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Rescue of a Herpes Simplex Virus Type 1 Neurovirulence Function with a Cloned DNA Fragment

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A herpes simplex virus type 1 (HSV-1) genetic function that is required for viral replication in the murine central nervous system was unambiguously localized. Thus, cosmid clones of either HSV-1 *Hin*dIII fragment C (0.64 to 0.87 map units) or fragment B (0.64 to 0.83 plus 0.91 to 1.0 map units) were employed to restore neurovirulence to an intertypic recombinant (RE6) that is specifically deficient in this property. The neurovirulent recombinants were generated in cell culture by cotransfecting the clone fragments and unit-length RE6 DNA and then selected in mouse brains. Either fragment efficiently conferred neurovirulence to RE6, demonstrating that no short region unique sequences are required. Analyses of the genomic structures of the neurovirulent recombinants showed that, in every case, HSV-1 information from 0.71 to 0.83 map units was incorporated into the RE6 genome. Cleavage of *Hin*dIII fragment C with *Eco*RI eliminated its capacity to rescue RE6. Virulence could be restored by the addition of HSV-1 *Bam*HI fragment L (0.71 to 0.74 map units) that spans an *Eco*RI site at 0.72 map units. The precise location of this HSV-1 neurovirulence function is discussed.

The identification of viral genes that control pathogenic properties at the level of the animal and the elucidation of their function are of fundamental importance to an understanding of the basic molecular mechanisms of disease. With respect to the natural history of herpes simplex virus (HSV), the nervous system is a critical target organ, and the general pathologic features of this viral-host interaction have long been appreciated (4). Thus, after acute infection at the surface of the body, the virus travels through axons and can establish latent infections in both the peripheral (11) and central (7) nervous systems. Viral reactivation produces secondary movement in axons, and clinically apparent disease that ranges in severity from relatively benign cutaneous lesions to acute fatal encephalitis may ensue.

It is of importance to determine the genetic properties of this virus that predispose it to invade and destroy nervous tissue. However, the viral genes that control either neuroinvasiveness or neurovirulence are little understood. It is known that the viral thymidine kinase gene is required for maximum demonstration of neurovirulence (3). Increased neurovirulence has been demonstrated in one avirulent natural isolate by serial passage of virus in the murine central nervous system (6). In this case, DNA from the virulent agent possessed an altered restriction enzyme profile, but whether this change is related to the increase in neurovirulence has not been established. Among wild-type and laboratory strains, it has been demonstrated that isolates of HSV, and even subclones of the same isolate, differ in degree of neurovirulence (2). However, in no instances have consistent changes in either viral proteins or DNA been detected.

Our previous studies described an HSV type 1 (HSV-1)–HSV-2 intertypic recombinant that is completely and specifically nonneurovirulent after intracranial inoculation of mice (12). This recombinant (RE6) is at least 10-million-fold less neurovirulent than either parental strain but replicates as well or better than the parents in all tested cells and tissues, with exception of the mouse brain.

To understand the basis of this property, we developed a technique that allows identification and localization of HSV genes which control pathogenic phenotypes displayed in vivo (13). This strategy involves cotransfecting tissue cultures with unit-length DNA from an HSV strain that lacks a biologically selectable marker with restriction endonuclease-fragmented DNA of a strain that displays the desired phenotype. Phenotypically positive recombinants are then selected in vivo in the tissue of interest. This procedure has the advantage of eliminating the background of avirulent parental virus as well as selecting against any recombinants that replicate poorly at the temperature of the mouse. Utilizing this approach, our previous results are here confirmed and extended by demonstrating the rescue of RE6 neurovirulence with cloned HSV-1 DNA fragments.

Previous results demonstrated that an electrophoretically purified HSV-1 DNA fragment (*Hin*dIII fragment B, 39 kilobases, 0.64 to 0.83 plus 0.91 to 1.0 map units [m.u.]) contains the type 1 gene or genes which restore neurovirulence to RE6. To unequivocally confirm the role of HSV-1 *Hin*dIII fragment B in HSV neurovirulence, it was necessary to utilize a cloned DNA fragment for rescue. Since the fragment size is large, the cosmid cloning vector PHC-79 was used. The cloning procedure employed was essentially as described (5) except for certain modifications designed to select cosmids that had incorporated the large *Hin*dIII fragments of strain 17 syn$^+$. When 12 cosmids were analyzed, two contained HSV-1 *Hin*dIII fragment B (39 kilobases, 0.64 to 0.83 plus 0.91 to 1.0 m.u.), two *Hin*dIII fragment A (41.5 kilobases, 0.25 to 0.52 m.u.), and eight *Hin*dIII fragment C (35 kilobases, 0.64 to 0.87 m.u.). Despite a *recA*$^-$ strain of *Escherichia coli* being used as the recipient strain, four of the cosmids contained detectable permutations when analyzed with six restriction endonucleases.

Two of HSV-1 *Hin*dIII fragment C cosmids (PHC 17-3 and PHC 17-11) and one of the HSV-1 *Hin*dIII fragment B cosmids (PHC 17-1) were then tested for their ability to

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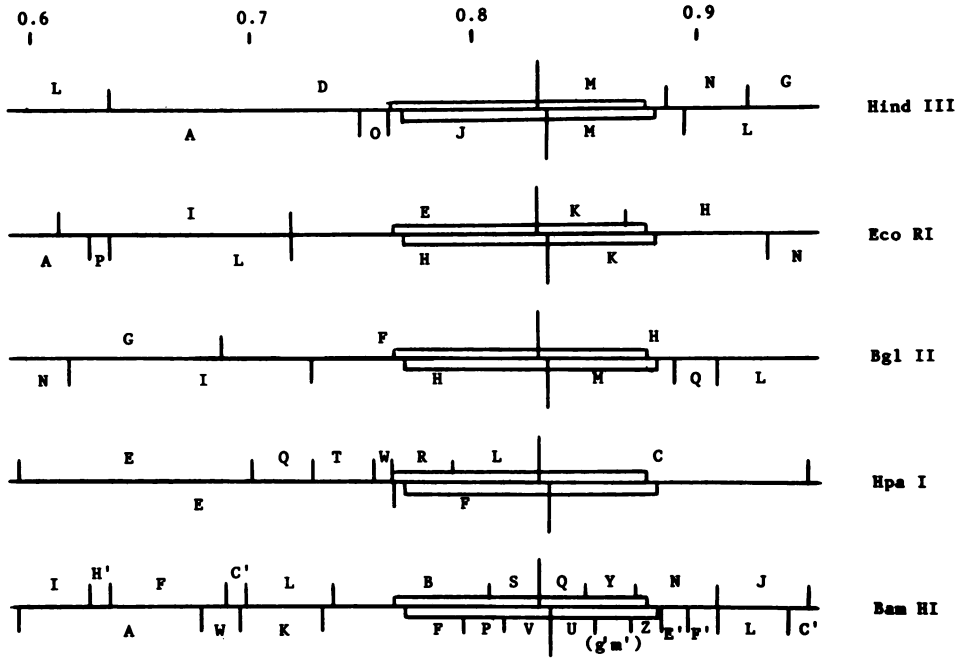

FIG. 1. Reference map of restriction endonuclease sites for HSV-1 (strain 17 syn$^+$) and HSV-2 (strain HG52) in the region 0.6 to 0.95 m.u. The genome of HSV is shown schematically as a single horizontal line, and the repetitive sequences flanking the long and short regions are depicted as open boxes. The joint between the long and short regions is indicated by an extended vertical line at about 0.83 m.u. The restriction enzyme sites for *Hin*dIII, *Eco*RI, and *Bgl*II, *Hpa*I, and *Bam*HI are shown as short vertical lines, with HSV-1 sites along the top and the HSV-2 sites along the bottom. Fragment names (capital letters) are as described in the text.

confer neurovirulence. Initially, cosmid PHC 17-11 (17 syn$^+$ *Hin*dIII fragment C) was employed as the donor DNA, and under the cotransfection and in vivo selection conditions described previously (13), this clone conferred neurovirulence upon RE6 with high efficiency. In three separate neurovirulence rescue experiments (of 10 transfection plates each), between 70 and 100% of the cultures contained virus that killed mice. Virus was obtained from the mouse brains at necropsy, plaque purified, and employed to generate PFU per 50% lethal dose (PFU/LD_{50}) ratios.

Neurovirulence of the various plaque isolates was analyzed by the generation of PFU/LD_{50} ratios after direct intracranial inoculation as described previously (12). During the course of these studies, the neurovirulent strain of HSV-1 (17 syn$^+$) consistently produced ratios of between 5 and 15. In contrast, mice could not be killed with the avirulent recombinant RE6, even at inoculation titers of 4.1×10^7, the highest stock titer produced. Most of the neurovirulent recombinants generated with cloned *Hin*dIII fragment C yielded ratios of approximately 10^3, an example of which is recombinant RC 14.1, which gave a ratio of 1.1×10^3 in this assay. However, some tested recombinants, such as RC 18.1 (1.4×10^4) and RC 19.2 (2.2×10^5), gave higher ratios.

The quantitative differences between PFU/LD_{50} ratios established by these recombinants and the wild-type virus can be explained in two obvious but not exclusive ways. First, the rescue of RE6 may be a multigenic event and various amounts of the *Hin*dIII C fragment could have been incorporated between 0.72 and 0.83 m.u. Second, the *Hin*dIII C fragment employed could contain mutations that would affect either neurovirulence or general viral replication. Other possibilities and the basis for variation between recombinants derived from the same clone will be discussed below.

To determine whether differences in the amount of HSV-1 *Hin*dIII fragment C incorporated into the NV-1A$^+$ recombinants could account for the variation in observed lethality, the genomic structure of three of the plaque-purified neurovirulent recombinants was analyzed with six restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Xba*I, *Hpa*I, and *Bgl*II). These restriction enzymes were chosen to conveniently differentiate between HSV-1 and HSV-2 DNA in the region of 0.7 to 0.83 m.u. in which the original genomic exchange between type 1 and type 2 occurred in RE6. A reference map for these enzymes in this region is shown in Fig. 1. RC 14.1, RC 18.1, and RC 19.2 were chosen for analysis as they reflected the full range of recovered neurovirulent phenotypes.

The *Hpa*I digests are the easiest to interpret, and an example of an 0.8% agarose gel of such a digest is shown in Fig. 2. It is apparent from these data that all of the neurovirulent recombinants selected in vivo incorporated HSV-1 DNA between 0.71 and 0.83 m.u. *Hpa*I fragments corresponding in size to *Hpa*I fragments Q (0.713 to 0.737 m.u.), T (0.737 to 0.759 m.u.), W (0.759 to 0.764 m.u.), R (0.764 to 0.788 m.u.), and L (0.788 to 0.827 m.u.) are apparent. Some heterogeneity can be seen in the *Hpa*I fragment R and L bands, but this region is known to vary even among plaque isolates of the same strain (8). These data were entirely consistent with those obtained from analysis with the other five restriction endonucleases employed and indicate that, in every case, the entire region from 0.71 to 0.83 m.u. was replaced by type 1 information.

These findings were confirmed by Southern blot analysis (10) in which nick-translated cloned HSV-1 probes labeled with [^{32}P]dCTP or biotin-conjugated dUTP were used. Representatives of such blots are shown in Fig. 3. In Fig. 3A, 17 syn$^+$, RC 14.1, RC 18.1, RC 19.2, and RE6 were cleaved with *Hpa*I, blotted, and hybridized with a ^{32}P-labeled clone

of 17 syn$^+$ *Bam*HI fragment L (0.71 to 0.745 m.u.). The radioactive bands in the figure confirm that all three NV-1A$^+$ recombinants incorporated type 1 DNA corresponding to *Hpa*I fragments Q (0.713 to 0.737 m.u.) and T (0.737 to 0.759 m.u.). The same DNAs were cleaved with *Bam*HI and again hybridized to the 17 syn$^+$ *Bam*HI fragment L probe (Fig. 3B). Here, it is apparent that all three NV-1A$^+$ recombinants have lost the fusion fragment RE6 *Bam*HI fragment LIK2 and regained a normal sized 17 syn$^+$ *Bam*HI fragment L. Thus, in every case, the original site of the crossover at ~0.72 m.u. in RE6 has been replaced. Within the limits of resolution of these procedures, the HSV-1 neurovirulence function that rescues RE6 is between 0.71 and 0.83 m.u.

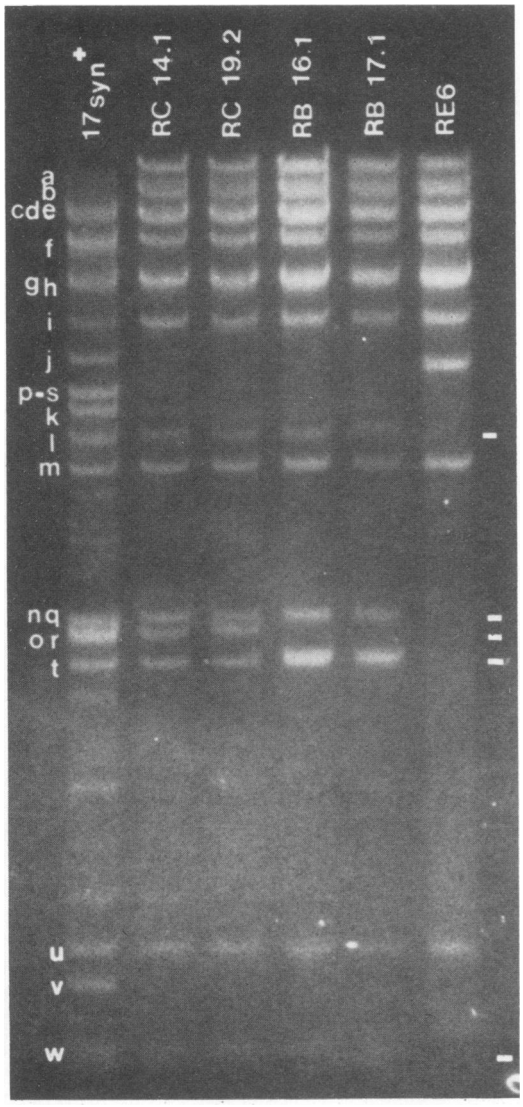

FIG. 2. Electropherogram of HSV *Hpa*I DNA fragments. DNA from HSV strains 17 syn$^+$, RE6, and neurovirulent recombinants was digested with *Hpa*I and electrophoresed in a 0.8% agarose gel as described in the text. To the right of the lane depicting RE6 DNA, white bars mark HSV-1 bands that are present in all of the neurovirulent recombinants but not in the parental RE6. These bands correspond to HSV-1 *Hpa*I fragments L (0.788 to 0.827 m.u.), Q (0.713 to 0.737 m.u.), T (0.737 to 0.759 m.u.), W (0.759 to 0.764 m.u.), and R (0.764 to 0.788 m.u.). Note that the RE6 fragment that comigrates with 17 syn$^+$ *Hpa*I fragment J is actually the fusion fragment RE6 *Hpa*I Q1E2.

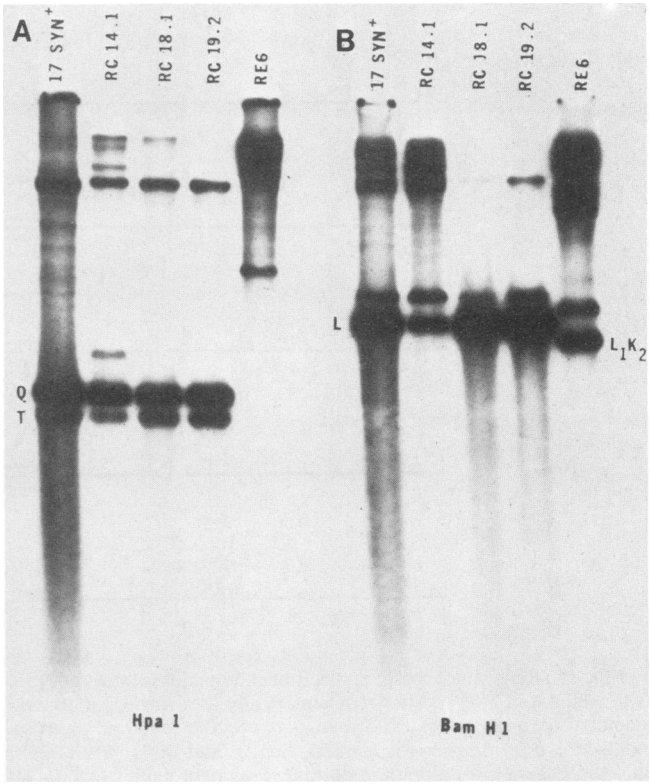

FIG. 3. Southern blot analysis of HSV strains 17 syn$^+$, RE6, and neurovirulent recombinants. (A) DNAs were digested with *Hpa*I, electrophoresed, and blotted to nitrocellulose filters as described previously (13). The filters were hybridized to a nick-translated clone probe of HSV-1 *Bam*HI fragment L (0.71 to 0.74 m.u.). Note that all the neurovirulent recombinants have acquired *Hpa*I fragments Q (0.713 to 0.737 m.u.) and T (0.737 to 0.759 m.u.). (B) The DNAs were cleaved with *Bam*HI and again probed with HSV-1 *Bam*HI fragment L. Note that all the recombinants have lost the fusion fragment LIK2 present in RE6 and regained a normal 17 syn$^+$ *Bam*HI L fragment.

As mentioned previously, an alternative explanation for the variation in virulence observed in the recombinants might be a hidden mutation or permutation in PHC 17-11. To test this possibility, two additional cosmid clones were employed in rescue experiments. The first one tested was an additional cosmid containing *Hin*dIII fragment C (PHC 17-3), which contained no detectable permutations after cleavage with six restriction endonucleases. In a total of four such experiments of 20 plates each, no successful rescue of neurovirulence was detected. Two of the experiments were controlled with transfections employing PHC 17-11 (see above). In these cultures, 7 of 10 plates and 9 of 10 plates killed mice. Therefore, despite PHC 17-3 containing no detectable permutations, it must contain a defect that prevents the restoration of neurovirulence to RE6. One should note that such a defect need not reside in a gene controlling HSV neurovirulence per se; a mutation in any essential gene in this region could result in the failure of recombinants to replicate.

PHC 17-1, a clone of the 17 syn$^+$ *Hin*dIII fragment B, was tested next. In two transfection experiments, 9 of 10 and 10 of 10 of the tested cultures contained neurovirulent recombinants. Two unrelated isolates were plaque purified and analyzed for genomic structure and neurovirulence. Both RB 16.1 and RB 17.1 have incorporated HSV-1 sequences

TABLE 1. Rescue of RE6 neurovirulence with subfragments of HSV-1 *Hin*dIII fragment C

Expt	PHC 17-11 (*Hin*dIII fragment C) cleaved by[a]:			
	*Hin*dIII	*Hin*dIII plus *Eco*RI	*Bam*HI-L alone	PHC 17-11 *Hin*dIII plus *Eco*RI plus HSV-1 *Bam*HI-L
1	10/10	0/10	ND	ND
2	9/10	0/20	0/40	ND
3	10/10	0/20	0/20	3/10
4	10/10	0/20	ND	3/10

[a] Rabbit skin cells were cotransfected with RE6 DNA and HSV-1 *Hin*dIII fragment C cleaved from the vector with *Hin*dIII, with both *Hin*dIII and *Eco*RI, with HSV-1 *Bam*HI fragment L alone, or with *Hin*dIII-*Eco*RI-digested *Hin*dIII fragment C plus HSV-1 *Bam*HI fragment L. Mice were inoculated with the progeny of transfection cultures, and when they died from encephalitis, virus was isolated from the brain tissue and reinoculated at 10^5 PFU per mouse. When these mice also died, the transfection culture was scored as positive for neurovirulent recombinants. Values are the number of transfection cultures positive over number tested. ND, Not done.

between 0.71 and 0.83 m.u. (see Fig. 2). One should note that, in both cases, *Hpa*I fragment R (0.71 to 0.735 m.u.) is smaller than normal, a change that is not evident in the cosmid DNA (data not shown). The PFU/LD_{50} ratios of RB 16.1 and RB 17.1 were 1.1×10^4 and 1.4×10^4, respectively.

In an effort to map more closely the defective neurovirulence function of RE6, HSV-1 *Hin*dIII fragment C was cleaved with both *Hin*dIII and *Eco*RI and used in rescue experiments. *Eco*RI cleaves the *Hin*dIII C fragment in two places: at 0.72 and 0.85 m.u. on the viral genome. It should be noted that all portions of the fragment (*Hin*dIII-*Eco*RI D through E, 0.64 to 0.72 m.u.; *Eco*RI E plus K, 0.72 to 0.85 m.u.; and *Eco*RI-*Hin*dIII K through M, 0.85-0.87 m.u.) were present in the transfection cocktail. This cleavage completely eliminated the ability to restore neurovirulence to RE6. Of a total of 60 transfection plates tested, none contained neurovirulent recombinants. The simplest explanation of these data is that an HSV-1 neurovirulence function resides at, or close to, one of these *Eco*RI sites.

The *Eco*RI site at 0.72 m.u. resides close to the original point of crossover between HSV-1 and HSV-2 DNA sequences in RE6 (13). To determine whether a permutation introduced at this site in RE6 is involved in its avirulent properties, HSV-1 *Bam*HI fragment L (0.71 to 0.745 m.u.) that spans the *Eco*RI site at 0.72 m.u. was cloned. Since it was shown above that some clones may contain permutations that preclude rescue, five independent clones of *Bam*HI fragment L were derived and pooled for use in these rescue experiments.

As previously, the viral progeny of the transfection cultures were injected intracranially into mice, and when the mice died, virus was recovered from brain tissue and reinjected at 10^5 PFU per mouse. When these mice also died, the culture was scored as positive for containing neurovirulent recombinants.

As before, HSV-1 *Hin*dIII fragment C was highly efficient in restoring neurovirulence to RE6, and greater than 90% of these cultures contained neurovirulent recombinants (Table 1). When cleaved with *Eco*RI, this fragment was not able to rescue RE6 (no neurovirulent recombinants in 60 plates). However, when HSV-1 *Bam*HI fragment L was added to the *Eco*RI-cleaved *Hin*dIII C fragment, 30% of the cultures contained neurovirulent virus. Since *Bam*HI fragment L was capable of restoring the ability of the *Eco*RI-cleaved *Hin*dIII fragment to rescue RE6, intact HSV-1 sequences near the *Eco*RI site at 0.72 m.u. must have been required. Whether the *Bam*HI L fragment was incorporated into these neurovirulent recombinants could not be determined since recombination near the *Eco*RI site of the cleaved cosmid would generate a fragment of nearly the same size as intact *Bam*HI fragment L, and this fragment would still contain HSV-2 information.

When the *Bam*HI L fragment was employed alone in cotransfections, no neurovirulent recombinants were recovered. It should be noted that signs of central nervous system disease were detected in some of the mice inoculated with these cultures, and ~10% of these cultures were lethal (data not shown). However, neurovirulent virus could not be obtained from the brains of these mice at necropsy.

It was previously shown that the intertypic recombinant RE6 is specifically blocked from replication in mouse brains (12). Here, through the use of in vivo recombinant selection, neurovirulence was restored to RE6 with cosmid cloned HSV-1 DNA sequences comprising *Hin*dIII fragments B (0.64 to 0.83 plus 0.91 to 1.0 m.u.) and C (0.64 to 0.87 m.u.). Since both fragments rescue RE6, it is demonstrated that no unique short region sequences are required. These results unequivocally demonstrate that one or more HSV-1 gene function(s) residing in *Hin*dIII fragment C is associated with a greater than 100,000-fold increase in neurovirulence. Little is known about the in vivo functions of HSV genes encoded in this region. Genes that control syncytial formation in tissue culture cells have been mapped to this area (9). By utilizing this marker, it was found that intratypic recombinants of various syncytial morphology produced characteristic corneal lesions in rabbit eyes; however, in this instance, the neurovirulent nature of the syncytial parent was not transferred to the recombinants (1).

The PFU/LD_{50} ratios of neurovirulent recombinants generated with these fragments were higher than those observed with wild types and varied even when the same clone was employed in transfection experiments. Two possible reasons for this result were studied, and there are other possibilities. First, isolates of the same strain of HSV have been shown to vary over several orders of magnitude in neurovirulence, and the observed variation may reflect the natural variation in the RE6 population once the defect is repaired. Second, although HSV-1 information from 0.71 to 0.83 m.u. was incorporated into the RE6 genome in all cases, it is possible that a variable and undetectable amount of HSV-1 sequences was incorporated into the preexisting type 1 portions of the RE6 genome. In addition, multiple crossover events, resulting in small inserts of HSV-2 sequences, cannot be completely ruled out. Thus, more than one gene may be needed to quantitatively restore neurovirulence. Third, although the replication kinetics of these agents were not systematically investigated, at least one of the relatively avirulent recombinants (RC 19.2) replicates poorly in culture and thus would fit into an overall category of defectiveness for recombinant viruses. Several possible reasons for such agents were previously discussed (13), and they could result from either permutations introduced at the time of transfection or recombination or be due to preexisting mutations in the employed cloned fragments. In this latter case, such mutations must exist since one *Hin*dIII fragment C clone employed would not rescue RE6 despite its presenting a normal restriction enzyme fragment profile upon cleavage with six endonucleases.

The data presented here also suggest that the studied neurovirulence defect resides near the *Eco*RI site at 0.72

m.u. since cleavage of *Hin*dIII fragment C with *Eco*RI eliminated the ability of the fragment to restore neurovirulence to RE6. This can be restored by the addition of 17 syn$^+$ *Bam*HI fragment L (0.71 to 0.745 m.u.). Since this *Eco*RI site lies very close to the original point of crossover between type 1 and type 2 information in RE6, a permutation introduced into RE6 at this point during the act of recombination is a likely cause of its nonneurovirulence.

The neurovirulence of RE6 could not be rescued with *Bam*HI fragment L alone, and it may be that some other region of *Hin*dIII fragment C is also involved. However, in HSV-1, the transcription map of the *Bam*HI L fragment is rather complex with several overlapping transcripts on opposite strands. The same is true of the transcript map of HSV-2 (R. L. Thompson and E. K. Wagner, work in progress), and in this instance, incorporation of the *Bam*HI L fragment would disrupt at least two HSV-2 mRNAs. This may not be compatible with successful replication of the virus.

It should be emphasized that HSV neurovirulence and neurotropism are almost certainly multigenic functions. The defect studied here is one that specifically precludes replication in mouse brains. Localization of this defect to a small region of the RE6 genome will make it possible to analyze the transcripts encoded by RE6 in this region and to compare them with those encoded by HSV-1 and HSV-2. Further experimentation should then lead to an understanding of the peptide product and in vivo function of this gene.

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