence. Of greater significance, however, was the fact that one virus (R13-1) isolated in this experiment did

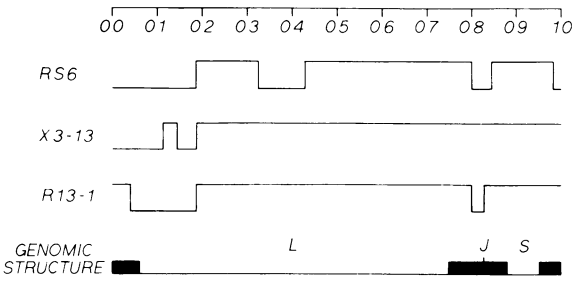

FIG. 1. Genomic structures of HSV intertypic recombinants RS6, X3-13, and R13-1 in prototype orientations. The top line of each genomic structure indicates regions encoded by HSV-1 DNA (17 syn$^+$), and the bottom line represents regions encoded by HSV-2 DNA (HG_{52}). The scale at the top displays genomic map coordinates. The structure of the HSV genome is shown at the bottom. The inverted repeats (▬), joint region (J), and long (L) and short (S) unique regions are also indicated.

not demonstrate the X3-13 HSV-1 region at 0.11 to 0.14 m.u. and was weakly neurovirulent (see below). The genomic structure of the intertypic recombinant virus R13-1 is illustrated in Fig. 1.

Comparative phenotypes of R13-1, 17 syn$^+$, and HG_{52}. (i) LD_{50} after intracranial inoculation. To establish the neurovirulence of R13-1 relative to that of its wt virus counterparts (strains 17 syn$^+$ and HG_{52}), we inoculated 6-week-old male outbred Swiss Webster mice intracranially with 0.03 ml of serial 10-fold dilutions (five mice per dilution) of virus. Deaths resulting from encephalitis were scored for 21 days postinfection, and at the end of the experiment, 50% lethal doses (LD_{50}s) were calculated by the method of Reed and Muench (14). The LD_{50} of R13-1, 17 syn$^+$, and HG_{52} were 3.5×10^4, 2.0, and 1.3 PFU, respectively, indicating that R13-1 was approximately 10,000-times less neurovirulent than the wt viruses from which it was derived.

Since R13-1 had extensive genomic replacements of RS6 HSV-2 DNA with HSV-1 DNA, it was potentially a virus with a neurovirulence defect only in the region from 0.11 to 0.14 m.u. If this was so, we could more definitively establish the role of this genomic region in HSV neurovirulence and then functionally characterize the gene(s) involved. We began by establishing pertinent biochemical and biological phenotypes.

(ii) TK expression. Because of the established correlation between TK expression and neurovirulence in mice (4), we examined TK expression by R13-1 by performing a TK enzyme assay of viruses R13-1, 17 syn$^+$ (TK positive), and TK^{-7} (TK negative). LM(TK$^-$) cells in monolayers were mock infected or infected with R13-1, 17 syn$^+$, or TK^{-7}. After 1 h of adsorption at 37°C, infection continued for 5 h at 38.5°C (normal body temperature for mice). The cells were then harvested and lysed, and the cytoplasmic extracts were used to test for TK enzyme activity. The results (Table 1) indicate that R13-1 expressed wt levels of TK in murine cells maintained at the normal body temperature for mice. Therefore, the low neurovirulence of R13-1 cannot be attributed to reduced TK expression.

(iii) Replication kinetics in vitro and in vivo. A trivial explanation for the reduced neurovirulence of R13-1 would be generalized defective replication in mouse cells at the normal body temperature for mice. Therefore, we compared the multistep replication kinetics of R13-1 to those of its wt parental viruses in primary MEFs at 38.5°C. MEFs were infected with 0.01 PFU of R13-1, 17 syn$^+$, or HG_{52} per cell;

TABLE 1. Expression of HSV-specific TK measured by phosphorylation of [*methyl*-^{3}H]thymidine[a]

Virus	Phosphorylation of thymidine (cpm)[b]
17 syn$^+$	215,600
R13-1	247,600
TK^{-7}	16,000
None (mock infected)	19,900

[a] Mock-infected- and infected-cell cytoplasmic extracts were derived for assay at 5 h postinfection from LM(TK$^-$) cells infected with 5 PFU per cell. Cells were incubated at 38.5°C (normal body temperature for mice).

[b] Counts per minute of [*methyl*-^{3}H]thymidine phosphorylated per 30 min per milligram of protein at 38.5°C.

at 0, 24, 48, 72, and 96 h postinfection, the cells were harvested and stored frozen at −70°C for later titration. R13-1 displayed intermediate replication kinetics in MEFs (Fig. 2A), similar to those reported previously for virus RS6 (6), suggesting that the reduced neurovirulence of R13-1 was not caused by generalized replicative defects in mouse cells at the normal body temperature for mice.

Previous work with the intertypic viruses RE6 (18) and RS6 (6) indicated that reduced neurovirulence in mice correlates with an inability to replicate to wt virus titers in the mouse brain, and we expected that R13-1 would display similar replication deficiencies in these tissues. To test this, 6-week-old male outbred Swiss Webster mice were inoculated intracranially with 3×10^3 PFU of R13-1, 17 syn$^+$, or HG_{52}. This viral dose represented approximately 0.1 LD_{50} of R13-1 and 10^3 LD_{50}s of 17 syn$^+$ and HG_{52}. At 0, 14, 24, 39, 48, 72, 96, and 144 h postinfection surviving mice were killed, and brains were removed and stored frozen at −70°C

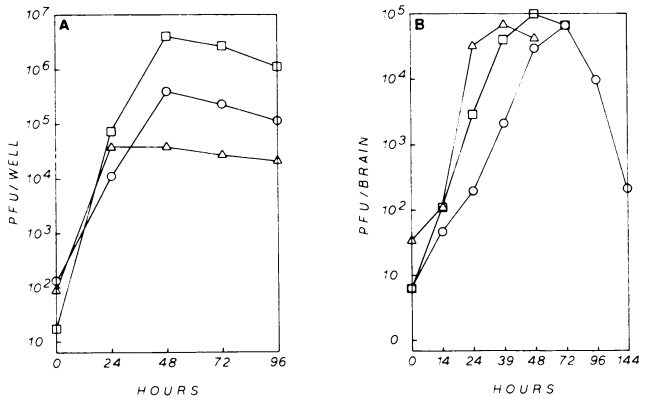

FIG. 2. (A) Replication kinetics of HSV R13-1, 17 syn$^+$, and HG_{52} in primary MEFs at 38.5°C in vitro. A total of 10^5 MEFs in 24-well tissue culture plates were infected with 10^3 PFU of virus, and at the indicated times postinfection, the cells were harvested (two wells per time point per virus) by scraping with the end of a plastic 5-ml pipette and frozen at −70°C for later titration. At the time of titration, the cells were frozen and thawed twice to release viruses. (B) Replication kinetics in mouse brains in vivo. Six-week-old male Swiss Webster mice were inoculated intracranially with 3×10^3 PFU of virus, and at the indicated times postinfection, surviving mice (two mice per time point per virus) were killed by cervical dislocation, and their brains were removed and stored at −70°C. For titration of viruses, the brains were homogenized as 10% (wt/vol) solutions, and the homogenates were centrifuged in a Sorvall SS-34 rotor (3,000 × *g* for 5 min at 4°C). The resulting supernatants were titrated for virus on rabbit skin cells. Symbols: ○, R13-1; □, 17 syn$^+$; △, HG_{52}.

for later titration of virus. The results of this experiment are presented in Fig. 2B. During the course of the experiment, all mice inoculated with HG_{52} or 17 syn^+ died from encephalitis by 48 and 72 h, respectively, but no mice inoculated with virus R13-1 died. Nevertheless, the results show that, although the replication kinetics of R13-1 slightly lagged behind those of the wt viruses, peak viral yields were equivalent and no fatal encephalitis was produced. These analyses of virus R13-1 suggest that it carries a genetic defect which dissociates neurovirulence from high-titer replication in the brain.

Physical localization of the genetic defect in R13-1. (i) Rescue with the 17 syn^+ *Eco*RI-d clone (0.079 to 0.192 m.u.). Since the genomic structure of R13-1 indicated that its genetic defect(s) might be located in the region from 0.11 to 0.14 m.u., we performed a marker rescue experiment using a cloned HSV-1 DNA fragment spanning this region. Specifically, we cotransfected unit-length R13-1 DNA and cloned HSV-1 (17 syn^+) *Eco*RI-d (0.079 to 0.192 m.u.) DNA onto five independent cell cultures. We also performed two control experiments because the mutagenicity of DNA transfection (1) and the selective conditions of brain passage (7) could have revealed that the phenotype studied was influenced by these procedures. First, for neurovirulence comparisons, virus R13-1 DNA was transfected alone, and the resulting viruses were passaged twice through mouse brains (Table 2). Second, we cotransfected unit-length R13-1 DNA with the cloned HSV-1 (17 syn^+) *Eco*RI g DNA fragment (0.192 to 0.300 m.u.) onto five cultures and tested progeny. This transfection controlled for an HSV-1 DNA fragment similar in size to *Eco*RI-d and cloned into the same plasmid vector.

We were able to recover virus from injected mouse brains (see Materials and Methods) with all five transfection plates containing the *Eco*RI-d clone and two of five transfection plates containing the *Eco*RI-g clone. The five *Eco*RI-d-derived (D1 through D5) and one of the two *Eco*RI-g-derived (G5) viruses were then plaque purified, and LD_{50}s were determined. The LD_{50}s after intracranial inoculation of mice with control virus R13-1, wt virus 17 syn^+, viruses D1 through D5, and control virus G5 are shown in Table 2. Although the control viruses (R13-1 and G5) displayed as much as a 25-fold increase in neurovirulence over that of the original R13-1 stock virus (LD_{50}, 3.5×10^4 PFU), only the five viruses derived from the cotransfection of unit-length R13-1 DNA with the cloned *Eco*RI-d DNA displayed LD_{50}s similar to that of 17 syn^+.

To investigate whether viruses D1 through D5 were recombinants, we examined their DNAs by restriction enzyme and Southern blot analyses. The restriction enzyme *Bam*HI was chosen for these analyses because in the HSV-2 region of R13-1 overlapping the HSV-1 *Eco*RI d fragment, there were several HSV-2 *Bam*HI fragments whose disappearance would indicate replacement of HSV-2 DNA with HSV-1 DNA (Fig. 3). The *Bam*HI-digested DNAs of viruses D1 through D5 flanked by DNA of R13-1 and HG_{52} (HSV-2) are shown in Fig. 4A. By this analysis, it was found that a prominent genomic difference between these viruses and R13-1 was the presence of one of two novel high-molecular-weight fragments (cxh or cxh', Fig. 3). A Southern blot (Fig. 4B) of the gel shown in Fig. 4A, probed with the *Eco*RI d fragment, indicated that these novel high-molecular-weight bands resulted from the loss of HSV-2 (HG_{52}) *Bam*HI fragments c (0.048 to 0.099 m.u.), x (0.099 to 0.115 m.u.), and h (0.115 to 0.154 m.u.). Also important was the fact that only virus D5 lost the HSV-2 *Bam*HI q fragment (0.154 to 0.179 m.u.). The latter finding suggested that to generate recombinant viruses with wt neurovirulence, replacement of R13-1 HSV-2 DNA with HSV-1 DNA only within the left portion of the *Eco*RI d fragment was required.

(ii) Rescue with subclones of the 17 syn^+ *Eco*RI-d clone. The results presented above strongly suggested that the HSV-1 DNA sequences needed to rescue the reduced neurovirulence of R13-1 were located in the left-hand side of the *Eco*RI d fragment. Since there is a single *Bam*HI site (0.143 m.u.) located near the middle of the *Eco*RI d fragment, we cleaved the *Eco*RI d fragment with *Bam*HI and *Eco*RI and subcloned the resulting 9.1-kilobase *Eco*RI/*Bam*HI fragment dc and the 7.8-kilobase *Bam*HI/*Eco*RI fragment ad, representing the left and right halves, respectively, of *Eco*RI-d (Fig. 3B). Fragments dc (0.079 to 0.143 m.u.) and ad (0.143 to 0.192 m.u.) were then used in marker rescue experiments with unit-length R13-1 DNA. Ten of ten dc and seven of ten ad transfection plates produced virus. Of these virus-positive plates, 8 of 10 dc and 2 of 7 ad transfection plates yielded viruses after mouse brain passage (see Materials and Methods). The eight dc (viruses 62-3 through 62-10) and two ad (viruses 37-6 and 37-7)-derived viruses were plaque purified and examined for neurovirulence by intracranial inoculation of mice. The LD_{50}s of these viruses and the control virus R13-1 (Table 2) are shown in Table 3. Four of the eight dc-derived viruses (62-5, 62-6, 62-9, and 62-10) exhibited wt neurovirulence, but neither of the two ad-derived viruses displayed increased neurovirulence beyond that exhibited by the control virus, R13-1. Again, as was indicated by the genomic structures of recombinant viruses derived from *Eco*RI-d transfections, only the left side of the *Eco*RI d fragment was involved in generating viruses with wt neurovirulence.

To determine whether the four viruses just described displayed a recombinant genomic structure similar to any of the *Eco*RI-d-clone-derived viruses (D1 through D5), we analyzed their DNAs with the restriction enzyme *Bam*HI (Fig. 5A). All of the viruses which did not exhibit neurovir-

TABLE 2. Neurovirulence of HSV R13-1, 17 syn^+, five viruses (D1 through D5) derived from cotransfection of unit-length R13-1 DNA and the 17 syn^+ *Eco*RI d fragment clone, and one of two viruses derived from cotransfection of unit-length R13-1 DNA and the 17 syn^+ *Eco*RI g fragment clone[a]

Virus	Genotype[b]	LD_{50} (PFU)
R13-1[c]		1.4×10^3
17 syn^+		2.3
D1	R	6.0
D2	R	5.8
D3	R	7.3
D4	R	<3.4
D5	R	7.6
G5	NR	2.1×10^3

[a] Mice were inoculated intracranially with serial 10-fold dilutions (five mice per dilution) of each virus, and deaths resulting from encephalitis were scored for 21 days post infection. LD_{50}s were determined by the method of Reed and Muench (14).

[b] Genomic structure compared with that of R13-1, determined by analysis with *Bam*HI. R, Recombinant; NR, identical to R13-1.

[c] As a control, unit-length R13-1 DNA was transfected alone onto rabbit skin cells. The resulting viruses were collectively inoculated into two mice, and after these mice succumbed to encephalitis, their brains were homogenized and the resulting brain homogenates (cleared by centrifugation) were reinoculated into two more mice. The R13-1 virus used in this experiment was a stock prepared from the viruses recovered from the brain of one of the two mice which died from encephalitis.

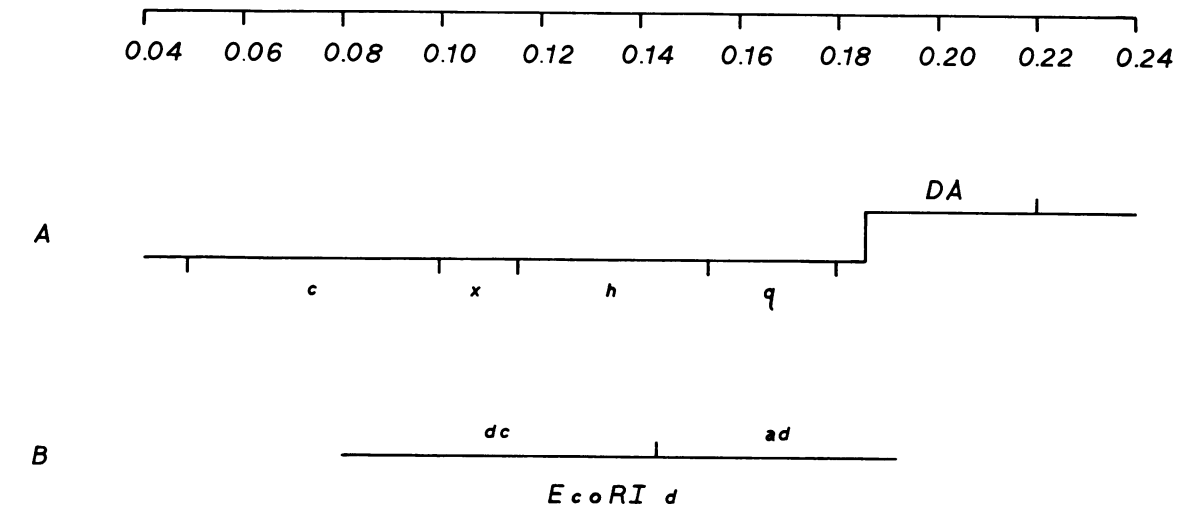

FIG. 3. (A) HSV-2 (bottom line) and HSV-1 (top line) *Bam*HI sites and DNA fragments encoded within the region from 0.040 to 0.240 m.u. of the R13-1 genome. Lowercase letters designate HSV-2 (HG_{52}) fragments, and fragment DA (0.179 to 0.221 m.u.) is a hybrid (HSV-1 and HSV-2) fragment formed by the R13-1 intertypic crossover at approximately 0.184 m.u. The high-molecular-weight fragment cxh (0.048 to 0.154 m.u.) is formed when recombination with the *Eco*RI d fragment (0.079 to 0.192 m.u.) causes the loss of the two *Bam*HI sites which define HSV-2 fragment × (0.099 to 0.115 m.u.). Alternatively, if both of these sites are lost and the HSV-1 *Bam*HI site (0.143 m.u.) of *Eco*RI fragment d is acquired, fragment cxh′ (0.048 to 0.143 m.u.) is produced. (B) The HSV-1 (17 syn^+) *Eco*RI d fragment. The single HSV-1 *Bam*HI site (0.143 m.u.) within the *Eco*RI d fragment separates the *Eco*RI/*Bam*HI dc (0.079 to 0.143 m.u.) and *Bam*HI/*Eco*RI ad (0.143 to 0.192 m.u.) fragments.

ulence characteristics resembling those of wt viruses (Table 3) displayed DNA profiles identical to that of R13-1 by this analysis (data not shown). In contrast, viruses 62-5, 62-6, and 62-10 demonstrated recombinant genotypes resembling those of viruses D2 and D3 derived from the *Eco*RI-d transfections. Southern blot analysis (Fig. 5B) of the gel shown in Fig. 5A, probed with the *Eco*RI-d clone, confirmed this conclusion and also indicated that recombinant virus 62-6 was contaminated with parental virus R13-1. By this analysis, virus 62-9 resembles R13-1.

DISCUSSION

Using the intertypic recombinant virus R13-1, we identified a HSV gene function or functions which significantly affect the ability of HSV to elicit a fatal encephalitis after intracranial inoculation of mice. Since the virus into which this gene was placed already had the ability to replicate to high titer in the mouse brain, the function is not related to high-titer replication in this organ. Also important is the fact that the defect associated with this novel phenotype mapped to a region of the HSV genome not previously known to influence neurovirulence (0.079 to 0.143 m.u.). With respect to the last point, only viruses derived from the cotransfection of unit-length R13-1 DNA with either the HSV-1 *Eco*RI d (0.079 to 0.192 m.u.) or the HSV-1 *Eco*RI/*Bam*HI dc (0.079 to 0.143 m.u.) DNA fragment exhibited both wt neurovirulence and recombinant genotypes, as determined by analysis with the restriction enzyme *Bam*HI. Although one of these viruses (virus 62-9) resembled parental virus R13-1 by this analysis, it seems likely that this virus is also a recombinant, with undetected HSV-1 inserts within the region of interest. Although we cannot rule out multiple genetic lesions being responsible for the phenotype of R13-1, one genetic defect may be involved, since a single, cloned DNA fragment returned the neurovirulence of R13-1 to levels characteristic of a wt virus.

In a general sense, the work presented in this report was a continuation of genetic and biological studies of the intertypic recombinant RS6. Our previous work with RS6 indicated that one or more genetic defects associated with its reduced neurovirulence reside within the region from 0.79 to 0.83 m.u. and that another lesion might also be present in the region from 0.11 to 0.14 m.u.; however, our attempts to increase the neurovirulence of RS6 with cloned HSV-1 DNA spanning this region were unsuccessful. A possible explanation for this result is that other genetic defects of RS6 masked the importance of this region. It has been observed that replacement of HSV-2 DNA with HSV-1 DNA in specific genomic regions is always linked to increased neu-

TABLE 3. Neurovirulence of HSV R13-1, eight viruses (62-3 through 62-10) derived from cotransfection of unit-length R13-1 DNA and the 17 syn^+ *Eco*RI/*Bam*HI dc fragment clone, and two viruses (37-6 and 37-7) derived from cotransfection of unit-length R13-1 DNA and the 17 syn^+ *Bam*HI/*Eco*RI ad fragment clone[a]

Virus	Genotype[b]	LD_{50} (PFU)
R13-1[c]		5.7×10^2
62-3	NR	$>1.1 \times 10^3$
62-4	NR	3.1×10^3
62-5	R	<4.6
62-6	R	9.1
62-7	NR	6.6×10^2
62-8	NR	$>3.6 \times 10^3$
62-9[d]	NR	8.3
62-10	R	<2.5
37-6	NR	$>3.2 \times 10^3$
37-7	NR	5.7×10^2

[a] Mice were inoculated intracranially with serial 10-fold dilutions (five mice per dilution) of each virus, and deaths resulting from encephalitis were scored for 21 days postinfection. LD_{50}s were determined by the method of Reed and Muench (14).

[b] Genomic structure compared with that of R13-1, determined by analysis with *Bam*HI. R, Recombinant; NR, identical to R13-1.

[c] See Table 2, footnote *c*, for the derivation of this virus.

[d] It is likely that the high neurovirulence of this virus resulted from recombinational events with the *Eco*RI/*Bam*HI dc fragment which were not detected by analysis with *Bam*HI.

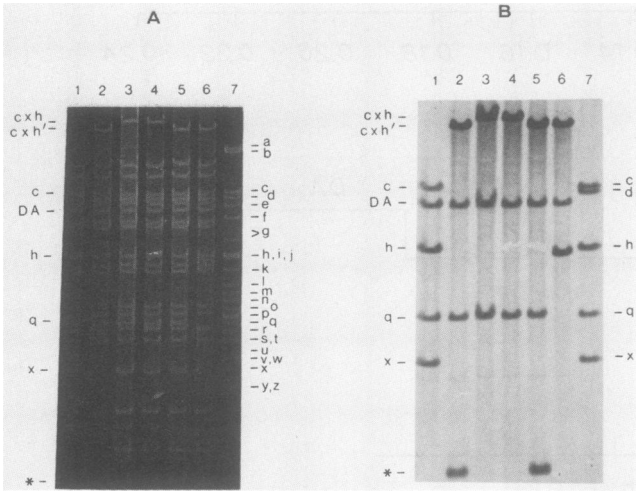

FIG. 4. Agarose gel electropherogram (A) and Southern blot analysis (probe, HSV-1 *Eco*RI-d [0.079 to 0.192 m.u.]) (B) of *Bam*HI-generated DNA fragments of R13-1 (lane 1), five viruses (D1 through D5; lanes 2 through 6, respectively) derived from the cotransfection of unit-length R13-1 DNA and the 17 syn$^+$ (HSV-1) *Eco*RI d fragment clone, and HG_{52} (lane 7). HSV-2 (HG_{52}) fragments are labeled on the right side of each panel, and R13-1 and recombinant-derived fragments are labeled on the left. Viruses D1 through D5 have lost HSV-2 (HG_{52}) *Bam*HI fragments c (0.048 to 0.099 m.u.), x (0.099 to 0.115 m.u.), and h (0.115 to 0.154 m.u.), which resulted in the high-molecular-weight fusion fragments (cxh [0.048 to 0.154 m.u.] and cxh' [0.048 to 0.143 m.u.]) at the tops of the lanes. The low-molecular-weight fragment (*[0.143 to 0.154 m.u.]) in the Southern blots of viruses D1 and D4 was generated when intertypic recombination occurred between the HSV-1 *Bam*HI site (0.143 m.u.) of *Eco*RI-d and the leftmost HSV-2 *Bam*HI site (0.154 m.u.) of fragment q (0.154 to 0.179 m.u.). Only virus D5 has lost the HSV-2 *Bam*HI q fragment as a result of intertypic recombination within this fragment, which produced a hybrid fragment (0.143 to 0.179 m.u.) with a size similar to that of HSV-2 fragment h. Fragment DA (0.179 to 0.221 m.u.) represents a hybrid band derived from HSV-2 fragment d (0.179 to 0.226 m.u.) and HSV-1 fragment a (0.143 to 0.221 m.u.) and was generated by the R13-1 intertypic crossover at approximately 0.18 m.u.

rovirulence of intertypic recombinants selected for this phenotype (6, 17, 19). Therefore, we reasoned that if we could isolate an RS6-derived recombinant virus in which all RS6 HSV-2-encoded regions were replaced with HSV-1 DNA except in the general region from 0.11 to 0.14 m.u., such a virus would display reduced neurovirulence. A virus with these characteristics is R13-1. We have assumed that because of the parentage of R13-1, the genetic lesion associated with reduced neurovirulence was inherited from RS6. Consistent with this idea is the fact that heterologous DNA replacement (HSV-2 DNA with HSV-1 DNA) in the general region from 0.11 to 0.14 m.u. was required to generate viruses with wt neurovirulence from both RS6 and R13-1. The nature of this genetic defect is not known, but several possible explanations for defects arising within intertypic recombinant viruses have been suggested (18).

The genetic stability of the neurovirulence phenotype of R13-1 was somewhat different than in our previous studies. The extremely stable, low neurovirulence of viruses RE6 and RS6 (6, 19) after transfection and multiple brain passage was not found for virus R13-1. Here, neurovirulence increased 25- to 60-fold after transfection and two passages through mouse brains. Since a single genetic defect would increase the probability of producing neurovirulent revertants, an explanation for the observed stability differences might be that R13-1 possesses only one genetic defect, whereas RE6 and RS6 carry at least two lesions associated with their reduced neurovirulence. In support of this notion, the neurovirulence of RE6 or RS6 was not returned to wt levels by a single cloned HSV DNA fragment as it was for R13-1.

The reason for the paradoxical phenotype of R13-1 (low neurovirulence and high-titer brain replication) has yet to be determined but might reflect an altered cellular tropism. For example, R13-1 may replicate efficiently in some brain cells but be restricted within others, and only the latter would be essential for survival of the animal. Precedence for such a model comes from studies with reoviruses (21) in which the avirulent serotype 1 replicated exclusively in ependymal cells lining the brain ventricles, whereas the virulent serotype 3 replicated in neurons. Other possible mechanisms include restricted spread (independent of replication) to critical regions or viral replication not resulting in the death of some cells. In any case, it seems likely that R13-1 can be studied in combination with a standard virus to define cells of the mouse brain crucial to the appearance of HSV-induced encephalitis.

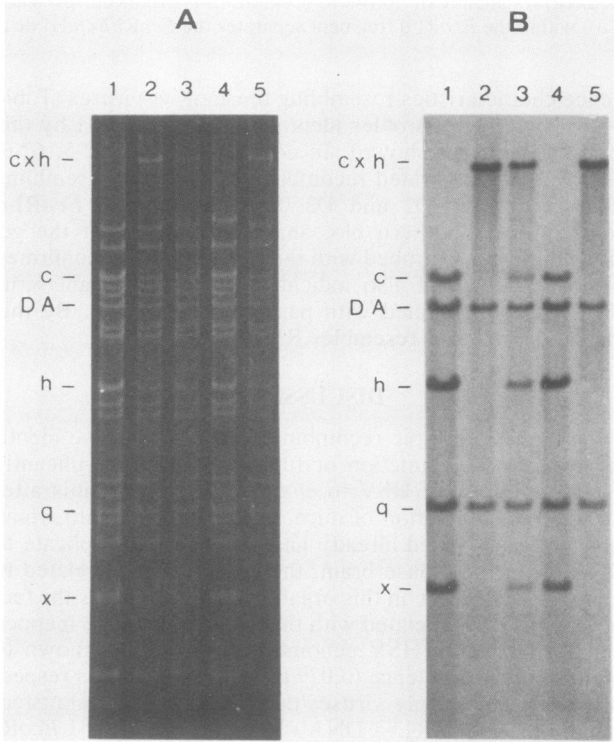

FIG. 5. Agarose gel electropherogram (A) and Southern blot analysis (probe, HSV-1 *Eco*RI-d [0.079 to 0.192 m.u.]) (B) of *Bam*HI-generated DNA fragments of R13-1 (lane 1) and four viruses (62-5, 62-6, 62-9, and 62-10; lanes 2 through 5, respectively) derived from the cotransfection of unit-length R13-1 DNA and the 17 syn$^+$ *Eco*RI/*Bam*HI dc fragment (0.079 to 0.143 m.u.) clone. R13-1 and recombinant-derived fragments are labeled on the left side of each panel. Viruses 62-5 and 62-10 are recombinants which lost HSV-2 (HG_{52}) *Bam*HI fragments c (0.048 to 0.099 m.u.), x (0.099 to 0.115 m.u.), and h (0.115 to 0.154 m.u.), and this resulted in the high-molecular-weight fusion fragment cxh (0.048 to 0.154 m.u.) at the tops of the lanes. The Southern blot analysis of virus 62-6 indicated that it is a virus mixture containing both a recombinant virus (similar to viruses 62-5 and 62-10) and parental virus R13-1. Virus 62-9 resembles virus R13-1.

Finally, known genetic functions within the region from 0.079 to 0.143 m.u. can be considered. Although little is known about this region, three complementation groups (1-10, 1-6, and 1-26) defined by temperature-sensitive mutations (2, 11, 12, 22) have been discerned, and several transcripts (20) map to this general area. The 1-10 gene is important early in the viral replication cycle and is essential for DNA synthesis (11), the 1-6 gene has recently been implicated in processing or packaging of viral DNA (15), and the 1-26 gene appears necessary late in infection (2). In addition, a structural gene, VP 18.8 (9), is also encoded here. Whether these or other as yet unknown genes are concerned with the gene function described in this study can be investigated with additional, refined marker rescue experiments.

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