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Fine Mapping and Molecular Cloning of Mutations in the Herpes Simplex Virus DNA Polymerase Locus

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Mutations in five phenotypically distinct mutants derived from herpes simplex virus type 1 strain KOS which lie in or near the herpes simplex virus DNA polymerase (*pol*) locus have been fine mapped with the aid of cloned fragments of mutant and wild-type viral DNAs to distinct restriction fragments of 1.1 kilobase pairs (kbp) or less. DNA sequences containing a mutation or mutations conferring resistance to the antiviral drugs phosphonoacetic acid, acyclovir, and arabinosyladenine of *pol* mutant PAA^{r}5 have been cloned as a 27-kbp *Bgl*II fragment in *Escherichia coli*. These drug resistance markers have been mapped more finely in marker transfer experiments to a 1.1-kbp fragment (coordinates 0.427 to 0.434). In intratypic marker rescue experiments, temperature-sensitive (*ts*), phosphonoacetic acid resistance, and acyclovir resistance markers of *pol* mutant *ts*D9 were mapped to a 0.8-kbp fragment at the left end of the *Eco*RI M fragment (coordinates 0.422 to 0.427). The *ts* mutation of *pol* mutant *ts*C4 maps within a 0.3-kbp sequence (coordinates 0.420 to 0.422), whereas that of *ts*C7 lies within the 1.1-kbp fragment immediately to the left (coordinates 0.413 to 0.420). *ts*C4 displays the novel phenotype of hypersensitivity to phosphonoacetic acid; however, the phosphonoacetic acid hypersensitivity phenotype is almost certainly not due to the mutation(s) conferring temperature sensitivity. The *ts* mutation of mutant *ts*N20—which does not affect DNA polymerase activity—maps to a 0.5-kbp fragment at the right-hand end of the *Eco*RI M fragment (coordinates 0.445 to 0.448). The mapping of the mutations in these five mutants further defines the limits of the *pol* locus and separates mutations differentially affecting catalytic functions of the polymerase.

Herpes simplex virus (HSV) induces a novel DNA polymerase activity which resembles mammalian DNA polymerase α in several respects (31), yet it can be distinguished from cellular polymerases by a variety of criteria including sensitivity to certain antiviral drugs or their triphosphate derivatives (9, 21, 35, 47). Considerable evidence indicates that HSV specifies this polymerase activity and that the activity is essential for viral replication. This evidence rests on studies of a variety of temperature-sensitive (*ts*) and drug-resistant mutants of HSV which specify altered polymerase activities (1, 9, 11, 16, 19, 23–25, 29, 42, 43, 45). Of these, the *ts* mutants have been assigned to complementation groups 1-3, 1-4, 1-14, and 2-3; however, based upon their close proximity on the physical map of the viral genome and the overlapping patterns of complementation displayed by several of them (4, 5, 48; S. K. Weller, D. P. Aschman, W. R. Sacks, D. M. Coen, and P. A. Schaffer, Virology, in press; this work), they may be regarded as affecting one function, DNA polymerase (*pol*).

The location of these *pol* mutations defines the *pol* locus, the precise limits of which remain unknown. One approach to defining these limits is to map mutations within the *pol* locus precisely. By definition, the *pol* locus must at least span the distance between mutations within it. Thus, based on previous intertypic marker rescue experiments mapping *pol* mutations of *ts*C7 (KOS) and *paa*^{r}-1 (strain 17) (4, 5), the *pol* locus would span coordinates 0.417 and 0.422 (all coordinates have been adjusted to those assigned to strain KOS by Parris et al. [39] and Weller et al. [in press]). This would correspond to a distance of 0.7 kilobase pairs (kbp). Several lines of evidence indicate that the HSV polymerase is a polypeptide of about 150,000 daltons (16, 28, 40); thus, this distance would probably represent only a small fraction of the coding capacity of the *pol* locus.

Similarly, the location of mutations in non-*pol* genes can provide limits beyond which the *pol* locus could not extend without overlapping these other genes.

Additionally, it might reasonably be expected that mutations located at different sites within the *pol* locus would affect different functional sites on the polymerase molecule. Drug resistance mutations should be especially useful in identifying such sites in that mutations conferring resistance to specific drugs should affect those functional sites which interact with the drug. For example, mutants resistant to the pyrophosphate analog phosphonoacetic acid (PAA) would be expected to specify polymerases affected in pyrophosphate exchange and release (16, 32), whereas mutants resistant to acyclovir (ACG) or arabinosyladenine (araA), which are deoxyribose purine analogs altered in their sugar moieties, would be expected to specify polymerases affected in sugar recognition at the deoxyribonucleoside triphosphate binding site (9, 19, 21, 29, 38). The fine mapping of mutations differentially affecting these functions should then aid in the correlation of polymerase structure and function.

The definition of the limits of the *pol* locus and the dissection of functional sites of the polymerase can be facilitated by the use of cloned DNA fragments for fine physical mapping of mutations. Such an approach has been employed for other HSV genes by Preston (41) and by Spang et al. (50) and Weller et al. (52), who conducted intratypic marker rescue experiments with cloned wild-type viral DNA fragments to map *ts* mutations to different regions of the genes encoding the immediate early polypeptide ICP4 and the major DNA-binding protein, respectively. Additionally,

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the availability of cloned DNA fragments from mutant viruses would be valuable for physical mapping of drug resistance mutations by marker transfer (9, 11, 27) and to enable the analysis of these mutations at the base-pair and amino acid levels by DNA sequencing.

Four *pol* mutants of HSV type 1 (HSV-1), strain KOS, which vary in their drug resistance phenotypes and thus can be expected to specify DNA polymerases differentially affected in their functional properties, are PAA^{r}5, *ts*D9, *ts*C4, and *ts*C7 (1, 4, 9–12, 19, 26, 30, 43–45, 49; this work). We report here the fine physical mapping of the mutations of these four *pol* mutants and the non-*pol* mutant *ts*N20 (KOS) (1, 48; Weller et al., in press) to distinct fragments of 1.1 kbp or less. The drug resistance markers of PAA^{r}5 were mapped by marker transfer with the aid of a cloned DNA fragment containing the PAA^{r}5 mutation(s). The *ts* and certain drug resistance mutations of *ts*D9, *ts*C4, *ts*C7, and *ts*N20 were mapped by intratypic marker rescue with the aid of cloned wild-type strain KOS DNA fragments. The fine mapping of these mutations further defines the limits of the *pol* locus and separates mutations differentially affecting catalytic functions of the polymerase.

(Portions of this study were presented at the International Acyclovir Symposium, September 1981, and the Sixth Cold Spring Harbor Meeting on Herpesviruses, September 1982.)

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (Vero), human epidermoid carcinoma number 2 cells (HEp-2), and primary rabbit kidney cells (RK) were propagated and maintained as described previously (52). Stocks of wild-type HSV-1, strain KOS, and mutants *ts*C4, *ts*C7, *ts*D9, *ts*N20, and PAA^{r}5 were prepared in Vero cells as described previously (48).

Drugs. Disodium PAA, the generous gift of Abbott Laboratories (North Chicago, Ill.); ACG, kindly provided by Burroughs Wellcome Co. (Research Triangle Park, N.C.); and araA (Calbiochem, La Jolla, Calif.) were prepared as described previously (9, 10).

Virus assays. Virus assays were performed in Vero cell monolayers by a plaque method utilizing a 2% methylcellulose overlay (17). Viruses were assayed for drug resistance by overlaying infected cells with methylcellulose containing the concentrations of drugs indicated in the text, tables, and figures. The permissive and nonpermissive temperatures for assays of *ts* mutants were 34 and 39°C, respectively.

Bacterial strains and plasmids. Plasmid pKC7, which contains a single *Bgl*II site in a gene conferring kanamycin resistance (46), and plasmids p1BD1 and p1BI1, which contain the strain KOS *Bgl*II D and I fragments, respectively, inserted into the pKC7 *Bgl*II site (33), were propagated in *Escherichia coli* LE392 and were the generous gift of W. C. Leung (University of Alberta, Edmonton, Alberta, Canada). Plasmid pBR325, which contains a single *Eco*RI site in a gene conferring chloramphenicol resistance (2), and plasmids pSG3, pSG5, pSG17, and pSG18, which contain the strain KOS *Eco*RI L, O, M, and F fragments, respectively, inserted into that site (22), were carried in *E. coli* DH-1. These plasmids were kindly provided by R. Sandri-Goldin and M. Levine (University of Michigan, Ann Arbor). Plasmid pKEF-P1, generously supplied by N. DeLuca, contains strain KOS DNA extending from the *Pst*I site at coordinate 0.398 to the *Eco*RI site at coordinate 0.422. pSG18-SA contains the largest *Sal*I subfragment of the strain KOS *Eco*RI F fragment and was the kind gift of D. Knipe, Harvard Medical School, Boston, Mass. Plasmid pKOS29 was derived from a "shotgun" cloning of KOS *Bam*HI fragments into pBR322 (7). It contains the *Bam*HI Q fragment and a smaller fragment of 2.8 kbp which is as yet unidentified but which does not share sequences with the *Eco*RI M or F fragments. *E. coli* HB101 (3) was used for bacterial transformation. Plasmids pPA17 and pPA22 were propagated in strain HB101 and are described below. Map locations of the HSV sequences contained in the various plasmids are given in Fig. 1 and 3 and in the accompanying text.

DNA isolation. HSV DNA was prepared from virions produced by KOS- or mutant-infected HEp-2 or RK cells as described previously (39). Plasmid DNA was prepared in either of two ways. For small-scale preparations, DNA was isolated from 10-ml bacterial cultures after chloramphenicol amplification essentially as described by Davis et al. for 1-ml cultures (15). When larger amounts of purer DNA were required, DNA was isolated from 250-ml cultures of bacteria after chloramphenicol amplification as described by Clewell and Helinski (6). The plasmid DNA was further purified by CsCl-ethidium bromide centrifugation; ethidium bromide was removed by extraction with *n*-butanol saturated with 5 M NaCl, 10 mM Tris-hydrochloride, and 1 mM EDTA (pH 8.5), and CsCl was removed either by dialysis against 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) or by ethanol precipitation (14). pPA22 DNA prepared by both procedures was active in marker transfer experiments.

Restriction endonuclease analysis. Restriction enzymes were obtained from New England Biolabs (Beverly, Mass.) and were used according to the recommendations of the supplier. Restriction fragments were separated on horizontal 0.6 to 1.5% agarose slab gels cast in 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, and 0.5 μg of ethidium bromide per ml and were visualized by UV illumination. Bacteriophage lambda DNA (New England Biolabs) digested with *Hin*dIII was used as the standard for determining the molecular weights of DNA fragments.

Molecular cloning. pKC7 DNA and PAA^{r}5 DNA were digested to completion with *Bgl*II, mixed in a mass ratio of 1:15, and ethanol precipitated. The resulting precipitate was suspended in water and heated at 70°C for 5 min to inactivate any residual *Bgl*II activity. The mixture was adjusted to 50 mM Tris-hydrochloride (pH 7.8), 10 mM $MgCl_2$, 20 mM dithiothreitol, and 1 mM ATP (ligation buffer), and T4 DNA ligase (the kind gift of R. Kolodner, Dana-Farber Cancer Institute) was added, giving a final DNA concentration of about 50 μg/ml. The mixture was incubated for 1 h at 12.5°C and then diluted 45-fold with ligation buffer, and additional ligase was added. Ligation was allowed to proceed overnight under these conditions, which favor self-circularization of the initial ligated products (18). The mixture was stored at −20°C.

Portions of this ligation mixture were adjusted to 10 mM in $CaCl_2$ and used to transform strain HB101 by the procedure of Morrison (36). Transformed cells were spread onto agar plates containing 100 μg of ampicillin per ml to yield about 100 colonies per plate. After small colonies formed, the colonies were replica plated onto plates containing 100 μg of ampicillin and 100 μg of kanamycin per ml and onto plates containing only ampicillin. Putative Amp^r Kan^s colonies were picked and retested for drug sensitivity. Amp^r Kan^s colonies were grown in 1- to 1.5-ml cultures and screened for the presence of plasmids bearing inserted *Bgl*II fragments by the procedure of Davis et al. (15).

Isolation of DNA fragments. Restriction enzyme-generated DNA fragments were isolated from agarose gels essentially

as described by Vogelstein and Gillespie (51). Briefly, fine particles of 325-mesh glass were heated to near boiling in 50% nitric acid and rinsed extensively in glass-distilled water. Agarose gel slices containing restriction fragments were dissolved at 37°C in 2 ml of 6.1 M sodium iodide saturated with sodium sulfite per g of gel slice. The DNA was then adsorbed to the treated glass overnight at 0°C. The glass was recovered by centrifugation and washed once with the sodium iodide solution and then twice with 50% ethanol–10 mM Tris (pH 7.5)–0.1 M NaCl–1 mM EDTA at 0 to 4°C. The DNA was eluted twice at 37°C for 30 min with 2 to 3 volumes of 10 mM Tris (pH 7.5)–1 mM EDTA per volume of glass.

Marker transfer and rescue. Marker transfer experiments were performed by transfecting RK cells in suspension as described previously (39) with a mixture of infectious KOS DNA and either plasmid DNA, isolated restriction fragments, or PAA^{r}5 DNA, digested with appropriate restriction enzymes. Unless otherwise noted, before use in marker transfer experiments, restriction digests were routinely heat inactivated at 70°C for 5 min, deproteinized by phenol extraction, extracted with chloroform, and ethanol precipitated. The precipitated DNA was resuspended in 10 mM Tris (pH 7.5)–1 mM EDTA before use. In transfection mixtures, restriction fragments were present in fivefold excess or greater relative to the amount of analogous wild-type viral sequences contained in the infectious KOS DNA.

These procedures were developed because it was found that marker transfer was much less efficient when undigested plasmid DNA was used than when the inserted DNA was cleaved free of the vector or when the plasmid DNA was linearized by an enzyme which cut the plasmid once within vector sequences. This result is analogous to that obtained by Preston for marker rescue of *ts* mutants by plasmid DNAs (41). Additionally, if DNA digested with certain restriction enzyme preparations was heat inactivated but not deproteinized before transfection, both the infectivity of the KOS DNA to which digests were added and the subsequent efficiency of marker transfer decreased. This effect could not be ascribed to the restriction enzyme buffer used.

Progeny virus from transfection was assayed for drug resistance as described above.

Marker rescue experiments were performed in an analogous fashion (52) except that infectious *ts*C4, *ts*C7, *ts*D9, or *ts*N20 DNA was mixed with either plasmid DNA or isolated restriction fragments. Progeny virus from transfection of RK or Vero cells was assayed at 34 and 39°C.

RESULTS

Molecular cloning and mapping of PAA^{r}5. (i) Cloning of the PAA^{r}5 *Bgl*II D fragment containing the drug resistance markers. PAA^{r}5 is resistant to PAA, ACG, and araA due to a mutation or mutations in the *pol* locus (9–11, 19, 26). Previous marker transfer experiments with gel-purified DNA fragments had localized the drug resistance markers of PAA^{r}5 to the *Hpa*I B fragment (coordinates 0.339 to 0.464) (9; unpublished data). The *Hpa*I B fragment overlaps the *Bgl*II D and I fragments of PAA^{r}5 (22, 39) (Fig. 1). To map the PAA^{r}5 markers more finely, we cloned the *Bgl*II D and I fragments of PAA^{r}5 into the *Bgl*II site of plasmid pKC7, yielding the hybrid plasmids pPA22 and pPA17, respectively. Their identity was confirmed by double digestion with *Bgl*II and *Eco*RI, which yielded fragments which comigrated with those obtained by cleavage of plasmids p1BD1 and p1BI1 (data not shown), which contain the wild-type *Bgl*II D and I fragments, respectively (33).

To determine whether the plasmids contained drug resistance mutations, each was cleaved with *Bgl*II and tested in marker transfer experiments by cotransfection with infectious, drug-sensitive KOS DNA. Progeny were assayed for drug resistance. As shown in Table 1, pPA22 DNA transferred resistance to PAA, ACG, and araA. No transfer was detected with pPA17 DNA. Thus, pPA22 contained the mutation(s) responsible for the drug resistance phenotypes of PAA^{r}5, mapping these markers to the *Bgl*II D fragment and narrowing the limits for these phenotypes to sequences between the left-hand end of the *Bgl*II D fragment and the right-hand end of the *Hpa*I B fragment (0.417 to 0.464) (Fig. 1). (Unless otherwise indicated, the terms mutation and marker will be used to signify one or more mutations which confer a given phenotype.)

(ii) Fine mapping of the PAA resistance marker of PAA^{r}5. To map the location of the mutation(s) responsible for the drug resistance phenotypes of PAA^{r}5 more finely, pPA22 DNA was first digested with *Bam*HI, *Kpn*I, *Sal*I, and *Eco*RI to see whether sufficient marker transfer for further analysis could be obtained after digestion with these enzymes. In this experiment, the most efficient transfer of PAA resistance relative to that seen with no fragment added was observed after digestion with *Bam*HI and *Kpn*I (Table 2), and thus, these two enzymes were suitable for fine mapping of the drug resistance mutation.

The location of the *Bam*HI and *Kpn*I restriction sites in pPA22 and their positions on the HSV-1 genome were next determined. The resulting restriction maps and map coordinates are shown in Fig. 1. (These maps confirm the map presented by Chartrand et al. [4] for this part of the genome of strains KOS and 17; however, we would call the KOS *Bam*HI fragment which they termed "r", "Q" [52]. The *Bam*HI maps of strains KOS and 17 differ, however, in several respects from those described by Locker and Frenkel for strains F and Justin [34].)

*Bam*HI and *Kpn*I restriction fragments of pPA22 DNA were purified from agarose gels and tested for marker transfer. As seen in Fig. 2, *Kpn*I fragment *d* and *Bam*HI fragment *d* transferred PAA resistance at substantially higher frequencies than the other fragments. This result was reproduced four times. The *Kpn*I *d* and *Bam*HI *d* fragments of pPA22 overlap by about 1.1 kbp within the KOS *Bgl*II D and *Hpa*I B fragments at coordinates 0.427 to 0.434 (Fig. 1).

We found a rough correlation between marker transfer and rescue efficiencies and the size of the transferring or rescuing fragment, as noted previously by Knipe et al. (27) and Preston (41). An exception to this correlation arose reproducibly, however, when *Eco*RI-digested DNAs were used to transfer the PAA^{r}5 PAA resistance marker (compare transfer efficiencies in Table 2 with fragment sizes in Fig. 1). This observation suggests that reductions in efficiencies of marker transfer and rescue after restriction enzyme digestion should not be interpreted solely to indicate that a mutation lies close to a particular restriction enzyme site.

(iii) Cotransfer of ACG and araA resistance markers with PAA resistance. To determine whether the ACG resistance and araA resistance markers of PAA^{r}5 mapped to the same 1.1-kbp region as PAA resistance, we tested 20 PAA-resistant plaque isolates derived from marker transfer experiments conducted with the *Kpn*I *d* fragment and the *Bam*HI *d* fragment for resistance to PAA, ACG, and araA. Eight of the 10 isolates derived from marker transfer with the *Kpn*I *d* fragment (Fig. 1 and 2) exhibited levels of resistance to all three drugs similar to those of PAA^r 5 in terms of plating efficiency (Table 3) and plaque size. One isolate, Kp5, was

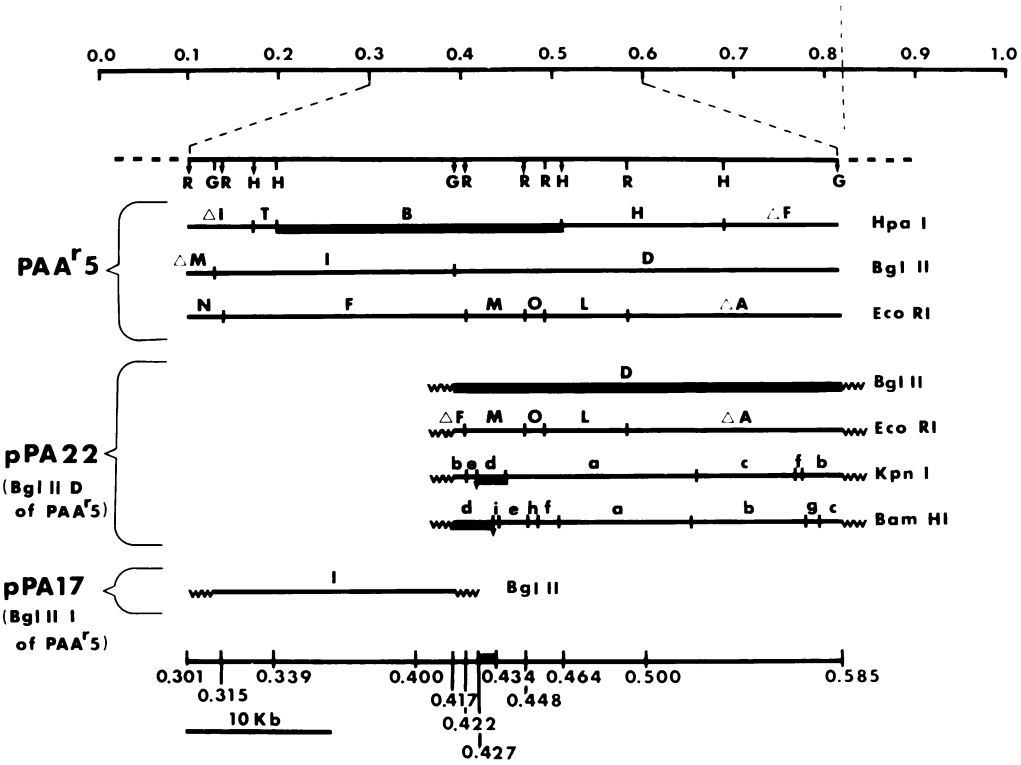

FIG. 1. Summary of mapping of PAA^{r}5 markers. The numbers on the top and bottom lines are physical map coordinates of HSV-1 PAA^{r}5 (KOS) DNA in the prototype arrangement. In the second line, the region of the genome from coordinates 0.3 to 0.6 is expanded to show the locations of restriction sites (*Hpa*I, H; *Bgl*II, G; *Eco*RI, R). On this line and on lines 8 and 9 arrows correspond to coordinates presented on the bottom line. The next three lines show the locations and designations of restriction fragments generated by these enzymes in this region of the genome. The next four lines show the location and designation of restriction fragments generated by *Bgl*II, *Eco*RI, *Kpn*I, and *Bam*HI from plasmid pPA22. The next line gives the location and designation of the *Bgl*II I fragment of PAA^{r}5 DNA contained in pPA17. Wavy lines indicate bacterial plasmid sequences. Restriction fragments designated with capital letters refer to the size of fragments in the entire PAA^{r}5 genome; lowercase letters refer to the size of fragments in a particular plasmid. The symbol △ indicates that only a portion of the fragment is contained in the plasmid. The restriction sites were aligned by analysis of single, double, and triple restriction digests of plasmids pPA22, pSG17, pSG3, and pSG5. The locations of the PAA^{r}5 drug resistance markers from experiments described in reference 9 and in Table 1 and Fig. 2 of this work are indicated by bold lines (PAA^{r}5 *Hpa*I B, pPA22 *Bgl*II D, *Kpn*I *d*, and *Bam*HI *d* fragments). The region of overlap of these fragments is indicated by a bold line between coordinates 0.427 and 0.434 on the bottom line.

considerably less resistant to both PAA and ACG than PAA^{r}5; a second isolate, Kp6, appeared as PAA resistant but less ACG and araA resistant.

The 10 plaque isolates derived from marker transfer with the *Bam*HI *d* fragment exhibited levels of PAA and ACC resistance similar to those of PAA^{r}5 in terms of plating efficiency (Table 3) and plaque size. Of these 10, 2, Ba3 and Ba4, exhibited less than half the plating efficiency of PAA^{r}5 in 150 μM araA; however, it is not clear whether these differences are meaningful since there is considerable variability in assays of araA resistance (9; also compare experiments 1 and 2 in Table 3).

Thus, for each set of plaque isolates, at least 8 to 10 behaved in a manner similar to PAA^{r}5 with regard to ACG and araA resistance. Thus, both the *Kpn*I *d* and *Bam*HI *d* fragments also transferred resistance to ACG and araA, mapping these markers to the 1.1-kbp sequence common to

TABLE 1. Marker transfer with cloned *Bgl*II fragments of PAA^{r}5 DNA[a]

DNA transfected	Efficiency of plating (%) in:		
	1.1 mM PAA	250 μM araA	10 μM ACG
KOS DNA	0.064	0.056	0.68
KOS DNA + pPA22 DNA[b]	3.7	0.26	3.2
KOS DNA + pPA17 DNA[c]	0.058	0.043	0.56

[a] Marker transfer experiments were performed as described in the text, and progeny were assayed for drug resistance in the presence of the indicated drug concentrations.

[b] pPA22 consists of the PAA^{r}5 *Bgl*II D fragment cloned into pKC7.

[c] pPA17 consists of the PAA^{r}5 *Bgl*II I fragment cloned into pKC7.

TABLE 2. Marker transfer of PAA resistance with restriction digests of pPA22 DNA[a]

pPA22 DNA digested with:	Efficiency of plating (%) in 1.1 mM PAA
*Bgl*II	2.5
*Eco*RI	0.05
*Bam*HI	0.16
*Kpn*I	0.12
*Sal*I	0.03
No pPA22 DNA	0.01

[a] KOS DNA alone or mixed with pPA22 DNA digested with the indicated restriction enzymes was used to transfect RK cells in marker transfer experiments. The progeny of the transfections were assayed for resistance to 1.1 mM PAA.

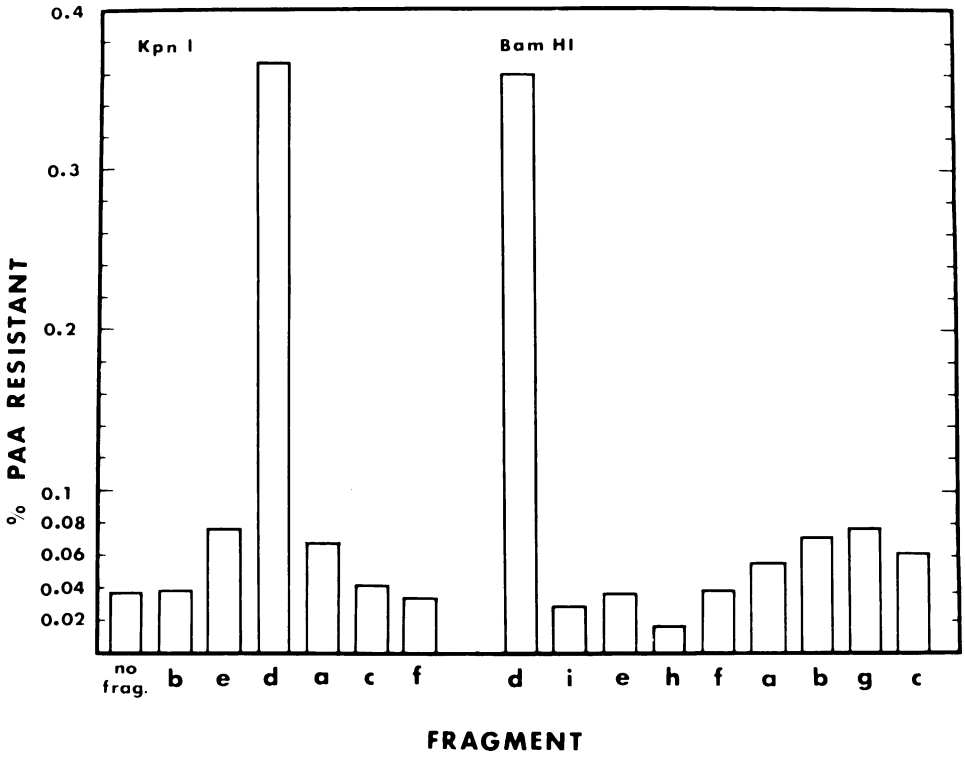

FIG. 2. Marker transfer of PAA resistance with restriction fragments of pPA22. Marker transfer with the *Kpn*I and *Bam*HI restriction fragments of pPA22 depicted in Fig. 1 (lines 8 and 9) was conducted as described in the text. The percentage of the progeny resistant to 1.1 mM PAA resulting from transfections conducted with the indicated fragment is plotted in bar graph form.

the two fragments (coordinates 0.427 to 0.434). Two isolates which exhibited diminished resistance to one, two, or all three drugs were present in both sets of plaque isolates. These isolates were most likely spontaneous PAA-resistant mutants derived during transfection; such mutants could be expected to occur at a frequency of 10 to 20% (compare the frequencies of PAA-resistant progeny with no fragment versus the *d* fragments in Fig. 2). We cannot exclude the possibility, however, that they resulted from separation during marker transfer of two or more mutations within the *Kpn*I *d* and *Bam*HI *d* fragments, thus conferring different degrees of resistance to the various drugs.

Fine mapping of the markers of *ts*D9. (i) Mapping the mutation conferring the *ts* phenotype of *ts*D9. Mutant *ts*D9 is temperature sensitive, PAA resistant, and ACG resistant, but sensitive to araA (1, 9, 26, 43, 44, 49). Its *ts* and PAA resistance markers were mapped by intertypic marker rescue by Chartrand et al. (4) to coordinates 0.421 to 0.440. These sequences overlap the junction between HSV-1 (KOS) *Eco*RI fragments F and M. To determine which *Eco*RI fragment contains the *ts*D9 *ts* mutation, we performed marker rescue experiments by using cloned DNA molecules containing either the *Eco*RI F (pSG18) or M (pSG17) fragments (Fig. 3). Only pSG17 containing the *Eco*RI M fragment rescued *ts*D9 efficiently (Table 4).

As a first step in mapping the mutation of *ts*D9 more finely, the *Kpn*I and *Sal*I restriction sites in pSG17 were determined (Fig. 3). Fragments generated by these enzymes from pSG17 were then isolated from agarose gels and tested for their ability to rescue the *ts* mutation of *ts*D9. The large *Kpn*I *a* fragment containing both ends of *Eco*RI-M and the entire pBR325 vector and the *Sal*I *b* fragment derived from the left-hand end of *Eco*RI-M in the prototype arrangement of HSV DNA (Fig. 3) generated large numbers of ts^+ recombinants when cotransfected with infectious *ts*D9 DNA (Table 4). These data suggested that the *ts*D9 *ts* mutation lies between the leftmost *Eco*RI site and the leftmost *Kpn*I site in *Eco*RI-M. This suggestion was confirmed in a third experiment in which pSG17 DNA was digested with *Eco*RI and *Kpn*I, and the three resulting fragments derived from *Eco*RI-M were tested for their ability to rescue *ts*D9. Only the leftmost 0.8-kbp *Eco*RI-*Kpn*I *d* fragment (Fig. 3) generated substantial numbers of ts^+ recombinants (Table 4), mapping the *ts* mutation of *ts*D9 to this fragment, coordinates 0.422 to 0.427.

(ii) Mapping drug resistance markers of *ts*D9 within the 0.8-kbp *Eco*RI-*Kpn*I fragment. To test whether the same fragment which rescued the *ts*D9 *ts* mutation also converted the *ts*D9 PAA resistance phenotype to PAA sensitivity as predicted by previous coreversion and mapping studies (4, 26, 39, 43, 44), we tested a ts^+ plaque isolate, $D9^+$E/K4, derived by rescue of *ts*D9 with the 0.8-kbp *Eco*RI-*Kpn*I fragment for its sensitivity to PAA. This isolate, $D9^+$E/K4, displayed PAA sensitivity similar to that of the wild-type virus, (50% inhibitory concentration [ED_{50}], 270 μM) (Fig. 4). Similar results were obtained with other ts^+ isolates derived from marker rescue experiments (data not shown). Thus, both the PAA resistance and *ts* markers of *ts*D9 map within the same 0.8-kbp *Eco*RI-*Kpn*I fragment (coordinates 0.422 to 0.427).

Similarly, to test whether a mutation conferring the ACG resistance displayed by *ts*D9 maps within the same 0.8-kbp fragment, we tested $D9^+$E/K4 for sensitivity to ACG. The isolate was more sensitive to ACG than *ts*D9 (ED_{50}s of 12 and 36 μM, respectively) but still substantially more resis-

TABLE 3. Cotransfer of ACG and araA resistance markers of PAA^{r}5 with PAA resistance[a]

Expt	Virus	Efficiency of plating (%) in:		
		1.1 mM PAA	10 μM ACG	150 μM araA
1[b]	PAA^{r}5	77	41	24[c]
	KOS	0.035	0.45	1.3
	Kp1	78	59	17
	Kp2	67	62	14
	Kp3	55	50	23
	Kp4	71	64	19
	Kp5	37	2.1	ND[d]
	Kp6	63	17	5.6
	Kp7	73	35	19
	Kp8	94	38	16
	Kp9	81	32	15
	Kp10	87	36	30
2[e]	PAA^{r}5	71	45	67[c]
	KOS	0.07	0.76	0.70
	Ba1	65	50	45
	Ba2	59	55	67
	Ba3	66	49	28
	Ba4	80	47	33
	Ba5	68	46	41
	Ba6	98	70	37
	Ba7	56	51	50
	Ba8	61	67	63
	Ba9	67	80	38
	Ba10	79	79	38

[a] KOS, PAA^{r}5, and plaque isolates from the marker transfer experiment shown in Fig. 2 were tested for resistance to the indicated concentrations of antiviral drugs.

[b] Plaque isolates derived from marker transfer experiments conducted with the *Kpn*I *d* fragment of pPA22 (Fig. 2).

[c] Variability in the sensitivity of HSV to araA in different assays has been noted by us and by others (9, 37). This variability appears to be due to various levels of adenosine deaminase in cells and serum rather than to variability in the potency of different batches of araA.

[d] ND, Not determined.

[e] Plaque isolates derived from marker transfer experiments conducted with the *Bam*HI *d* fragment of pPA22 (Fig. 2).

tant than KOS (ED_{50} of 0.9 μM) (Fig. 5). Thus, a mutation conferring ACG resistance in *ts*D9 maps within coordinates 0.422 to 0.427 but does not account for all of the ACG resistance of this mutant. It is likely that the remaining ACG resistance is due to the thymidine kinase deficiency of *ts*D9 (12, 49). Regardless, drug resistance markers of *ts*D9 map to a different fragment in the *pol* locus than those of PAA^{r}5 (Fig. 6).

Fine mapping of the markers of *ts*C4. (i) Mapping the *ts* mutation of *ts*C4. The *ts* mutation of *ts*C4 was previously localized in intertypic marker rescue experiments by Chartrand et al. (4) to map coordinates 0.413 to 0.438, a sequence which overlaps the *Eco*RI F-M junction. To determine which *Eco*RI fragment rescued the *ts*C4 *ts* mutation, we performed marker rescue experiments. pSG18, which contains the *Eco*RI F fragment, rescued *ts*C4 efficiently, whereas pSG17, which contains the *Eco*RI M fragment, did not (Fig. 3 and Table 4). The map limits were refined further by experiments in which *ts*C4 was rescued by pKEF-P1, by pKOS29, and by the *Sal*I *c* subfragment of pKOS29 (Fig. 3 and Table 4). In contrast, pSG18-SA, which lies adjacent to the left of this *Sal*I fragment, failed to rescue *ts*C4. These data then map the limits of the *ts*C4 *ts* marker to the 0.3-kbp sequence between the leftmost *Sal*I site in the *Bam*HI Q fragment contained in pKOS29 and the *Eco*RI F-M junction (coordinates 0.420 to 0.422) (Fig. 3).

(ii) *ts*C4 hypersensitivity to PAA and marginal resistance to ACG. *ts*C4 was originally described as exhibiting wild-type PAA sensitivity on the basis of its efficiency of plating in 100 μg of PAA per ml (4, 26, 44). We sought to examine its relative sensitivity to PAA in greater detail by using several concentrations of drug in plaque-reduction assays. Interestingly, as shown in Fig. 4, *ts*C4 was, in fact, more sensitive to PAA than KOS, exhibiting an ED_{50} of 98 μM—a value nearly threefold lower than that for KOS (270 μM). We term this phenotype PAA hypersensitivity (PAA^{hs}).

*ts*C4 has been reported to be ACG resistant, with an ED_{50} nearly 10 times greater than that of KOS (49). We attempted to reproduce this result. However, in six separate plaque-reduction assays directly comparing KOS and *ts*C4, we found that *ts*C4 was only marginally resistant to ACG. Figure 5 shows the average values from the six assays. Interpolation of the data in Fig. 5 yields an average ED_{50} which is only about twofold greater than that for KOS (2 μM versus 0.9 μM). The largest difference in ED_{50}s we observed in any one assay was fourfold, and in one assay we observed no difference. Perhaps the difference between our data and those previously reported (49) is related to alterations in the pool sizes of phosphorylated ACG derivatives in different Vero cell lines upon HSV infection (20).

(iii) *ts*C4 PAA hypersensitivity and *ts* phenotypes apparently due to different mutations. To test whether the same fragments of DNA which rescued the *ts* marker of *ts*C4 yielded drug-sensitive recombinants, we tested three ts^+ plaque isolates derived from marker rescue of *ts*C4. The three isolates exhibited plating efficiencies at 39°C comparable to that of KOS (not shown). One plaque isolate, C4$^+$S1, derived by rescue of *ts*C4 with the *Sal*I *c* subfragment of pKOS29, and one derived by rescue with plasmid pKEF-P1, C4$^+$E1, remained roughly as PAA hypersensitive as *ts*C4 (Fig. 4). Additionally, the third plaque isolate, C4$^+$E2, derived by rescue of *ts*C4 by pKEF-P1, was markedly more PAA hypersensitive than *ts*C4 (Fig. 4). Thus, rescue of the *ts* mutation of *ts*C4 failed to convert the virus to the wild-type level of PAA sensitivity. Therefore, almost certainly, the two phenotypes cannot be due to the same mutation. Nevertheless, the additional hypersensitivity exhibited by isolate C4$^+$E2 (Fig. 4) raises the possibility that sequences contained in pKEF-P1 (i.e., sequences to the left of the *Eco*RI F-M junction) encode amino acids which can affect the recognition of PAA by HSV polymerase.

We also tested the same three plaque isolates for their ACG sensitivity, comparing them to KOS and *ts*C4. In this case, the differences between KOS and *ts*C4 were insufficient (Fig. 5) to consider the behavior of the plaque isolates as resembling that of *ts*C4 more than that of KOS or vice versa (data not shown).

Fine mapping of the mutation and drug sensitivities of *ts*C7. (i) Mapping the *ts* mutation of *ts*C7. Chartrand et al. mapped the *ts* mutation of *ts*C7 by intertypic marker rescue to map coordinates 0.403 to 0.417 (4). The right-hand coordinate of these limits was defined by the junction of the HSV-1 strain KOS *Bgl*II I and D fragments (4). However, this map limit rests on the structure of a single intertypic recombinant which could have become ts^+ via undetected crossovers or nonisogenic effects (discussed in references 5 and 7). Knopf et al. have subsequently reported that a *Bam*HI fragment of defective DNA derived from the nonisogenic HSV-1 strain ANG, corresponding to the strain KOS *Bam*HI Q fragment, rescued *ts*C7 (30), which would narrow the map limits of this

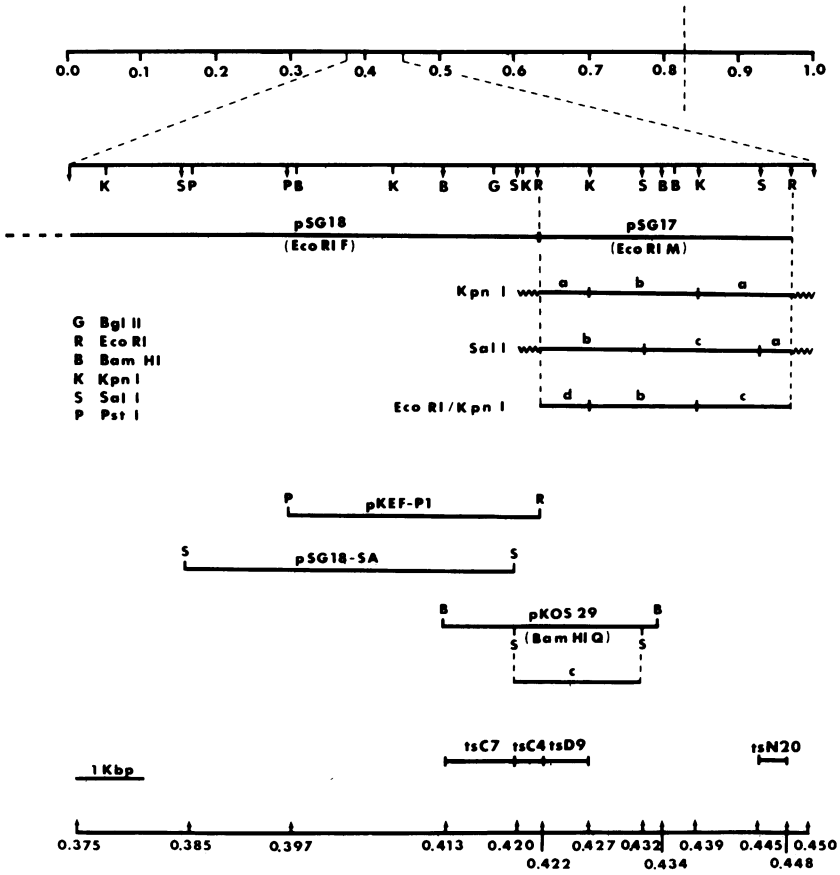

FIG. 3. Recombinant DNA plasmids containing HSV-1 strain KOS DNA and restriction fragments isolated from them. The numbers on the top and bottom lines are physical map coordinates of the HSV-1 strain KOS genome. The second line expands the region from coordinates 0.375 to 0.450 to show the location of restriction sites for enzymes *Bgl*II (G), *Eco*RI (R), *Bam*HI (B), *Kpn*I (K), *Sal*I (S), and *Pst*I (P). In the case of *Pst*I, we have only included sites between 0.375 and 0.422. Arrows on this line correspond to the coordinates presented on the bottom line. The HSV-1 strain KOS DNA insertions in plasmids pSG18, pSG17, pKEF-P1, pSG18-SA, and pKOS29 which overlap the region on the second line are indicated in the lower half of the figure. Below the lines indicating the DNA insertions in pSG17 and pKOS29 are the locations and designation of restriction fragments generated by particular enzymes for these DNAs. Fragments are designated by lowercase letters according to their size. Wavy lines indicate the presence of plasmid pBR325 sequences in fragments *Kpn*I *a*, *Sal*I *a*, and *Sal*I *b* of pSG17. The map location of *ts*C7, *ts*C4, *ts*D9, and *ts*N20 mutations derived from Table 4 are shown at the bottom.

marker to coordinates 0.408 to 0.417. We sought to map the mutation of *ts*C7 by intratypic marker rescue by using the wild-type DNA derived from its parental strain, KOS, to ensure that effects due to nonisogenicity or undetected recombination events would be avoided.

We first established the left-hand map limit for the *ts*C7 *ts* mutation by rescue of *ts*C7 with pKOS-29, which contains the *Bam*HI Q fragment, confirming the preliminary report by Knopf et al. (30) (Fig. 3 and Table 4). The right-hand limit was established by rescue of *ts*C7 with pSG18-SA but not by the *Sal*I *c* subfragment derived from pKOS-29, which had rescued *ts*C4 efficiently (Fig. 3 and Table 4). These data, then, locate the *ts*C7 *ts* mutation between the left-hand *Bam*HI site in the *Bam*HI Q fragment and the right-hand limit of pSG18-SA, a sequence of 1.1 kbp (Fig. 3, map coordinates 0.413 to 0.420).

We were unable to verify or contradict the mapping of the mutation of *ts*C7 to the left of the *Bgl*II I-D junction (0.417) as reported by Chartrand et al. (4), as neither the cloned *Bgl*II I or D fragments contained in plasmids p1BI1 and p1BD1 (33) rescued *ts*C7 in our experiments (data not shown).

(ii) Drug sensitivities of *ts*C7. *ts*C7 has previously been reported to be sensitive to araA and to ACG in plaque reduction assays (8, 49) and to PAA by comparing the efficiencies of plating in 100 μg of PAA per ml for KOS and *ts*C7 (4, 26, 44). We confirmed the wild-type levels of sensitivity of *ts*C7 to ACG and PAA by plaque reduction assays (ED_{50} of PAA for KOS and *ts*C7, 270 μM; ED_{50} of ACG for KOS and *ts*C7, 0.9 μM).

Fine mapping of the *ts* mutation of *ts*N20. Weller et al. (in press) have shown that the *ts* mutation of mutant *ts*N20 maps within the *Eco*RI M fragment, as do several *pol* mutations. Despite this map location, *ts*N20 can be characterized as a non-*pol* mutant in that it synthesizes approximately wild-type amounts of viral DNA and viral DNA polymerase at the nonpermissive temperature and it complements most *pol* mutants efficiently (1, 48; Weller et al., in press). To locate the *ts*N20 mutation more finely relative to the *pol* locus, marker rescue experiments were conducted with fragments

TABLE 4. Marker rescue of *ts*D9, *ts*C4, *ts*C7, and *ts*N20

Plasmid or fragment	Map coordinates	Marker rescue efficiencies[a] with DNA from:			
		*ts*D9[b]	*ts*C4[c]	*ts*C7[c]	*ts*N20[b]
pSG17	0.422–0.448	8.6[d]	<0.001	<0.008	8.5[c]
pSG18	0.315–0.422	0.04	240[d]	0.044[d]	ND[e]
pSG17					
*Kpn*I *a*	0.422–0.427, 0.439–0.448	23[d]	ND	ND	19[d]
*Kpn*I *b*	0.427–0.439	<0.01	ND	ND	<0.1
*Sal*I *a*	0.445–0.448	0.14	ND	ND	16[d]
*Sal*I *b*	0.422–0.432	2.4[d]	ND	ND	<0.03
*Sal*I *c*	0.432–0.445	<0.01	ND	ND	<0.13
*Eco*RI/*Kpn*I *b*	0.427–0.439	<0.02	ND	ND	ND
*Eco*RI/*Kpn*I *c*	0.439–0.448	<0.04	ND	ND	ND
*Eco*RI/*Kpn*I *d*	0.422–0.427	17[d]	ND	ND	ND
pKEF-P1	0.397–0.422	ND	4.6[d]	1.0[d]	ND
pSG18-SA	0.385–0.420	ND	<0.003	0.75[d]	ND
pKOS29	0.413–0.434	ND	10[d]	1.2[d]	ND
pKOS29 *Sal*I *c*	0.420–0.432	ND	0.68[d]	<0.018	ND
None		0.04	<0.006	<0.008	0.008

[a] Marker rescue was conducted with the indicated mutant DNAs, plasmid DNAs, or restriction fragments. Marker rescue efficiencies are expressed as progeny virus titer at 39°C/progeny virus titer at 34°C × 10^3.
[b] Conducted in RK cells.
[c] Average of experiments conducted with RK and Vero cells.
[d] Values considered to be positive.
[e] ND, Not done.

isolated from pSG17, which contains the *Eco*RI M fragment. The *Kpn*I *a* and *Sal*I *a* fragments of pSG17 rescued *ts*N20 efficiently (Fig. 3 and Table 4). These data map the *ts*N20 mutation to the 0.5-kbp *Sal*I-*Eco*RI fragment at the right-hand end of the *Eco*RI M fragment (Fig. 3) (coordinates 0.445 to 0.448). Thus, the *ts*N20 mutation lies between 1.4 and 3.2 kbp to the right of the drug resistance markers of PAA^{r}5 (Fig. 6).

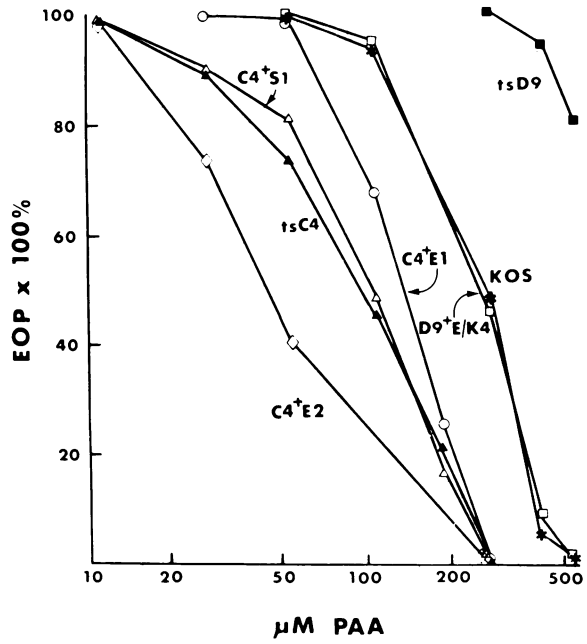

FIG. 4. Effect of various concentrations of PAA on the plating efficiency (EOP) of strain KOS, *ts*C4, *ts*D9, and recombinant viruses generated from them. Virus titers were determined at 34°C by a plaque method (17) under methylcellulose containing the indicated concentrations of PAA. Symbols: ■, *ts*D9; ✱, KOS; □, D9$^+$E/K4; ○, C4$^+$El; △, C4$^+$S1; ▲, *ts*C4; ◇, C4$^+$E2.

DISCUSSION

Limits of the *pol* locus. The data we have presented address two major issues: (i) definition of the limits of the *pol* locus and (ii) separation of *pol* mutations affecting different functional sites of polymerase.

In terms of the first issue, since the drug resistance markers of *pol* mutant PAA^{r}5 map at least 1.2 kbp from the right-hand limit of the *pol* mutation of *ts*C7 (KOS) (Fig. 6) and at least 1.5 kbp from the *ts*C7 mutation as mapped by Chartrand et al. (4), then the *pol* locus must span the 1.2-kbp or greater distance between them (Fig. 6). This 1.2 kbp clearly contains polymerase-coding sequences, since the *pol* mutations of *ts*C4 and *ts*D9 map within this region.

A recent report (13) suggests that the minimum limits of the *pol* locus could be extended about 0.5 kbp further to the right to an HSV type 2 strain HG52 *Bam*HI restriction site. This report provided map limits for a bromovinyldeoxyuridine resistance marker of *pol* mutant, *paa*^{r}-1, extending rightward 2.9 kbp from this HSV type 2 *Bam*HI restriction site. These limits, however, rested on the structure of a single intertypic recombinant. Should this report be confirmed in further genetic and biochemical studies, then it can be stated that at a minimum the *pol* locus must span at least 1.7 kbp between the *ts*C7 mutation and the bromovinyldeoxyuridine resistance marker of *paa*^{r}-1.

The 1.2-kbp distance between the *ts*C7 and PAA^{r}5 mutations which the *pol* locus must span could encode about 40,000 daltons of polypeptide. This represents slighly more than one quarter of the coding capacity required for the putative 150,000-dalton polymerase polypeptide (16, 28, 40); thus, it is likely that the *pol* locus must extend an additional 3.3 kbp to the left of the *ts*C7 mutation or to the right of the PAA^{r}5 markers or both. The non-*pol* *ts* mutation of mutant *ts*N20 maps to a 0.5-kbp *Sal*I-*Eco*RI fragment on the right-hand end of the HSV-1 *Eco*RI M fragment. Thus, we can conclude that the *pol* locus cannot extend the 3.2 kbp from the left most map limit of PAA^{r}5 to the right-hand end of the *Eco*RI M fragment without overlapping the gene defined by *ts*N20.

Weller et al. (52) have recently mapped the *ts* mutations of two non-*pol* mutants, *ts*A16 (KOS) and *ts*A24 (KOS), to the HSV-1 strain KOS *Bam*HI V fragment, the left-hand end of which lies 3.5 kbp from the right most map limit of *ts*C7. Thus, the *pol* locus cannot extend this distance without overlapping the gene defined by these mutants, which appears to encode the major HSV DNA-binding protein (50, 52). From the mapping of *ts*N20, *ts*A16, and *ts*A24, the *pol* locus appears at a maximum to be limited to the 8 kbp between the HSV-1 strain KOS *Bam*HI G-V junction to the HSV-1 strain KOS *Eco*RI M-O junction (Fig. 6).

Recent experiments by L. Holland and M. Levine (personal communication) have identified early 4.3- and 4.2-kilobase (kb) RNAs which span the sequences containing the *pol* mutations mapped here. One end of both these transcripts lies near the *Bam*HI site, which is the left most limit of the *ts*C7 marker (Fig. 6); the other end of both transcripts lies about 0.5 kbp to the right of the right most limit of the PAA^{r}5 markers. These two transcripts are thus good candidates for mRNAs which are translated into polymerase. The mapping of these transcripts and of the *pol* mutations they span suggest precise limits for the *pol* locus.

Holland and Levine (personal communication) have also mapped two late transcripts of 3.0 and 1.4 kb which share sequences with the right end of the *Eco*RI M fragment. Interestingly, of these two, only the 3.0-kb transcript spans the map limits of *ts*N20. Thus, this transcript is a candidate for the mRNA specified by the gene defined by this mutant. These data further suggest that a gene specifying the 1.4-kb transcript which is as yet undefined by mutation may lie between the *pol* locus and the gene defined by *ts*N20.

Mutations affecting different functional sites of polymerase. The mapping of the *ts* and drug resistance mutations of the four *pol* mutants constitutes steps in the identification of the various functional sites of the HSV polymerase. The four mutants exhibit distinct phenotypes with respect to their sensitivities to antiviral drugs. PAA^{r}5 is resistant to PAA, ACG, and araA; *ts*D9 is resistant to PAA and ACG, but not araA; *ts*C4 is hypersensitive to PAA and slightly ACG resistant; whereas *ts*C7 is sensitive to all three drugs. They can thus be expected to specify polymerases which are affected differently in those sites which interact with the drugs—the pyrophosphate exchange-release site and the nucleoside triphosphate binding site.

The three drug resistance markers of PAA^{r}5 map to the same 1.1-kbp region of DNA. All three drug resistance phenotypes may be due to a single mutation; however, some of the data presented in Table 3 could be interpreted to favor the possibility that the different phenotypes are caused by two or more closely linked mutations. Since the PAA^{r}5 markers are contained in a cloned DNA molecule, pPA22, this question can now be addressed by DNA sequencing analysis. This task may be made easier by recent experiments mapping an aphidicolin hypersensitivity mutation of PAA^{r}5 to a 0.8-kbp region within the 1.1-kbp region (8).

Previous studies have strongly supported the idea that the *ts* and PAA resistance phenotypes of *ts*D9 were due to a single mutation (4, 26, 39, 43, 44). The mapping here of both markers to a 0.8-kbp region of DNA lends further support to this concept. The data shown in Fig. 5 indicate that the same *pol* mutation probably confers ACG resistance as well. This would refute our earlier suggestion that *ts*D9 might contain a PAA resistance mutation which does not confer ACG resistance (10). Nevertheless, the *pol* mutation in *ts*D9 certainly affects sugar recognition at the polymerase nucleoside triphosphate-binding site differently than the mutation(s) in PAA^{r}5, as witnessed by the relatively weak ACG resistance and araA sensitivity of *ts*D9 (9).

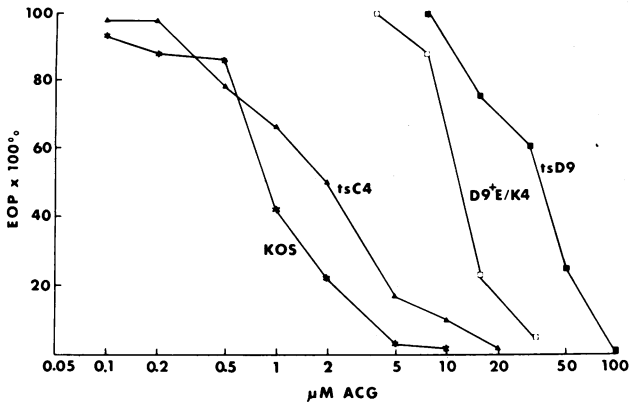

FIG. 5. Effect of various concentrations of ACG on the plating efficiencies (EOPs) of strain KOS, *ts*C4, *ts*D9, and recombinant virus D9$^+$E/K4. Virus titers were determined at 34°C by a plaque method under methylcellulose containing the indicated concentrations of ACG. Symbols: ■, *ts*D9; □, D9$^+$E/K4; ▲, *ts*C4; ✱, KOS.

Mutant *ts*C4 has previously been described as substantially ACG resistant, and it has been suggested that this phenotype is due to a *pol* mutation since *ts*C4 induces substantial levels of viral thymidine kinase (12, 49). Regrettably, the difference in ACG sensitivity between *ts*C4 and KOS which we detected did not permit unequivocal assignment of this phenotype to the *pol* locus. Indeed, *ts*C4 was only marginally ACG resistant such that it is possible that very slight alterations in thymidine kinase consistent with observed levels (12, 49) could account for this phenotype.

Somewhat more surprisingly, *ts*C4 exhibited the novel phenotype of PAA^{hs}. In contrast to the case with *ts*D9, the PAA^{hs} phenotype almost certainly cannot be due to the *ts*C4 *ts* mutation since *ts*$^+$ recombinant viruses generated by marker rescue remain PAA^{hs}. In fact, the PAA^{hs} marker cannot as yet be assigned to the *pol* locus. We think it likely that it will be, however, since all other alterations in PAA sensitivity to date have been due to *pol* mutations (4, 5, 16, 19, 24–26, 29, 39, 43). Nevertheless, the *ts* mutations of both *ts*C4 and *ts*C7 evidently do not themselves lead to altered PAA, ACG, or araA sensitivity.

Although alternate interpretations are available (see below), it is nevertheless tempting to speculate that the distinct locations of the mutations of *ts*D9 and PAA^{r}5 reflect the subdivision of the nucleoside triphosphate-binding site. Specifically, a mutation in *ts*D9 may alter an amino acid within that site without affecting the ability of the polymerase to accept araATP, whereas a PAA^{r}5 mutation might alter an amino acid lying nearby such that it can discriminate between araATP and dATP more stringently. Similarly, it may be that the *ts* mutations of *ts*C4 and *ts*C7, which evidently do not lead to altered PAA, ACG, or araA sensitivity, alter amino acids involved in polymerase assembly or folding or interactions with other proteins or DNA.

The map locations and phenotypes of four finely mapped drug-resistant *pol* mutants, PAA^{r}5 (KOS), *ts*D9 (KOS), paa^r-1 (strain 17), and paa^r-2 (HG52). (4, 5, 12, 13; this work), are shown in Fig. 6. (The map location of paa^r-1 was taken from the original intertypic mapping study [5], which included regions of uncertainty rather than from another study [29] which reported narrower limits.) The map limits of the drug resistance mutations of the four mutants vary and, in the

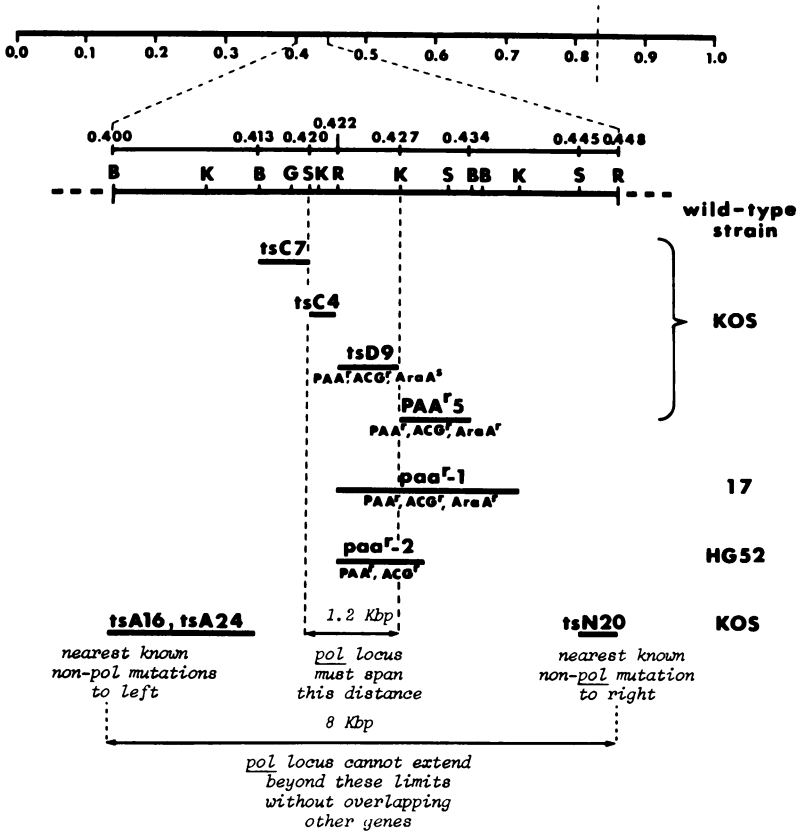

FIG. 6. Map location of selected mutations in or near the *pol* locus. The numbers on the top line are physical map coordinates of HSV-1 strain KOS DNA. The second line depicts the region of KOS DNA between coordinates 0.400 and 0.448, showing the location of restriction sites for *Bam*HI (B), *Kpn*I (K), *Bgl*II (G), *Sal*I (S), and *Eco*RI (R). The numbers denote physical map coordinates. The locations of mutations in mutants *ts*C7, *ts*C4, *ts*D9, PAA^{r}5, and *ts*N20 (this paper); *paa*^{r}-1 and *paa*^{r}-2 (4, 5, 12, 13); and *ts*A16 and *ts*A24 (52) are indicated by lines below. The PAA, ACG, and araA resistance phenotypes of *ts*D9, PAA^{r}5, *paa*^{r}-1, and *paa*^{r}-2 are indicated below the lines showing their map locations (the sensitivity to araA of *paa*^{r}-2 has not been reported). The wild-type strains from which the mutants were derived are indicated to the right. The dashed lines extending from a *Sal*I site and a *Kpn*I site on the top line indicate the right-hand limit of *ts*C7 and left-hand map limit of PAA^{r}5, respectively; the *pol* locus must span this 1.2-kbp distance (see text). *ts*A16 and *ts*A24 are the nearest known non-*pol* mutations to the left of the *pol* locus and *ts*N20 is the nearest to the right. The *pol* locus cannot extend the 8-kbp distance between their outer map limits without overlapping the genes defined by these mutations (see text).

case of PAA^{r}5 and *ts*D9, are separable; nevertheless, it is conceivable that these mutations all lie within a small region of DNA near the *Kpn*I site which separates the PAA^{r}5 and *ts*D9 mutations (Fig. 6). Since the drugs to which the mutants are resistant mimic polymerase substrates, it is possible to propose that this small region "defines an active center of the HSV DNA polymerase enzyme," as has been asserted for a 1.3-kbp region of DNA based on biochemical and genetic analysis of mutant *paa*^{r}-1 (29).

Such a proposal seems premature for several reasons. First, the mapping data summarized in Fig. 6 allow the possibility that the drug resistance markers of *ts*D9 and *paa*^{r}-2 lie as much as 2.5 kbp from those of *paa* ^{r}-1. Second, any particular mutation, including that of *paa*^{r}-1, might alter an amino acid other than those which actually interact with substrates. Instead, the mutation might affect the binding of antiviral drugs indirectly through changes in protein folding.

Third, it seems unlikely that any small, single, uninterrupted DNA sequence would define an active center. Rather, it seems likely that such an active center would be created by folding of portions of the polymerase polypeptide(s) encoded by different regions of the *pol* locus. Indeed, the drug resistance markers of *paa*^{r}-2 and *ts*D9 map outside the 1.3-kbp region of DNA which was stated to define an active center (29).

Further systematic studies of *pol* mutants and the *pol* gene product(s) are required to enable identification, mapping, and subdivision of the catalytic sites of HSV DNA polymerase. The use of molecular cloning for fine mapping and sequence analysis of drug resistance mutations should facilitate these goals.

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