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Genetics of Resistance to Phosphonoacetic Acid in Strain KOS of Herpes Simplex Virus Type 1

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A DNA$^-$ temperature-sensitive mutant of herpes simplex virus type 1 exhibiting thermolabile DNA polymerase activity, *ts*D9, was shown to be resistant to phosphonoacetic acid (PAA) when plated at the permissive temperature. *ts*$^+$ revertants of *ts*D9 were PAA sensitive and exhibited DNA polymerase activity intermediate between that of the wild-type virus and *ts*D9, indicating that both temperature sensitivity and sensitivity to PAA are controlled by the same gene. Since the position of *ts*D9 on the existing herpes simplex virus type 1 linkage map is known, the locus for PAA resistance—and therefore for the structural gene for viral DNA polymerase—has been identified.

Phosphonoacetic acid (PAA) is an effective inhibitor of the growth of herpes simplex virus (HSV) in cell culture (10) and in animals (6). Biochemical and genetic studies have demonstrated that the inhibitory effect of PAA is probably a consequence of its direct action on a DNA polymerase (DP) (3, 5) that is (i) at least partially encoded by the viral genome (1-3) and (ii) essential for virus replication (1, 2).

The availability of temperature-sensitive (*ts*) mutants of HSV type 1 (HSV-1) unable to synthesize viral DNA at the nonpermissive temperature and exhibiting temperature-sensitive viral DP activity (1) suggested the possibility that these mutants might also exhibit altered sensitivity to PAA.

The present report describes the resistance of mutant *ts*D9 and the sensitivity of *ts*$^+$ revertants of this mutant to PAA. These findings demonstrate that the lesion in *ts*D9 is in the structural gene for viral DP and that it affects the binding of PAA to the enzyme. These properties have permitted us to locate the gene for viral DP on the existing HSV-1 linkage map.

The isolation and characterization of *ts* mutants of HSV-1 strain KOS have been described (8). Spontaneous PAA-resistant (*paa*$^-$) mutants (*paa*$^-$3 and *paa*$^-$5) were picked from wild-type virus-infected Vero cell monolayers grown in the presence of 100 μg of PAA (a gift of Abbott Laboratories, North Chicago, Ill.) per ml and cloned twice in the presence of the inhibitor. The efficiency of plating (EOP) of *ts* and *paa*$^-$ mutants of PAA was tested by assaying mutant stocks at a permissive temperature (34°C) in the presence and absence of 100 μg of PAA per ml in Vero cell monolayers. The EOP was calculated as the ratio of the mutant titer (PFU per milliliter) in the presence of PAA to the titer in the absence of PAA.

To determine the relative intracellular thermal stability of the DP induced by the wild-type virus, *ts*D9, and revertants of *ts*D9, 6 × 10^6 to 8 × 10^6 human embryonic lung cells in 8-ounce (ca. 240 ml) prescription bottles were infected with 5 PFU of each virus per cell. After virus adsorption for 1 h at 37°C, infected cultures were maintained at 34°C in constant-temperature water baths (±0.1°C) until 11 h postinfection when half of the infected cultures were shifted to 39°C and half were maintained at 34°C. At the indicated times, cultures were harvested, and crude enzyme extracts were prepared as previously described (1). The DP activity of each extract was determined by a modification of the method of Aron et al. (1). Briefly, incubation mixtures (200 μl) contained 100 mM Tris-hydrochloride (pH 8.0), 3 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 5 mM dithiothreitol, 60 μg of DNase-activated calf thymus DNA, 0.01 mM each dATP, dCTP, and dGTP, 0.005 mM cold TTP (Sigma Chemical Co., St. Louis, Mo.), 10 μCi of [*methyl*-^{3}H]thymidine-5'-triphosphate ([^{3}H]TTP; 55 Ci/mmol, Schwarz/Mann, Orangeburg, N.Y.), and enzyme extract containing 30 to 70 μg of protein. Mixtures were incubated at 39°C for 30 min, spotted on Whatman GF/C glass-fiber filter squares, and dried. Filters were washed six times in cold 5% trichloroacetic acid containing 30 mM sodium pyrophosphate followed by two washes in cold ethanol. Filters were dried, and trichloroacetic acid-insoluble radioactivity was determined. Tritiated TTP incorporation was standardized for all

samples based upon protein content.

The EOP in PAA of the wild-type virus, 2 spontaneous PAA-resistant mutants, and 13 *ts* mutants representing 12 complementation groups is shown in Table 1. The EOP of the wild-type virus and of 12 of the 13 *ts* mutants tested ranged from 1.5×10^{-3} (*ts*F18) to 1.4×10^{-5} (*ts*B2), demonstrating their sensitivity to PAA. These values are in good agreement with results obtained in other laboratories (3, 4). The EOP value obtained for *ts*D9 (0.95), on the other hand, was similar to the values obtained for the PAA-resistant mutants, demonstrating the resistance of *ts*D9 to the inhibitor. It should be noted that the two *ts* mutants in complementation group C as well as the group D mutant are phenotypically DNA^- and that all three mutants exhibit thermolabile intracellular DP activity (1); however, only *ts*D9 was resistant to PAA.

To test further the relationship between PAA resistance and the *ts* mutation in *ts*D9, five spontaneous ts^+ revertants of this mutant were isolated. ts^+ revertants were picked from plaques appearing on monolayers infected with different preparations of *ts*D9 and incubated at the nonpermissive temperature (39°C). Revertants, which were thus independently derived, were cloned two or three times at 34°C in the absence of PAA and assayed for temperature sensitivity and PAA sensitivity (Table 2). With the loss of temperature sensitivity, all five revertants exhibited a simultaneous loss of resistance to PAA. Whereas the sensitivity of the mutants to PAA resembled that of the wild-type virus, *ts*D9 exhibited resistance to PAA.

TABLE 1. *EOP of wild-type virus, 2 paa⁻ mutants, and 13 ts mutants of HSV-1 strain KOS in the presence of PAA*

Virus	EOP[a]	PAA sensitivity
Wild type	3.5×10^{-4}	+
paa⁻3	9.4×10^{-1}	−
paa⁻5	9.3×10^{-1}	−
*ts*A1	4.5×10^{-5}	+
*ts*B2	1.4×10^{-5}	+
*ts*C4	2.0×10^{-5}	+
*ts*C7	6.0×10^{-4}	+
*ts*D9	9.5×10^{-1}	−
*ts*E6	7.0×10^{-5}	+
*ts*F18	1.5×10^{-3}	+
*ts*G8	3.8×10^{-4}	+
*ts*J12	1.9×10^{-4}	+
*ts*L14	2.5×10^{-4}	+
*ts*M19	5.0×10^{-5}	+
*ts*N20	1.3×10^{-4}	+
*ts*O22	4.5×10^{-4}	+

[a] EOP = [PFU/ml with PAA (100 μg/ml)]/[PFU/ml without PAA], when grown and assayed at 34°C.

TABLE 2. *Temperature sensitivity and PAA sensitivity of the wild-type virus, tsD9, and five revertants of tsD9*

Virus	EOP (PFU/ml)		Phenotype	
	39°C/34°C[a]	+PAA/−PAA (34°C)[b]	*ts*	*paa*
Wild type	6.2×10^{-1}	3.5×10^{-4}	+	+
*ts*D9	$<2.0 \times 10^{-5}$	9.5×10^{-1}	−	−
ts^+1	4.1×10^{-1}	6.0×10^{-5}	+	+
ts^+2	4.2×10^{-1}	$<1.2 \times 10^{-5}$	+	+
ts^+3	3.9×10^{-1}	2.0×10^{-5}	+	+
ts^+4	3.3×10^{-1}	5.0×10^{-4}	+	+
ts^+5	4.0×10^{-1}	$<4.0 \times 10^{-5}$	+	+

[a] Virus stocks were assayed at 34 and 39°C. EOP = (PFU/ml at 39°C)/(PFU/ml at 34°C).

[b] [PFU/ml with PAA (100 μg/ml)]/[PFU/ml without PAA], when grown and assayed at 34°C.

These observations strongly suggest that temperature sensitivity and PAA resistance in *ts*D9 are consequences of the same genetic defect.

The relative thermal stability of DP activity of the wild-type virus, *ts*D9, and four ts^+ revertants was examined by a modification of a procedure described previously (1). As previously demonstrated (1), the DP activity of the wild-type virus was shown to be stable after a shift-up to 39°C, in contrast to the extreme thermal sensitivity of *ts*D9 DP activity (Fig. 1). Thermostable revertants of *ts*D9 exhibited DP activities that were intermediate in thermal stability between that of the wild-type virus and *ts*D9. Thus, at 39°C revertants 1 and 3 exhibited DP activity that was more thermolabile than that of the wild-type virus, but less so than that of the parental virus *ts*D9. Revertants 2 and 5, on the other hand, exhibited DP activity that was as thermostable as that of the wild-type virus at 39°C. The variations in the thermal stability of DP activity observed and the intermediate nature of these patterns among the four revertants are precisely what one would expect to observe in the case of independently derived revertants resulting from forward mutations of the suppressor type (pseudorevertants). These data, therefore, further support the concept that the mutation in *ts*D9 resides in the gene for viral DP.

On the linkage map of HSV-1 strain KOS, *ts*D9 is located adjacent to the C cistron in a tight cluster of DNA^- mutants (9; J. T. Jofre, R. Courtney, and P. A. Schaffer, manuscript in preparation). Not only has the coincidence of the loci for PAA resistance and temperature sensitivity in *ts*D9 facilitated the localization of PAA resistance on the HSV-1 linkage map, but also it should prove advantageous in attempts

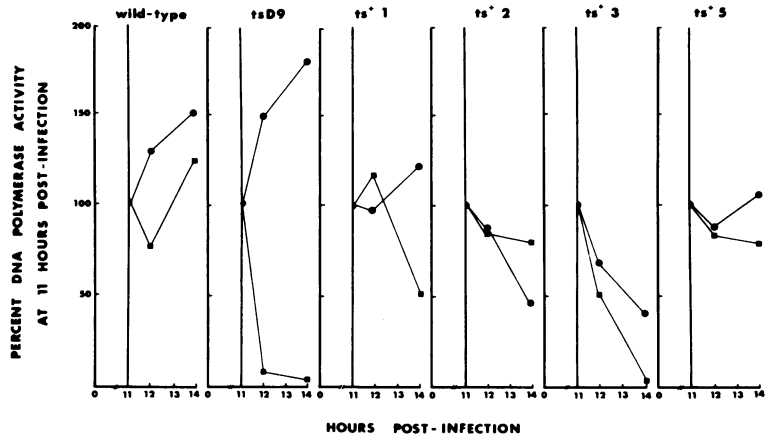

FIG. 1. *In vivo thermal stability of DP induced by wild-type virus, tsD9, and ts⁺ paa⁺ revertants (ts⁺1, -2, -3, and -5) of tsD9. Virus-infected human embryonic lung cells were incubated for 11 h at 34°C. At this time, half of the cultures were shifted to 39°C. Cells were harvested at 11, 12, and 14 h postinfection, and extracts from cells incubated at 34°C (●) and 39°C (■) were tested for DP activity.*

to place the PAA resistance locus on the physical map in marker-rescue experiments.

Although one locus for PAA resistance resides in the D cistron, the existence of more than one locus for PAA resistance has not been ruled out. If a second locus for PAA resistance were present at some distance from the D cistron, PAA-sensitive wild-type virus should be detected in the yields of crosses between mutants that are PAA resistant in different loci. To explore this possibility, PAA-resistant *ts*D9 was crossed with three PAA-resistant *ts*⁺ mutants at 34°C as previously described (9), and yields were tested for their content of PAA-resistant recombinants by plating in the presence and absence of PAA. The difference between the yield of virus in the absence of PAA (total virus) and in the presence of PAA (PAA-resistant virus) would represent PAA-sensitive virus.

The results of these tests are shown in Table 3. Since the yield of virus from crosses between PAA-resistant mutants was composed almost entirely of PAA-resistant virus, we conclude that among the mutants studied either resistance resides in only one locus or that it resides in two or more closely linked loci. Clearly, additional PAA-resistant mutants must be isolated and tested before this assumption can be stated with certainty.

It has been demonstrated previously that virus-induced DP is necessary for successful infection (1, 3, 7). Therefore, revertants of *ts*D9 encode either polymerases identical to that of the wild-type virus (true revertants) or mutant polymerases that can exist in a functional configuration at the nonpermissive temperature (pseudorevertants). In either case, the conformational change at the *ts*D9 locus also causes a drastic alteration in PAA sensitivity. That the mutation in *ts*D9 may lie in a modifier of DP and not in the structural gene of virus DP has not been ruled out. However, studies reported herein confirm that at least one polypeptide component of the virus-induced DP is the target for the action of PAA and that this polypeptide is virus coded.

TABLE 3. *Crosses between paa⁻ mutants*

Mutant pair	Virus yield (PFU/ml)		Ratio of *paa⁻* virus to total virus
	Total virus[a]	*paa⁻* virus[b]	
*ts*D9 × *ts*D9	2.3 × 10⁷	2.1 × 10⁷	0.91
*paa⁻*3 × *paa⁻*3	6.9 × 10⁷	6.6 × 10⁷	0.96
*paa⁻*5 × *paa⁻*5	6.2 × 10⁷	5.8 × 10⁷	0.94
*ts*D9 × *paa⁻*3	6.5 × 10⁷	6.2 × 10⁷	0.95
*ts*D9 × *paa⁻*5	3.0 × 10⁷	3.0 × 10⁷	1.00
*paa⁻*3 × *paa⁻*5	4.6 × 10⁷	4.5 × 10⁷	0.98

[a] Virus yield assayed at 34°C in the absence of PAA.

[b] Virus yield assayed at 34°C in the presence of 100 μg of PAA per ml.

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