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Functional and Molecular Analyses of the Avirulent Wild-Type Herpes Simplex Virus Type 1 Strain KOS

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It has been documented that KOS, a laboratory strain of herpes simplex virus type 1, is several orders of magnitude less neurovirulent than most other wild-type strains. Studies initiated to determine the functional nature of the block to neuroinvasiveness and to establish the genes involved have determined that, after footpad inoculation of mice, strain 17 syn$^+$ induced neuropathologic signs (paralysis) at titers of 10^2 and yielded a PFU/50% lethal dose ratio of 10^4. In contrast, KOS was not lethal and did not induce paralysis at inoculation doses of 10^8 PFU. This reduced neurovirulence of KOS could not be explained by the lack of thymidine kinase activity, its inability to replicate in mouse cells maintained in culture at 38.5°C, or its inefficient replication in nonneural tissues in vivo. Kinetic experiments tracing the virus through the nervous system after footpad inoculation showed that KOS was blocked at the level of the spinal ganglia. A cosmid library of strain 17 syn$^+$ was utilized in recombination and in vivo selection experiments with strain KOS to establish the genomic region involved in 17 syn$^+$ neuroinvasiveness. A cosmid clone containing the *Hin*dIII A fragment (0.25 to 0.53 map units) of strain 17 syn$^+$ in mixed transfections with full-length KOS DNA yielded recombinants with enhanced neuroinvasiveness.

After infection at the surface of the body, herpes simplex virus (HSV) travels through axons to ganglion cells in the host nervous system, where one of two events must ensue. Either the virus continues to replicate acutely in these cells or, as is more generally the case, a latent infection is established. Significant acute replication in the peripheral nervous system after a primary or reactivated infection can result in infection of the central nervous system (CNS), producing disease ranging in severity from transient transverse myelitis to fatal encephalitis (4). The ability of this virus to enter and replicate in the human nervous system is of obvious importance to an understanding of these aspects of herpetic disease.

The molecular events that control the neurotropism, neuroinvasiveness, and neurovirulence of HSV are largely unknown. The viral thymidine kinase gene (*tk*) has been shown to be essential for the maximum display of neurovirulence, but is is not required for virus replication in other somatic tissues (5). Thompson and Stevens (13) have identified at least one other HSV type 1 (HSV-1) function specifically associated with neurovirulence, which was localized to 0.7 to 0.83 map units (m.u.) on the HSV-1 genome (12, 14). Recently, it was demonstrated that the naturally avirulent HSV-1 strain Ang increased in virulence after serial passage in mouse brains in vivo (6). The restriction endonuclease pattern of the selected virulent Ang isolate differed from that of the avirulent parent, but it was not clear whether these changes were related to the virulence phenotypes observed. Finally, HSV strains and even subisolates of the same strain have been shown to vary in the capacity to induce disease in animal models (3). It has been shown that both avirulent and virulent isolates of the HSV-1 strain KOS exist (3). The basis for this difference was not determined, but it was demonstrated that the general replication kinetics of the isolates did not vary under single-step conditions in tissue culture cells.

In this report, we investigate a difference between two HSV-1 wild-type strains in the capacity to induce paralysis and death in mice after footpad inoculation. The virulent strain is 17 syn$^+$, an isolate which causes death by encephalitis after inoculation of about 10^4 PFU. In contrast, the avirulent strain KOS does not induce detectable disease after inoculation of as much as 10^8 PFU. To differentiate the peripherally avirulent isolate of KOS employed in this study from other existing isolates that vary in this property, it will hereafter be referred to as KOS(M). Strain KOS(M) is shown to be restricted in the peripheral nervous system and specifically at the level of the spinal ganglia. This strain replicates as well as the fully neurovirulent strain 17 syn$^+$ in mouse cells in culture and in peripheral tissues in vivo.

Experiments designed to identify the genes responsible for the difference in pathogenicity observed were initiated. As reported, it is possible to use cosmid clones of virulent HSV strains to localize HSV genetic functions that are responsible for pathogenic phenotypes displayed in animal models in vivo (12). In this report, a genomic library of the 17 syn$^+$ strain was employed in intratypic recombination experiments to donate at least one aspect of peripheral virulence to the KOS(M) strain. It was found that a clone of the *Hin*dIII A fragment (0.25 to 0.53 m.u.) of strain 17 syn$^+$ can confer enhanced peripheral neurovirulent character to strain KOS(M).

MATERIALS AND METHODS

Cells and virus culture. The techniques of tissue culture, virus isolation, propagation, and titration have been described previously (13). Unless otherwise indicated, the incubation temperature was 37.5°C. HSV-1 strain KOS(M) was obtained from M. Levine (Ann Arbor, Mich.) and plaque purified in our laboratory. The history of strain 17 syn$^+$ has been previously described (7). Outbred Swiss Webster mice were used in all experiments. Except where noted in the text, the animals were inoculated at 4 weeks of age. Inoculation

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procedures and quantiation of neurovirulence were as previously described (2, 9, 13).

Cosmid cloning of large HSV-1 fragments. The strategy used to specifically clone large (>30 kilobase) fragments of 17 syn$^+$ has been described previously (12). Some of the cosmids used in this study were generated by partial digestion of 17 syn$^+$ DNA with *Mbo*I, followed by size selection on sucrose gradients. These fragments were then cloned into the *Bam*HI site of the cosmid PHC-79. In these cases, it was not possible to specifically cleave the HSV-1 sequences from the vector, and therefore these cosmids were linearized with *Xba*I or *Cla*I prior to use in cotransfections. Such cosmids were named M17 followed by the colony number (e.g., M17-21). These cosmids were nick translated with [^{32}P]dCTP and Southern blotted against a series of HSV-1 subclones to map their location on the viral genome. These techniques have been described previously (13). Cosmids PHC-17-9 and PHC-17-11 are the 17 syn$^+$ *Hin*dIII A and C fragments, respectively, and the derivation and characterization of these clones has been described previously (12).

Restriction enzyme analysis. Restriction endonuclease digestions were performed as directed by the enzyme manufacturer (Bethesda Research Laboratories, Inc.). All HSV restriction fragments are referred to by their letter designation and map coordinates on the P arrangement of the HSV genome.

Generation of recombinant viruses. The cotransfection procedure employed to generate recombinants between HSV-1 strains KOS(M) and 17 syn$^+$ was essentially as described previously (12, 14), except that only 17 syn$^+$ sequences cloned into cosmid vectors were used as the donor DNA. Cosmid DNA (3 to 4 μg per plate) cleaved with the appropriate restriction enzyme and intact KOS(M) DNA (0.2 μg per plate) were used. In most experiments, carrier DNA was eliminated from the transfection cocktail.

Selection of neurovirulent recombinants. To avoid the problem of clonal relatedness of selected recombinants, only one neurovirulent isolate was derived from any one plate. After transfection, the viruses (200 to 300 PFU per plate) were allowed to amplify until the monolayers were destroyed. The cultures were then harvested, and the resulting stock was inoculated onto both rear footpads of 6-week-old outbred Swiss Webster mice (four per culture) which had been pretreated with 10% saline 6 h previously (8). Neurovirulent virus was isolated from the brain tissue of mice that died or displayed severe signs of CNS distress. The viruses were plaque purified, and the stocks were prepared and used as a means to determine pathogenicity and as a source of DNA for molecular analysis.

RESULTS

General properties of strain KOS(M). The following experiments were performed to investigate those properties of KOS(M) which pertain to its nonpathogenic character. Since the agent was plaque purified, it was necessary to determine that the avirulent phenotype reported was still maintained and that no defect in general replicative capacity had been inadvertently selected.

To quantitate the virulence of the agent, mice were inoculated via both rear footpads with serial 10-fold dilutions of either strain 17 syn$^+$ or KOS(M) (five mice per dilution). Under these conditions, KOS(M) did not cause any detectable signs of neurologic infection even at the highest titers inoculated (10^9 PFU/ml). This was true even when the mice were pretreated with 10% saline, a protocol that increases the peripheral neurovirulence of HSV about 100-fold (8). In contrast, strain 17 syn$^+$ produced a 50% lethal dose of $\sim 10^4$ PFU and paralyzed mice at lower dosages. With saline pretreatment, this strain killed some mice at 10 PFU.

Many avirulent isolates of HSV replicate poorly in any cell at the body temperature of mice (13). To determine whether this was the case for KOS(M), its ability to form plaques in tissue culture cells was first tested. A series of plaque assays was performed at 31 and 38.5°C in both rabbit skin cells and primary mouse embryo fibroblasts. In all cases, the 31°C/38.5°C ratios did not vary significantly from those of 17 syn$^+$ (data not shown) and revealed no detectable temperature sensitivity in the KOS(M) strain.

The ability of the KOS(M) strain to replicate in primary mouse cells maintained in tissue culture at 38.5°C was tested next. To approximate more closely the condition of natural infection, tissue cultures were infected at a very low multiplicity of infection (0.0001 PFU per cell) and analyzed for virus production over a period of days. KOS(M) replicated with kinetics identical to those of 17 syn$^+$ and reached equivalent titers (Fig. 1). Clearly, KOS(M) was not restricted in cultured mouse cells at 38.5°C.

Expression of the virus-encoded *tk* has been shown to be required for peripheral neurovirulence. The ara-T test (11) was used to determine expression of this gene in KOS(M). Tissue cultures were infected with 0.1 PFU of the virus strain per cell in the presence (50 μg/ml) or absence of ara-T. At 24 h, the titers of the stocks produced were analyzed by plaque assay. A thymidine kinase-negative mutant of 17 syn$^+$, TK-7, produced equivalent titers in both cultures (Table 1). In contrast, the titers of both 17 syn$^+$ and KOS(M) were reduced by about 5 orders of magnitude in the presence of ara-T. Thus, the isolate KOS(M) is at least as efficient at the production of thymidine kinase as is strain 17 syn$^+$.

Stability of the avirulent phenotype. The genetic stability of the avirulent phenotype of KOS(M) was determined after serial passage in various tissues. Repeated passage of the virus at 38.5°C in tissue culture cells did not increase its neuroinvasive capacity (data not shown). In addition, five serial passages of this agent in mouse brains in vivo did not reduce the 50% lethal dose after footpad inoculation. As will be seen below, attempts to isolate KOS(M) from brain tissue of mice inoculated via the footpad were generally unsuccessful. In those cases in which virus was recovered, the avirulent phenotype was displayed.

It has been demonstrated that the process of transfection is mutagenic (1). Since this procedure was used in these studies as reported below, the effect of transfection itself on the character of KOS(M) was examined. Viral stocks were obtained by transfecting tissue culture cells with KOS(M) DNA alone. As will be seen, stocks were often more neuropathogenic than the parental stock. In addition, in 7 of 7 attempts over the course of the experiments to be described, virus could be reisolated from the brain of animals with neuropathologic signs. Of the 7 plaque isolates obtained, 2 (designated K2 and K4) had 50% paralytic doses of 2×10^6 and 6×10^6 PFU, respectively; however, no animals died. Thus, these isolates were 20- to 50-fold more neuroinvasive than those of the nontransfected stock virus. As will be seen below, recombinant viruses were much more invasive than these isolates.

In vivo pathogenicity of KOS(M). Since no defect in the general replicative properties of KOS(M) could be detected, the replication kinetics of this agent were next compared with those of 17 syn$^+$ in various tissues after footpad inoculation. After inoculation of 10^6 PFU, both viruses

replicated to about 5×10^6 PFU/g in mouse feet by day 3 (Fig. 2A). A pronounced shoulder in the 17 syn$^+$ curve was seen between days 4 and 7. This probably represented retrograde passage of virus from productively infected cells in the sacral ganglia. Note that this shoulder was not present in the KOS(M) curve.

Following the natural route of infection toward the brain, virus was next recovered in the sciatic nerve (Fig. 2B). Here, a difference of several orders of magnitude was seen between the two strains. The 17 syn$^+$ reached titers of 3×10^5 PFU/g, whereas KOS(M) attained a titer of only 1.5×10^3 PFU/g in this tissue. Previous studies have suggested that the majority of the virus present in the sciatic nerve is the result of virions descending from infected ganglion cells (2). In agreement with this suggestion, a severe restriction of KOS(M) replication in sacral ganglia was found (Fig. 2C).

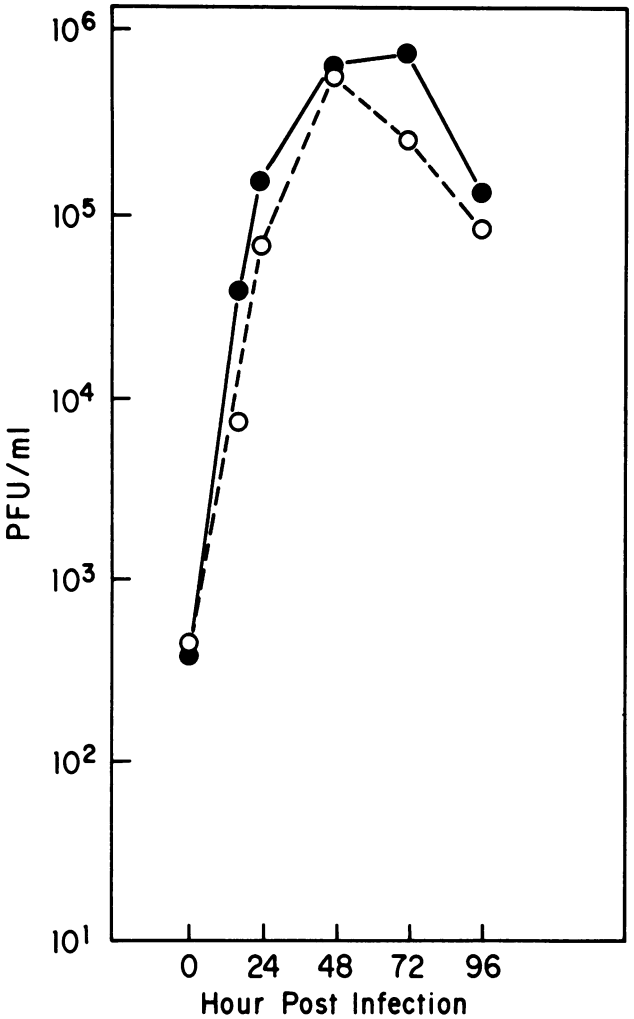

FIG. 1. Replication kinetics in mouse embryo cells. Mouse embryo cells were prepared as described previously (1) and plated into multiwell tissue culture dishes. Confluent cells were infected with either the KOS(M) or the 17 syn$^+$ strain of HSV-1 at a multiplicity of infection of 0.0001 PFU per cell and incubated at 38.5°C. At the indicated times, three wells were harvested, pooled, and frozen and thawed rapidly three times. The resulting stocks were quantitated on rabbit skin cells by plaque assay. ○, KOS(M); ●, 17 syn$^+$.

TABLE 1. Comparative thymidine kinase activity of HSV-1 strains[a]

Virus	Expt no.	Titer of virus (PFU/10^5 cells) treated:	
		With ara-T (50 μg/ml)	Without ara-T
TK-7	1	1.35×10^7	1.33×10^6
	2	1.40×10^6	3.0×10^6
17 syn$^+$	1	29	7.0×10^6
	2	0	1.6×10^6
KOS(M)	1	0	7.9×10^5
	2	0	1.5×10^5

[a] Rabbit skin cell monolayers were infected in the presence or absence of ara-T at a multiplicity of infection of 0.1. After 48 h, triplicate cultures were harvested and analyzed for virus content.

However, some virus reached the ganglion cells, and this virus was capable of some degree of replication.

The pattern of restriction continued to be observed when the kinetics of replication in the CNS were analyzed. No KOS(M) could be detected in the dorsal roots, but 17 syn$^+$ was present at peak titers of 5×10^4 PFU/g on day 7 (Fig. 2D). Again, it is likely that much of this virus was the result of productive infection of ganglion and spinal cord cells. In the spinal cord, KOS(M) was detectable at low levels on day 3 but cleared by day 4. In contrast, 17 syn$^+$ replicated to titers of 4×10^4 PFU/g on day 7 (Fig. 2E). Finally, although 17 syn$^+$ was present in the brains of mice on day 7 and most of these animals died between days 7 and 9 (Fig. 2F), no virus was recovered from the brains of mice inoculated with KOS(M), and none of these animals exhibited any signs of neurologic disease.

The preceding data demonstrated that the lack of peripheral neurovirulence of KOS(M) was a result of restricted replication at the level of the nervous system, but it was not clear whether this restriction occurred at entry into or replication in these cell types. To differentiate between these possibilities, the ability of KOS(M) to reach the mouse sacral ganglia after footpad inoculation was determined. Two days after mice were inoculated in the footpads with 10^7 PFU of either KOS(M) or 17 syn$^+$, the animals were sacrificed, and the sacral ganglia were removed intact and placed in organ culture. At various times, the cultures were analyzed for virus content. At the time of explantation, mice infected with either KOS(M) or 17 syn$^+$ had an equivalent amount of virus present in the spinal ganglia (Fig. 3). The physiological state of the ganglion cells was undoubtedly altered when the ganglia were explanted into culture, and this may account for the ability of KOS(M) to replicate efficiently in this tissue in vitro. However, this experiment does show that significant amounts of infectious KOS(M) reached these tissues at early times postinfection.

Analysis of the genes involved in the avirulence of KOS(M). Our studies of the replication efficiency of KOS(M) in tissue culture and in vivo clearly showed that this virus has a variation in at least one HSV-1 function that is required for efficient and prolonged replication in the intact nervous system. It was therefore of great interest to determine the genomic location of the gene or genes involved. Thompson et al. (12, 14) previously described a technique that allows the localization of genes controlling pathogenic phenotypes. The procedure involves the generation of random recombinant viruses between HSV strains that display different phenotypes, followed by the selection of virulent recombinants in the model animal.

To simplify analysis of the recombinants, cloned frag-

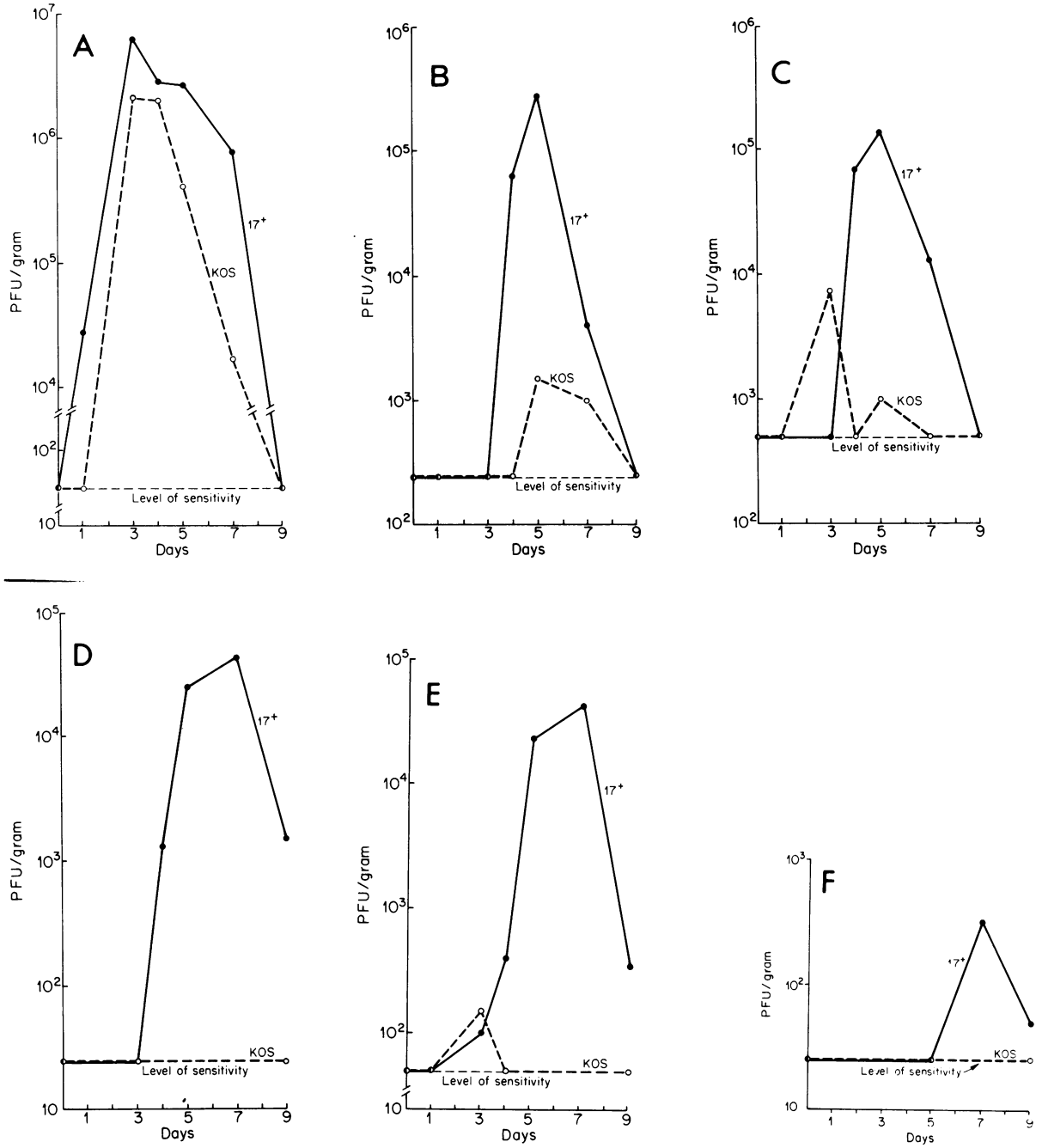

FIG. 2. Replication kinetics in vivo. Outbred Swiss Webster mice were inoculated via both rear footpads with 10^6 PFU of 17 syn$^+$ or KOS(M). At the indicated times, mice (three per datum point) were sacrificed, and various tissues were removed and stored at −70°C. These tissues were then homogenized as 10% suspensions and assayed for virus content. Tissues analyzed were both rear feet (A), both sciatic nerve trunks (B), lumbosacral ganglia from both sides of the spinal column (C), the dorsal roots associated with the examined ganglia (D), the entire spinal cord (E), and the entire brain, including the medulla (F).

ments were employed as the donor DNA. For this study, a genomic library of 17 syn$^+$ in cosmid vectors was constructed and used as the donor DNA in cotransfection experiments. The genomic location of the various cosmid DNAs employed is shown schematically in Fig. 4. One advantage of a cosmid library, such as that used here, is that the entire genome can be represented in a limited number of clones. In addition, the clones we have generated have overlapping ends. Therefore, the problems of low recombination frequency at the ends of individual clones and the possibility that specific genes that span the ends might be disrupted by recombination have been eliminated. Large clones may delete internal sequences during propagation, but this can be minimized by using a *rec*$^-$ bacterial host (12).

We first wished to determine whether the constructed library could restore neurovirulence to KOS(M). This was an important control, since Thompson et al. (12) had previously demonstrated that in some individual cases cosmid

clones can contain permutations that can preclude successful rescue of neurovirulence functions. The cosmid DNAs were cleaved with either *Xba*I (to linearize those cosmids generated with *Mbo*I partial digests) or *Hin*dIII (to cleave the viral sequences from the vector in the case of the 17 syn$^+$ *Hin*dIII A and C fragment cosmids). These DNAs were then pooled and coprecipitated with intact KOS(M) DNA onto subconfluent rabbit skin cell monolayers as described above. Control cultures consisted of transfection cultures that contained only KOS(M) DNA.

After the cytopathic effect in these cultures was complete, the plates were harvested independently, and the resulting viral stocks were analyzed for pathogenicity in mice. Two mice (pretreated with 10% saline) were infected in both rear footpads with the independently derived stocks of virus (~10^7 PFU per mouse). In this experiment, 8 of the 10 cultures generated with the cosmid DNAs caused paralysis of the inoculated mice. In contrast, none of the mice infected with the KOS(M) control cultures displayed any signs of nervous system infection.

The paralysis generated by the cotransfection of KOS(M) and the cosmid DNA was clearly statistically significant ($P < 0.01$). However, none of the transfection cultures tested was lethal, and there are several possible explanations for this result. First, the cosmid library employed might not have contained all the information of 17 syn$^+$ required for the complete rescue of KOS(M) neurovirulence. Second, one or more of the cosmids might have contained permutations in the HSV-1 sequences which affect lethality of the virus. Third, the background of KOS(M) virus present in the cultures could act in *trans* to inhibit the lethality of any fully virulent virus present.

To investigate the third possibility, the ability of KOS(M) to inhibit the pathogenesis of 17 syn$^+$ was tested when both viruses were present in the inoculum. Groups of five mice were infected with 10^7 PFU of KOS(M), 10^5 PFU of 17 syn$^+$, or a 1:1 mixture of the two stocks. The last contained a 100:1 excess of KOS(M) to 17 syn$^+$ virus. None of the mice infected with KOS(M) alone displayed any signs of CNS disease, whereas five of five mice infected with 17 syn$^+$ became paralyzed, and four of these mice died. In the group infected with the mixture, four of five mice displayed paralysis in both rear legs, but only one of the four mice eventually died. Therefore, at avirulent-to-virulent virus ratios of 100:1, an interference with the phenotype of the virulent agent was observed. This interference phenomenon was not investigated further, but a likely explanation is a heightened immune response caused by the increased antigenic burden at the time of infection.

Each of the cosmids was next tested individually in cotransfection experiments with KOS(M) DNA to determine which contained the 17 syn$^+$ sequences required to increase the virulence of KOS(M). The results of this experiment are presented in Table 2. Five independent transfection plates were generated with each of the eight cosmids used previously, and five were produced with KOS(M) DNA used alone as a control. The viral progeny of each of these plates was then inoculated into the footpads of four mice as previously described, and the mice were observed for pathologic signs. It is readily apparent from the data in Table 2 that only one transfection group yielded virus that was pathogenic, and this was the transfection containing PHC-17-9. This cosmid contains the *Hin*dIII A fragment (0.25 to 0.53 m.u.) of 17 syn$^+$. All five of these cultures caused significant paralysis, and one of the inoculated mice died.

It should be noted that cosmid M17-1, which is largely

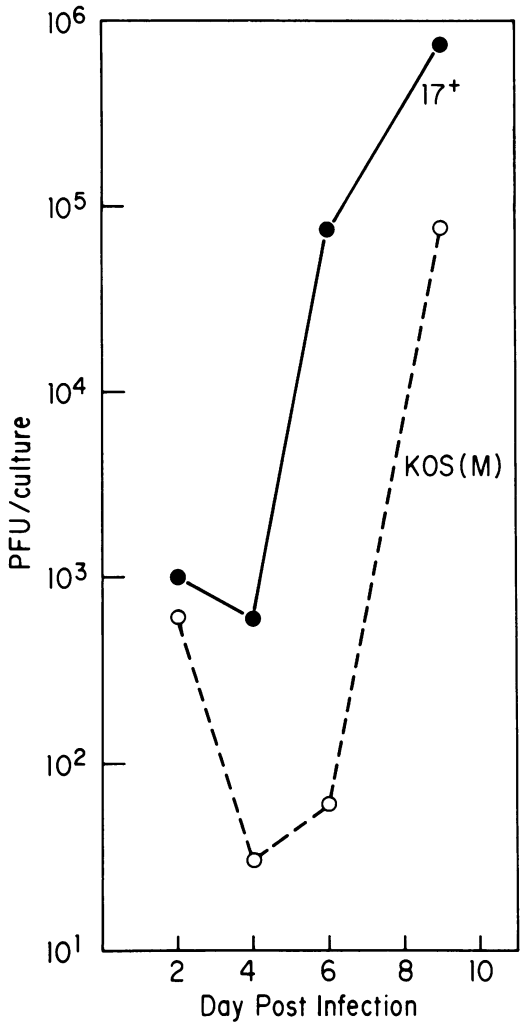

FIG. 3. Replication in spinal ganglia maintained in vitro. Mice were inoculated via the footpad with 10^7 PFU of either KOS(M) or 17 syn$^+$. After 48 h, the spinal ganglia of three mice per datum point were removed, pooled, and placed in organ culture. At the indicated times, ganglia were removed, homogenized in medium, and assayed for virus content.

colinear with *Hin*dIII fragment A (Fig. 4), did not rescue KOS(M) neurovirulence. Thompson et al. (12) have reported a similar phenomenon in another system and discussed several possible explanations for such a result. In the present case, the following points are germane. First, M17-1 was cleaved with *Xba*I prior to transfection. While we have not tested this possibility, it is possible that this treatment reduced the recombination frequency in the region of interest. Second, M17-1 may not contain all the information present in PHC-17-9. Third, this clone contains the replication origin of HSV-1, and this region is known to be unstable in a variety of vectors. Fourth, this clone may have been derived from a defective viral genome or may have undergone an undetected permutation during propagation.

The cotransfection was repeated with PHC-17-9 two more times, and the results are shown in Table 3. In the first experiment, young adult mice (~6 weeks old) were used as before, and again it can be seen that the cultures containing the *Hin*dIII A fragment were significantly more virulent than those generated with KOS(M) DNA alone. The second

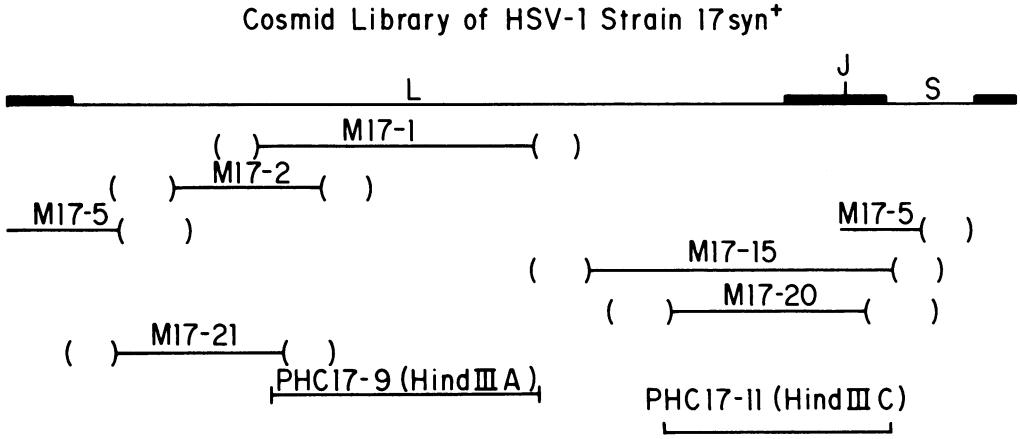

FIG. 4. Genomic location of the cosmids employed in this study. A genomic library of strain 17 syn$^+$ was produced in the cosmid vector PHC-79. The position of the various clones with respect to the prototype arrangement of the HSV-1 genome is shown above. Areas of uncertainty of the ends of the cloned sequences are shown as parentheses. The schematic of the HSV-1 genome shows the positions of the terminal and internal repeats as heavy lines, and the long unique, short unique, and joint regions are labeled L, S, and J, respectively.

experiment was performed with younger mice (~4 weeks old) in an effort to select lethal recombinant viruses. Here, it can be seen that KOS(M) was more virulent in the younger mice. However, 60% of these mice showed no pathologic signs, and no mouse died. In contrast, 100% of the mice receiving the recombinant cultures cotransfected with the *Hin*dIII A fragment and KOS(M) were paralyzed, and two of the five cultures tested were lethal. Clearly, the *Hin*dIII A fragment of 17 syn$^+$ is capable of providing the KOS(M) strain with the capacity to paralyze and kill mice.

Isolation and characterization of neurovirulent recombinants. To determine whether recombination with the *Hin*dIII A fragment of 17 syn$^+$ completely converted the phenotype of KOS(M) to virulence, virus was isolated from the brains

TABLE 2. Pathogenicity of transfection cultures generated with KOS plus individual cosmid DNAs

DNA and plate no.[a]	Pathologic signs[b]			DNA and plate no.	Pathologic signs		
	++	+	−		++	+	−
KOS				KOS + M17-20			
1	0	0	4	1	0	0	4
2	0	0	4	2	0	0	4
3	1	0	3	3	0	0	4
4	0	0	4	4	0	0	4
5	0	0	4	5	0	0	4
KOS + M17-1				KOS + M17-21			
1	0	0	4	1	0	2	2
2	0	0	4	2	0	0	4
3	0	0	4	3	0	2	2
4	0	0	4	4	0	0	4
5	0	0	4	5	0	0	4
KOS + M17-2				KOS + PHC-17-11 (*Hin*dIII-C)			
1	0	0	4	1	0	0	4
2	0	0	4	2	0	0	4
3	0	0	4	3	0	1	3
4	0	0	4	4	0	0	4
5	0	0	4	5	0	0	4
KOS + M17-5				KOS + PHC-17-9 (*Hin*dIII-A)			
1	0	0	4	1	4	0	0
2	0	0	4	2	1	2	1
3	0	2	2	3	0	4	0
4	0	0	4	4	2[c]	1	1
5	0	0	4	5	0	2	2
KOS + M17-15							
1	0	0	4				
2	0	0	4				
3	0	0	4				
4	0	0	4				
5	0	0	4				

[a] Four mice were used for each transfection plate.
[b] ++, Severe signs; +, observable signs: −, no observable signs.
[c] One mouse in the group died.

of mice which died or displayed severe signs of CNS distress for use in further pathogenetic analysis. In these experiments, virus isolated from the brains of mice inoculated with the KOS(M) cultures served as a control. It should be noted that about 10^4 PFU/g could be routinely isolated from the brain tissue of mice inoculated with the recombinant cultures. In contrast, very low titers of KOS(M) were recovered from brain tissue of animals which received the control inoculations. This result was consistent with previous data showing that KOS(M) rarely reached the brain after footpad inoculation (Fig. 2F).

After plaque purification, groups of four mice were again inoculated, and the pathogenicity of the plaque isolates was determined. The recombinant isolates (KA22 and KA24) had 50% paralytic doses of 8×10^2 and 3×10^3 PFU, respectively. In addition, these isolates were capable of inducing a lethal infection. For instance, when inoculated with 3×10^3 PFU of KA24, two of the four mice were killed. It was not possible to establish the 50% lethal dose with these isolates, as the lethality was inconsistent across several viral dilutions. This phenomenon was previously reported, and Thompson and Stevens (13) attribute it to the overall defectiveness of recombinant viruses. None of these isolates were as pathogenic as the 17 syn$^+$ parent, and this result will be discussed below. However, it is clear that the recombinant isolates were significantly more virulent than the viral stocks derived as described above by transfection with KOS(M) DNA alone. Both of the KOS(M) isolates had 50% paralytic doses of $>10^6$ PFU and were not lethal at any dilution.

Molecular evidence of recombination. It is not always possible to readily determine the locations of recombination events in a homotypic system. However, several of the isolates discussed in the preceding section were examined to determine whether there were restriction endonuclease fragment polymorphisms. This examination did confirm that recombinants between KOS(M) and 17 syn$^+$ were isolated from brain tissue. Figure 5 is an example of such an analysis. Here, DNA from strain 17 syn$^+$, KOS(M), two virulent recombinants (KA22 and KA24), and two KOS reisolates of slightly increased pathogenicity (K2 and K4) was examined by *Bam*HI and *Eco*RI restriction endonuclease digestion. It can be seen in the figure that the size of the *Bam*HI D fragment (0.460 to 0.521 m.u.) and the *Eco*RI L fragment (0.458 to 0.493 m.u.) varied between strain KOS(M) and strain 17 syn$^+$. This difference was evident in the cleavage pattern of cosmid PHC-17-9 as well (data not shown).

It can also be seen that both isolates derived from cotransfection with the *Hin*dIII A fragment displayed a restriction

TABLE 3. Pathogenicity of KOS + PHC-17-9 (Hind III-A) transfection cultures

Expt no.	DNA and plate no.[a]	Pathologic signs[b]			
		D	++	+	−
1	KOS				
	1	0	0	1	3
	2	0	0	0	4
	3	0	0	0	4
	4	0	1	0	3
	5	0	0	2	2
	KOS + *Hin*dIII-A				
	1	0	1	1	2
	2	0	3	1	0
	3	0	4	0	0
	4	0	1	1	2
	5	0	1	0	3
2	KOS				
	1	0	2	0	2
	2	0	1	0	3
	3	0	1	0	3
	4	0	2	0	2
	5	0	2	0	2
	KOS + *Hin*dIII-A				
	1	2	2	0	0
	2	0	3	1	0
	3	2	2	0	0
	4	0	2	2	0
	5	0	3	1	0

[a] Four mice were used for each transfection plate.
[b] D, Death; ++, severe signs; +, observable signs; −, no observable signs.

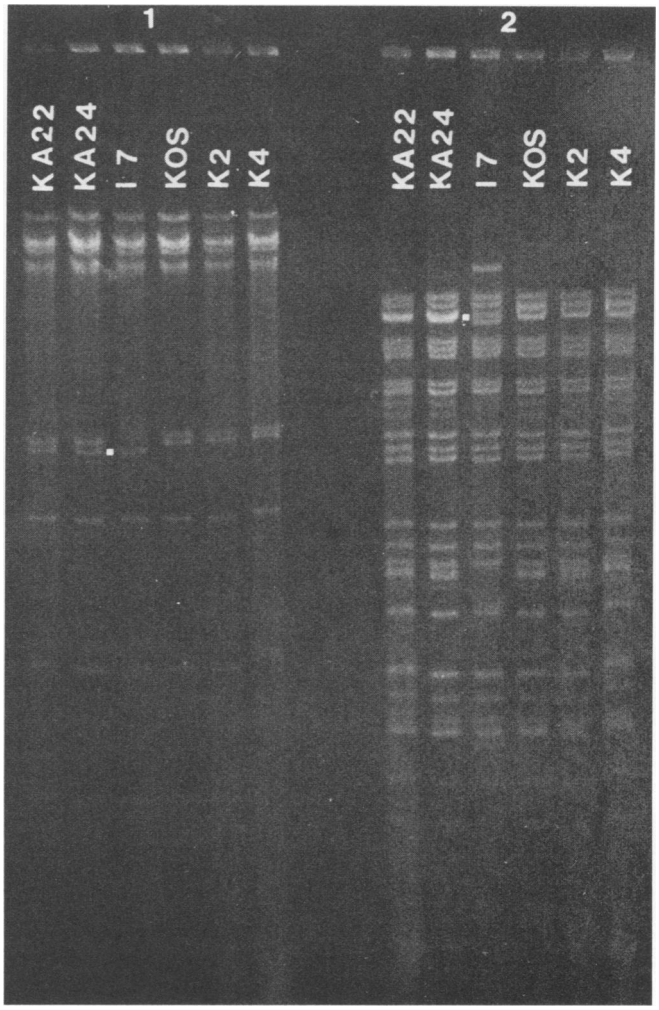

FIG. 5. Molecular evidence of recombination. DNA obtained from the wild-type strains 17 syn$^+$ and KOS(M), two neuroinvasive recombinants (KA22 and KA24), and two plaque isolates of KOS(M) reisolated from mouse brain tissues was analyzed to detect restriction endonuclease polymorphisms. In groups 1 and 2, the DNAs were cleaved with *Eco*RI and *Bam*HI, respectively, followed by agarose gel electrophoresis as described previously (13). Comparison of KA22 and KA24 with the KOS(M) lane reveals different migration patterns for *Eco*RI fragment L (0.458 to 0.493 m.u.) and *Bam*HI fragment D (0.460 to 0.521 m.u.). These fragments are marked to the right of the KA24 lane. The fragments now comigrate with the corresponding fragments of 17 syn$^+$.

enzyme fragment pattern intermediate between those of the parental strains. The migration of *Bam*HI fragment D and *Eco*RI fragment L of these isolates differed from that of strain KOS(M), and the *Bam*HI D fragment and the *Eco*RI L fragment comigrated with the 17 syn$^+$ and PHC-17-9 fragments. Neither of the isolates derived from KOS(M) DNA alone displayed any detectable polymorphisms. Because of the lack of readily detectable restriction endonuclease polymorphisms between these strains in the region 0.25 to 0.54 m.u., we did not determine the total extent of the genomic replacement.

DISCUSSION

In this report, the difference in peripheral neurovirulence between two wild-type strains of HSV-1 was analyzed. It was found that after footpad inoculation, the virulent strain 17 syn$^+$ produced paralysis and death in mice infected with as few as 10 PFU, and this strain yielded a 50% lethal dose of 10^4 PFU. In contrast, the avirulent strain KOS(M) did not induce death and only very rarely caused paralysis of mice even at inoculation titers of 10^9 PFU/ml, a result similar to that reported by others (3). This was the case even when mice were pretreated with 10% saline prior to infection, a process which enhances the virulence of HSV about 100-fold (8). Thus, these strains varied in the capacity to induce neurologic disease in mice after peripheral inoculation by at least 9 orders of magnitude.

The nature of the block in neurovirulence in strain KOS(M) was also investigated. This strain of HSV-1 induced levels of thymidine kinase in infected cells comparable to those induced by 17 syn$^+$ and replicated as well as the peripherally virulent strain in cultured primary mouse cells and nonneural mouse tissues in vivo. The restriction of the KOS(M) infection was first apparent at the level of the sacral ganglia, and after footpad inoculation of 10^6 PFU, KOS(M) was not capable of reaching the brain in detectable levels. It was shown that significant amounts of infectious KOS(M) enter the nervous system shortly after infection, and we suggest that it is the inability of this virus strain to replicate efficiently in these cells in vivo which is the cause of the avirulent phenotype of this isolate.

Through the use of in vitro generation of recombinants, followed by in vivo selection for neurovirulence, it has been demonstrated that 17 syn$^+$ sequences residing in a cloned *Hin*dIII A fragment (0.25 to 0.53 m.u.) can increase the peripheral pathogenicity of KOS(M) by at least 5 orders of magnitude. It should be noted that this is a different neurovirulence locus than that previously identified as required for replication in mouse brain tissue (12, 14). To our knowledge, this is the first time that a genetic function which contributes to a difference in pathogenicity between two wild-type strains of HSV-1 has been localized on the genome.

A number of HSV functions and the transcripts encoding them have been mapped to the *Hin*dIII A fragment (reviewed in reference 15), and the proteins encoded by some of these messages have been identified. Known genes include those coding for DNA polymerase, the DNA-binding protein, a part of the major capsid protein, glycoprotein B, and thymidine kinase. Of the genes encoded in the HSV-1 *Hin*dIII A fragment, only the viral *tk* has been previously implicated in the neurovirulence of HSV. It seems unlikely that this gene plays a role in the peripheral avirulent phenotype of KOS(M), since it has been shown that this agent produces levels of thymidine kinase equivalent to those induced by strain 17 syn$^+$. In addition, others have reported that mutant HSV strains that produce less thymidine kinase in the same assay as used here are considerably more neuroinvasive than KOS(M) (10).

Since the peripheral virulence of the recombinants was less than that displayed by 17 syn$^+$, it seems likely that more than one genetic function is required to completely alter the phenotype of KOS(M). Further recombination and selection experiments using the partially rescued recombinants as parental strains should allow detection of the additional sequences required. Finally, it should be possible to use subclones of the *Hin*dIII A fragment to localize further this HSV-1 virulence function, and these experiments are now in progress.

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