Molecular Genetics of Herpes Simplex Virus

III. Fine Mapping of a Genetic Locus Determining Resistance to Phosphonoacetate by Two Methods of Marker Transfer

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We have transferred a genetic locus determining resistance to phosphonoacetic acid (PAA') from one herpes simplex viral genome to another by two methods of marker transfer. One method requires recombination between an intact DNA molecule and a restriction endonuclease DNA fragment, whereas the other requires repair of a partial heteroduplex formed between the two DNA molecules. These two methods mapped the PAA' locus between positions 0.45 and 0.53 map units on the physical map of the viral DNA. Fine mapping of the PAA' locus showed that it maps at or near an EcoRI restriction endonuclease site at either 0.46 or 0.49 map units. We also describe and compare the two methods of marker transfer.

Phosphonoacetic acid (PAA) has been shown to inhibit the replication of herpesviruses in experimental animals and in cell cultures (22, 27). The drug has been shown to inhibit virus-specific DNA replication of these viruses both in infected cells (6, 9) and in cell-free extracts prepared from infected cells (1, 6, 9, 10, 19). This inhibition is believed to be due to the binding of the drug at the pyrophosphate end product site on the viral DNA polymerase (18).

Variants of herpes simplex virus (HSV) have been isolated which are resistant to PAA and replicate normally in its presence (6, 9, 12). Resistance to PAA segregated as a single genetic marker (PAA') in crosses involving these variants (9); furthermore, resistance to PAA in viral DNA synthesis in cell-free extracts correlated with the presence of the PAA' genetic marker (6, 9, 23). These results suggested that the PAA' genetic locus might lie within the gene for the viral DNA polymerase or one of its subunits or modifying proteins. The evidence that the PAA' locus is within the structural gene for the viral DNA polymerase is based on the observation that a PAA' ts mutant specifying a thermolabile DNA polymerase yielded revertants that were invariably both ts* and sensitive to PAA (PAA*) (12, 23a).

The genome of HSV is linear double-stranded DNA with a molecular weight of 96 million (28), consisting of two covalently linked components designated L and S. Each of these two components consists of unique sequences bounded by terminal inverted reiterated sequences (25, 28). An additional characteristic of HSV DNA is that viral DNA extracted from virions consists of four populations differing in the orientation of the L and S components (3, 8). On the basis of genetic linkage between the L and S components of the DNA of HSV-1 × HSV-2 recombinants, one form of the DNA was designated as the prototype (P) arrangement (21). The other three arrangements were designated as IS (inversion of S), IL (inversion of L), and ILS (inversion of both S and L). Map positions on the DNA are defined from left to right on the prototype arrangement of the molecule as 0 to 1.0 units (Fig. 1). The location of a PAA' locus on the physical map of the DNA was recently obtained by analysis of HSV-1 × HSV-2 intertypic recombinants constructed with PAA' as a selective marker (21). These studies mapped PAA' within map units 0.43 to 0.52. In this report we demonstrate that PAA' can be transferred from one viral genome to another by two marker transfer techniques. These techniques have not only allowed mapping of the PAA' locus to a position consistent with the above quoted results, but also allowed the fine mapping of PAA' to a position at or near a single restriction endonuclease cleavage site. In addition, we have used this genetic marker to characterize the two marker transfer techniques.

MATERIALS AND METHODS

Viruses and cells. The isolation and relevant properties of the virus strains used in this study, i.e., HSV-1(F) (4), HSV-1(HFEM) PAA' (21), and HSV-1(1061) PAA' (24), have been described previously.

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Virus stocks were prepared and titered on Hep-2 and Vero cells as described previously (21). Rabbit skin cells were originally obtained from J. McLaren.

**Preparation of viral DNAs and restriction endonuclease fragments.** Intact viral DNA was prepared from capsids purified from the cytoplasm (7) of Vero cells infected with HSV-1(F) or HSV-1(1061) or by preparative centrifugation in NaCl density gradients (29) of lysates of Vero cells infected with HSV-1(HFEM). Restriction endonucleases were purified by the method previously described (21).

DNA digests were fractionated on 0.45% agarose cylindrical gels as described previously (7, 13). DNA fragments for marker transfer were extracted from the gel slices by KI solubilization of the agarose matrix and adsorption of the DNA to hydroxypatite (13). DNA fragments prepared for re-digestion were purified by KI solubilization of the agarose matrix, dialysis of the KI, centrifugation to remove agarose (7), and n-butanol extraction to concentrate the DNA in the supernatant fluid. Isolation of labeled DNA fragments showed that the former procedure yielded 50 to 60% recovery of the DNA, whereas the latter yielded 80 to 90% recovery.

**Marker transfer by transfection of DNA mixtures.** Approximately 0.1 μg of intact HSV-1(F) DNA was mixed with 0.1 to 0.2 μg of an individual restriction endonuclease fragment. This mixture was diluted 1:1 with sterile 2 × HEPES-buffered saline (16 g of NaCl, 0.74 g of KCl, 0.25 g of Na2HPO4·2H2O, 2.0 g of dextrose, and 10.0 g of N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid [HEPES; Calbiochem, Elk Grove Village, Ill.] per liter, pH 7.05) containing 30 μg of salmon sperm DNA/ml. Each mixture was brought to a volume of 0.5 ml with 1 × HBS containing 15 μg of salmon sperm DNA/ml. After gentle mixing, 2 M CaCl2 (AnalaR; BDH Chemicals) was added to a final concentration of 0.125 M. The remainder of the transfection was performed as described by Graham and Van der Eb (5). The resulting suspension was then layered onto rabbit skin cell monolayers. After a 30-min incubation, the monolayers were overlaid with Dulbecco’s modification of Eagle medium supplemented with 2% inactivated fetal calf serum. The

**Fig. 1.** (A) Schematic drawing of the structure of HSV DNA and the Hau1 restriction endonuclease cleavage map of the four isomers of HSV-1(MP) DNA. (B) Restriction endonuclease cleavage maps of the P arrangement of HSV-1(1061) DNA. The thick lines denote the boundaries of the initial mapping of PAA' to the Hau1/BglII-F fragment and show the region emphasized in the fine mapping. HSV-1(1061) DNA differs from HSV-1(MP) DNA in the loss of the Hau1 M-N cleavage site and slight expansion of both ends of the S component.
cultures were incubated for 4 h at 37°C, and the medium was then changed to Dulbecco’s modification of Eagle medium supplemented with 6% inactivated fetal calf serum. The cultures were further incubated at 37°C for 3 to 4 days, harvested, and titered on Vero cell monolayer cultures in the presence and absence of 100 μg of PAA/ml.

Marker transfer by transfection of partial heteroduplexes. Approximately 0.5 μg of an individual restriction endonuclease fragment was mixed with 1.0 μg of HSV-1(F) DNA purified from viral capsids. The DNA was denatured by the addition of 0.1 volume of 1 M NaOH; after 5 min at room temperature, the mixture was placed on ice and neutralized by the addition of 0.1 volume of 1 M KH₂PO₄. Then 5 M NaCl was added to give a final Na⁺ concentration of 1 M. The final volume of this reaction mixture was 1.0 ml. Annealing of the DNA was carried out for 0 to 11.5 h at 42°C. Samples were quenched on ice, sterile salmon sperm DNA was added to a final concentration of 15 μg/ml, and the mixtures were dialyzed against one change each of 0.01 M Tris-hydrochloride, 0.001 M EDTA, pH 7.4, and 1 × HEPES-buffered saline. After dialysis against 1 × HEPES-buffered saline CaCl₂ was added (to 0.13 M), and the transfection procedure described above was followed.

Specific infectivity of HSV DNA. The specific infectivity of native HSV DNA isolated from either cytoplasmic viral capsids or infected cell lysates was 10² to 4 × 10⁵ PFU/μg of viral DNA. Denaturation of fresh preparations of capsid DNA reduced the specific infectivity to 10 to 15% of the infectivity of the native DNA as observed by others (26). Denaturation of DNA isolated by NaI equilibrium density gradient centrifugation completely eliminated its infectivity (data not shown). Reannealing of denatured DNA as for formation of heteroduplexes (12 h) generally did not reduce infectivity more than an additional 25%.

Quantitation of experimental data. The fraction of progeny conferring the PAA’ marker was determined from plaque counts obtained by plating serially diluted samples. In most instances relevant plaque counts were determined from two serial dilutions, and at least 10 plaques for negative transfer or 50 plaques for positive transfer were observed in the presence of PAA and 100 to 200 plaques were observed in the absence of PAA. It was convenient to present the data in the form of the fraction carrying the PAA’ marker rather than the actual plaque counts.

The fraction of plaques formed by a PAA’ virus in the presence of PAA varied in different experiments from 10⁻² to 10⁻⁷. We do not know the basis of this variability. For this reason each marker rescue experiment with all of the fragments was done at one time in the same batch of cells, and all assays of the progeny from a given experiment were done at the same time. Repeated marker transfer experiments yielded comparable results. The results presented in this paper represent individual experiments that were not summed or averaged with other experiments or assays.

RESULTS

Transfer of PAA’ phenotype. The purpose of the first series of experiments was to determine whether resistance to PAA could be transferred from one viral genome to another by specific recombination between intact DNA and a restriction endonuclease fragment. In these experiments rabbit skin cells were transfected with mixtures consisting of 0.1 μg of intact DNA extracted from HSV-1(F) PAA’ viral capsids and 0.5 μg of fragments generated by cleavage of HSV-1(1061) PAA’ DNA with BglII, HsuI, XbaI, HpaI, or EcoRI restriction endonuclease. The cultures were harvested 3 days after transfection, and the progeny virus was titered in Vero cell cultures in the presence and absence of PAA. As shown in Table 1, all digests except that generated by EcoRI were capable of transferring the PAA’ phenotype to HSV-1(F) virus. Rabbit skin cell cultures transfected with restriction endonuclease fragments, but without the intact DNA, did not yield infectious progeny; this was consistent with electrophoretic analysis of the HSV-1(1061) PAA’ DNA digests showing the absence of intact DNA or products of incomplete digestion (data not shown).

Efficiency of transfer as a function of fragment concentration. In these experiments we transfected rabbit skin cells with mixtures consisting of 0.1 μg of HSV-1(F) DNA and various amounts of XbaI digests of HSV-1(HFEM) PAA’ DNA. The results shown in Fig. 2 indicate that the fraction of viral progeny derived from these transfected cells that is resistant to PAA is proportional to the XbaI fragment concentration within the range tested (0 to 0.5 μg). In other experiments we observed that the fraction of PAA’ progeny was proportional to the DNA fragment concentration up to 1 μg of fragment DNA (not shown).

Transfer of PAA’ phenotype by partial heteroduplex formation. The objective of these experiments was to determine whether resistance to PAA could be transferred from one viral genome to another by repair of a partial heteroduplex formed between an intact single strand of HSV-1(F) DNA and a strand of DNA from a restriction endonuclease fragment bearing the PAA’ marker. We denatured and reannealed for various periods of time mixtures of intact HSV-1(F) PAA’ DNA and fragment DNA generated by digestion of HSV-1(HFEM) PAA’ DNA.

<table>
<thead>
<tr>
<th>DNA</th>
<th>% PAA’</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1(F) DNA alone</td>
<td>0.12</td>
</tr>
<tr>
<td>HSV-1(F) DNA + EcoRI fragments</td>
<td>0.13</td>
</tr>
<tr>
<td>HSV-1(F) DNA + XbaI fragments</td>
<td>2.9</td>
</tr>
<tr>
<td>HSV-1(F) DNA + HpaI fragments</td>
<td>0.4</td>
</tr>
<tr>
<td>HSV-1(F) DNA + HsuI fragments</td>
<td>10.0</td>
</tr>
<tr>
<td>HSV-1(F) DNA + BglII fragments</td>
<td>1.4</td>
</tr>
</tbody>
</table>
DNA with XbaI. Rabbit skin cells were then transfected with the resulting duplex DNA, and the progeny virus was tested to determine the fraction having the PAA' marker. We observed that the frequency of PAA' progeny increased approximately linearly with time of renaturation to approximately 7 h (Fig. 3A). However, the frequency of transfer reached a maximum at approximately 7 h of renaturation because further incubation did not increase the level of transfer.

To determine the concentration dependence of this transfer method, we reannealed various amounts of HSV-1(HFEM) PAA' DNA fragments with HSV-1(F) DNA for 11.5 h. We observed an increase in transfer with increasing amounts of fragments (Fig. 3B). Therefore, the assay can measure the presence of DNA bearing the PAA' marker.

Mapping of the gene specifying PAA' by marker transfer with specific fragments. In a preceding section it was shown that digestion of HSV-1 DNA with HsiI or BglII restriction endonucleases allowed the transfer of the PAA' phenotype to HSV-1(F) virus. In this series of experiments, we mapped the location of the gene specifying the PAA' phenotype by transfection of rabbit skin cells with either a mixture of intact HSV-1(F) DNA and individual fragments generated by digestion of HSV-1(1061) PAA' DNA with both HsiI and BglII restriction endonucleases or a partial heteroduplex formed between the intact HSV-1(F) DNA and the individual fragments. As shown in Table 2, the HsiI/BglII-

F fragment was the most efficient in transferring PAA' by both protocols. Although fragments E and G also showed some transfer, their efficiency was much lower and probably represented contamination from the neighboring F fragment in the gels. In this experiment the purified fragment showed a two- to threefold decrease in the level of PAA' transfer relative to an equivalent amount of unfractionated fragments (Table 2). This decrease is probably due to chemical damage to the fragment during isolation. We conclude that the PAA' locus maps between 0.41 and 0.52 map units (Fig. 1).

Fine mapping of the PAA' locus. To further map the PAA' locus, we isolated the HSV-1(1061) HsiI/BglII-F fragment and cleaved it with HpaI restriction endonuclease. As shown in Fig. 1 and 4, HpaI cleaves once within this fragment, yielding fragments with molecular weights of $6.6 \times 10^6$ (fragment 1) and $4.6 \times 10^6$ (fragment 2). These fragments were separated...
TABLE 2. Marker transfer of PAA' with HsuI/BglII fragments of HSV-1(1061) DNA

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Frequency of PAA' × 10^3</th>
<th>Recombination</th>
<th>Heteroduplex</th>
</tr>
</thead>
<tbody>
<tr>
<td>F DNA alone</td>
<td>0.1</td>
<td>ND^a</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII mix</td>
<td>7.7</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglIII-E</td>
<td>0.7</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-F</td>
<td>0.9</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-G</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-H</td>
<td>0.4</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-I</td>
<td>0.3</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-J</td>
<td>0.2</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-K</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-L, M</td>
<td>0.2</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-N</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-O</td>
<td>0.4</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-P</td>
<td>0.3</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-Q</td>
<td>0.4</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>F + HsuI/BglII-R</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

^a ND, Not determined.

on a 1% agarose slab gel (Fig. 4), and the two fragments were extracted from the gel bands and tested for transfer of PAA' by recombination.

The HpaI fragment with a molecular weight of 6.6 × 10^6 (fragment 1) transferred the PAA' phenotype, and therefore PAA' maps within the map positions 0.46 to 0.53. Furthermore, EcoRI cleaves only at two sites within this region, at position 0.49 and at position 0.46 near the HpaI cleavage site. It seems likely that the PAA' locus maps at 0.49 map units because HpaI digests transferred the marker but EcoRI did not. However, the precision of mapping of these restriction endonuclease cleavage sites is not sufficient to rule out the possibility that EcoRI cleaves slightly to the right of the HpaI cleavage site at 0.46. Thus, the PAA' locus probably maps at or near an EcoRI site at 0.49 map units or, less likely, at 0.46 map units.

**Efficiency of transfer as a function of fragment size.** Because we had located the PAA' locus on fragments of different sizes, we examined the relationship between the efficiency of transfer by DNA recombination and the size of the donor fragment. PAA' maps on the *HsuI/BglII-F* fragment and therefore on the *HsuI-A* (26 × 10^6) and *BglII-D* (16.8 × 10^6) fragments. Furthermore, it maps on the *HpaI-H* (6.6 × 10^6) fragment and therefore on the *XbaI-E* (18.5 × 10^6) fragment. Figure 5 shows the relationship between the size of the donor fragment and the efficiency of transfer as shown in Table 1. In general, increasing the size of the fragment increases the efficiency of rescue. However, the relationship between fragment size and efficiency of transfer seems to be an exponential one. In addition, there may be a lower limit on the size of the fragment capable of transferring the genetic marker. In this case the curve would not extrapolate to the origin as shown in Fig. 5.

**DISCUSSION**

In this study we have used two marker transfer methods to map the genetic locus determining PAA resistance to the *HsuI/BglII-F* fragment which arises from positions 0.41 to 0.52 map units of the viral DNA molecule. By further cleaving this fragment with *HpaI*, we were able...
to restrict the boundaries of this locus to positions 0.46 to 0.53. Also, because EcoRI digestion of HSV-1(1061) PAA' DNA inactivates transfer of the PAA' phenotype, we reasoned that the PAA' locus maps at or near an EcoRI restriction endonuclease cleavage site at 0.46 or 0.49 map units. Therefore, by combining both positive and negative transfer data, we have determined a precise location for the PAA' genetic locus.

The PAA' locus was previously mapped to the positions 0.43 to 0.52 map units by analysis of HSV-1 x HSV-2 recombnants (21). Results presented here confirm and extend those results. We have also studied the linkage relationship between the marker transfer of the PAA' locus and the syn3 locus, one of the loci determining polykaryocyte plaque morphology (24). The syn3 locus maps at 0.30 to 0.42 map units (24). We concluded from the linkage studies that cleavage of the PAA' syn3 DNA with XbaI restriction endonuclease eliminated linked transfer of the two markers (unpublished data). Therefore, the syn3 locus and the PAA' locus must be separated by the XbaI cleavage site at 0.45 map units, and PAA' must map to the right of this position. This conclusion is consistent with our other results.

Comparison of methods of marker transfer. We have used two methods for the marker transfer mapping of the PAA' locus. The first depends on recombination in the infected cell between an intact genome and a restriction endonuclease fragment bearing the genetic marker. This method requires few manipulations of the DNA, and we have observed that as much as 10% of the progeny virus bear the marker introduced on a DNA fragment (Fig. 2; reference 15). This represents a very high recombination frequency. We do not know whether this recombinational event is due entirely to host-cell enzymes or whether viral gene products are involved. Theoretically, there is no upper limit to the size of the DNA which can be transferred by recombination. In fact, we have observed transfer of DNA greater than 5 \times 10^6 in molecular weight (15). This method can also lead to insertion of transferred sequences as well as homologous replacement of sequences (15).

The second method requires repair of a partial heteroduplex formed between intact DNA and a restriction endonuclease fragment bearing the genetic marker (11, 16, 17). In most experiments the heteroduplex method yielded a higher level of transfer (Fig. 2 and 3; reference 24), probably because formation of the heteroduplex insures that cells receiving an intact DNA strand also receive the marker DNA. By this method we have achieved a yield of 35% PAA' progeny using a 1.5-fold excess of fragments. If the intact strands were saturated with fragment strands, the theoretical yield of PAA' progeny would be 50% (20). Thus, we are close to the theoretical yield of PAA' progeny by this method. Formation of an infectious heteroduplex does require viral DNA that contains intact single strands. The best source of intact viral DNA, cytoplasmic virions, contains only 10 to 20% intact strands (14). For this reason, this method requires more full-size duplex DNA and fragments to achieve the desired number of infectious events. Viral DNA purified from infected cell lysates by NaI density gradient centrifugation is not suitable for this method because it contains no intact single strands (unpublished data).

We do not know how large a continuous stretch of DNA can be transferred by transfection of a partial heteroduplex. Marker rescue studies with papovavirus DNA heteroduplexes have shown that two markers show linked transfer only if they are within approximately 100 base pairs of each other (20), probably because the length of DNA required during excision repair in mammalian cells is approximately 100 base pairs (2). If the HSV DNA heteroduplex is processed by a mechanism similar to that used on the papovavirus heteroduplex, we might expect that the continuous stretches of DNA that can be transferred from one genome to another are quite small.

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