JOURNAL OF VIROLOGY, Aug. 1979, p. 265-276
0022-538X/79/08-0265/12$02.00/0

Vol. 31, No. 2

Physical Mapping of *paa*^{r} Mutations of Herpes Simplex Virus Type 1 and Type 2 by Intertypic Marker Rescue

PIERRE CHARTRAND, NIGEL D. STOW, MORAG C. TIMBURY, AND NEIL M. WILKIE*

Medical Research Council Virology Unit and Department of Virology, University of Glasgow, Glasgow G11 5JR, Scotland

Received for publication 15 January 1979

Mutations (*paa*^{r}) in herpes simplex virus (HSV) which confer resistance to phosphonoacetic acid involve genes associated with virus-induced DNA polymerase activity. Two mutants of HSV (HSV-1 *ts*H and HSV-2 *ts*6) produce a thermolabile DNA polymerase activity. In this study, the *ts* lesions present in these mutants and those present in two independent phosphonoacetic acid-resistant mutants of HSV-1 and HSV-2 (*paa*^{r}-1 and *paa*^{r}-2) have been physically mapped by restriction endonuclease analysis of recombinants produced between HSV-1 and HSV-2 by intertypic marker rescue. All four mutations mapped within a 3.3-kilobase pair region around map unit 40. The accuracy of the method is reflected by the mapping results for *ts*H and *paa*^{r}-2, which were found to lie in the same 1.3-kilobase pair region. *paa*^{r}-1 was found to lie to the right of *ts*6. Virus-induced DNA polymerase is thought to have a molecular weight of 150,000, necessitating a gene with a coding capacity of 4.6 kilobase pairs. The four mutations mapped in this study all lie within a region smaller than this, but the results do not yet prove that all four lesions reside in this or any single gene.

Phosphonoacetic acid (PAA) inhibits herpes simplex virus (HSV) replication in vivo (17), and Mao et al. (12) showed that the drug blocks the activity of virus-induced DNA polymerase in vitro. Mutants of HSV-1 and HSV-2 which are resistant to the drug (PAA^r) have been isolated in several laboratories (6-8), and studies using these mutants have indicated a direct involvement of virus-induced DNA polymerase activity in the mechanism of inhibition.

Hay and Subak-Sharpe (6) first showed that the DNA polymerase activity induced by PAA^r mutants was more resistant to PAA in vitro than that of wild-type HSV. Resistance to increasing concentrations of the drug can be acquired by a multiple-step process (implying several consecutive mutations), and the increased level of resistance in vivo is reflected in the resistance of DNA polymerase activity in vitro (7). Genetic analysis of these consecutive mutations indicated very close linkage on the viral genome. Joffre et al. (8) also reported the inability to segregate the lesions of three independently isolated PAA^r mutants. The most direct evidence that the PAA resistance mutation (*paa*^{r}) occurs in the structural gene of the virus-induced DNA polymerase comes from the analysis of HSV-1 *ts*D9 (8, 20, 21). This temperature-sensitive (*ts*) mutant is resistant to PAA, although it was not selected for that phenotype, and revertants for the *ts* phenotype also revert to PAA sensitivity (PAA^s). The DNA polymerase activity induced by HSV-1 *ts*D9 is thermolabile in vivo (1, 8). Recently, Purifoy et al. (20) have shown that extensively purified DNA polymerase activity of HSV-1 *ts*D9 is both thermolabile and PAA^r, whereas the DNA polymerase activity of a revertant, under the same conditions, is thermostable and PAA^s.

Analysis of intertypic recombinants between HSV-1 and HSV-2 has proved to be a precise method for the physical mapping of HSV genetic markers, including a *paa*^{r} mutation (16) and virus-induced polypeptides (10, 14-16, 19, 24, 29). In particular, intertypic marker rescue has been highly developed as a means of physically mapping genetic loci (24). In this communication we describe the precise physical mapping by intertypic marker rescue of *paa*^{r} mutations of HSV-1 and HSV-2 and two *ts* mutations, *ts*H (HSV-1) and *ts*6 (HSV-2). Mutants carrying the *ts*H and *ts*6 markers have been shown previously to induce thermolabile DNA polymerase activities (5; I. K. Crombie, Ph.D. thesis, University of Glasgow, Glasgow, Scotland, 1975).

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney (BHK-21

C13) cells were grown in Eagle medium supplemented with 10% tryptose phosphate broth and 10% calf serum (11). Cell monolayers (2×10^6 to 4×10^6 cells) in 50-mm-diameter plastic petri dishes (Flow Laboratories, Inc.) were used throughout.

The *ts* mutants of HSV-1 strain 17 (*ts*H) and of HSV-2 strain HG52 (*ts*6) have been described previously (13, 25, 26). The HSV-1 strain 17 (ts^+ paa^r-1) and HSV-2 strain HG52 (ts^+ paa^r-2) mutants were those isolated and described by Hay and Subak-Sharpe (6).

Virus DNA preparation and production of HSV DNA fragments. Virion DNA was prepared from cells infected at 31°C at a multiplicity of infection of 10^{-2} as described by Wilkie(27).

The preparation of restriction endonucleases *Xba*I, *Hin*dIII, *Eco*RI, *Bgl*II, and *Hpa*I and the conditions used for the cleavage of HSV-1 and HSV-2 DNA were as described previously (2, 28).

Intertypic marker rescue and analysis of recombinants. We have previously described in detail the technique for intertypic marker rescue (24). Briefly, BHK cells are coinfected at the nonpermissive temperature with 0.4 μg of intact DNA of a *ts* mutant of one serotype and 0.4 μg of unseparated restriction endonuclease fragments of DNA from ts^+ virus of the other serotype. DNA infections are carried out by the Stow and Wilkie (23) modification of the technique of Graham and Van der Eb (4). ts^+ virus progeny from such crosses are plaque purified three times at 38.5°C, and the parental origin of restriction endonuclease sites in the DNA is determined (19, 24). The rationale behind the analysis of such recombinants is that DNA sequences from the ts^+ parent carrying a ts^+ gene sequence should replace the DNA sequences of the *ts* parent at the site of the *ts* lesion. In the present study, the DNA from the ts^+ parents carried the nonselected marker (paa^r) for resistance to 100 μg of PAA per ml. ts^+ recombinants were tested for the presence of this marker by determining their relative efficiency of plaquing (EOP) in the presence and absence of the drug at 38.5 and 31°C.

The recombinants described in this study were all derived from independent crosses and were therefore known to be clonally unrelated. Recombinants R6-17, R6-18, and R6-19, which were isolated from three well-separated plaques from the same dish and could have been related, nevertheless proved to have different genome structures (see Fig. 7).

Physical maps of HSV-1 and HSV-2. Figure 1 shows the physical maps of HSV-1 (strain 17) and HSV-2 (strain HG52) for the restriction endonucleases *Xba*I, *Hin*dIII, *Eco*RI, *Bgl*II, *Hpa*I, *Kpn*I, and *Bam*HI in the region 30 to 50 map units of the genome (24; A. Davison, personal communication). One map unit is equivalent to 10^6 daltons (1.7 kilobase pairs) and the genome orientation is as described previously (24, 29). Each map indicates the position of the restriction sites and corresponding fragments, above for HSV-1 and below for HSV-2. At the top of Fig. 1 is a summary of all the restriction sites combined from the seven maps. The numbers correspond to the numbers given to each restriction endonuclease, and the sites are aligned with the corresponding ones from each map. The order of these sites for each serotype was deduced by recleavage of individual fragments (3, 28; A. Davison, personal communication). The alignment of HSV-1 restriction sites with respect to HSV-2 restriction sites is the result of DNA/DNA hybridization experiments (N. M. Wilkie and R. Cortini, unpublished data) and from the study of the genome structure of the recombinants presented in this paper. This alignment cannot, therefore, be considered to be completely accurate, although the aligned maps shown in Fig. 1 are consistent with all of the recombinant structures presented herein.

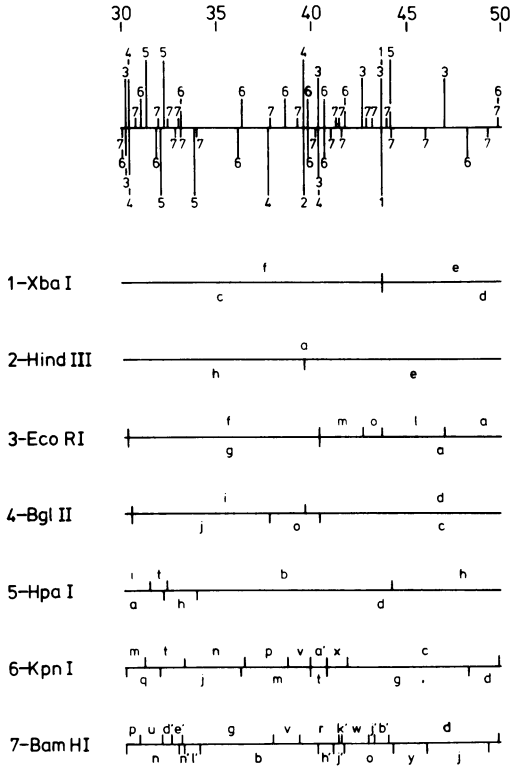

FIG. 1. *Physical maps of HSV-1 (strain 17) and HSV-2 (strain HG52) for seven restriction endonucleases (see text).*

RESULTS

Phenotypic characterization of intertypic recombinants. In marker rescue experiments in which PAA is used as a selection for recombinants, we have found a high background of PAA^r virus, which makes mapping very difficult (N. D. Stow, Ph.D. thesis, University of Glasgow, Glasgow, Scotland, 1978). For this reason, the paa^r mutations were mapped as nonselected markers in recombinants produced by intertypic crosses between intact *ts* PAA^s virus DNA and unseparated restriction fragments of ts^+ PAA^r virus DNA, using ability to grow at 38.5°C as the selective pressure.

Tables 1 and 2 list the 40 recombinants ana-

lyzed in this study, give the restriction endonucleases used to produce fragments of the *ts*$^+$ DNA, and show the relative EOP 38.5°C/31°C and the relative EOP in the presence and absence of PAA for each recombinant and their parents. The relative EOP 38.5°C/31°C values ranged from 0.4 to 5.0, indicating that every recombinant was *ts*$^+$. The relative EOP ± drug values for PAA^r recombinants ranged from 0.2 to 1.7, whereas PAA^s recombinants gave values from 10^{-4} to 10^{-3}. The relative EOP ± drug determinations shown in Tables 1 and 2 were carried out at 31°C, but similar values were obtained at 38.5°C.

Restriction endonuclease mapping of intertypic recombinants. The method of determining the parental origin of DNA sequences in intertypic recombinants by restriction endonuclease analysis has been described previously (19, 24, 29). Three factors must be considered when interpreting the results. First, the presence of DNA from the rescuing fragment will only be detected provided that the restriction endonuclease profile of the putative recombinant genome is altered from that of the parental genome. Second, definition of the length and position of the inserted rescuing sequences depends on the alignment of the physical maps for HSV-1 and HSV-2 DNA (see above). Third, in cases in which the size of the recombinant genome is unaltered and only loss of restriction endonuclease sites can be found, we assume replacement of genomic sequences of the mutant by rescuing DNA sequences at those sites.

TABLE 1. *Intertypic recombinants: R6 series*[a]

Recombinant	*ts*$^+$ DNA fragments produced by:	Titer at 31°C (PFU/ml)	Relative EOP 38.5°C/31°C	Relative EOP ± drug[b]
R6-3	*Hin*dIII	1.0×10^9	0.5	0.9
R6-4	*Hin*dIII	1.9×10^9	0.9	0.5
R6-5	*Hin*dIII	1.3×10^9	1.2	1.6
R6-6	*Hin*dIII	9.4×10^8	1.1	1.7
R6-7	*Hin*dIII	5.0×10^8	1.2	1.4
R6-8	*Xba*I	9.9×10^8	1.3	1.3
R6-9	*Xba*I	5.7×10^8	0.8	1.1
R6-10	*Xba*I	1.9×10^9	0.8	0.8
R6-11	*Xba*I	5.9×10^8	1.1	1.0
R6-13	*Hpa*I	6.6×10^8	1.0	1.7
R6-14	*Hpa*I	6.7×10^8	0.6	0.4×10^{-3}
R6-15	*Hpa*I	1.4×10^9	0.6	1.1
R6-17	*Eco*RI	1.1×10^9	1.0	$<10^{-4}$
R6-18	*Eco*RI	2.6×10^8	0.5	0.7
R6-19	*Eco*RI	7.7×10^8	0.5	0.5
R6-20	*Eco*RI	1.1×10^9	0.9	0.5
R6-21	*Eco*RI	8.3×10^8	0.7	0.2×10^{-3}
R6-22	*Eco*RI	1.0×10^9	1.1	0.4×10^{-3}
R6-23	*Eco*RI	6.3×10^8	1.3	0.7×10^{-3}
R6-24	*Eco*RI	9.6×10^8	0.4	0.3
R6-26	*Bgl*II	6.2×10^8	1.2	0.4×10^{-4}
R6-27	*Bgl*II	1.2×10^9	0.8	0.2×10^{-3}
R6-28	*Bgl*II	1.0×10^9	1.3	1.3
R6-29	*Bgl*II	7.6×10^8	0.6	0.9
R6-30	*Bgl*II	3.8×10^8	1.2	1.1×10^{-3}
R6-31	*Bgl*II	6.4×10^8	0.8	1.1
R6-32	*Bgl*II	1.2×10^9	0.5	0.5
R6-33	*Bgl*II	9.0×10^8	1.1	1.0
R6-34	*Bgl*II	6.6×10^8	1.7	0.6
R6-35	*Bgl*II	1.4×10^9	0.6	0.7
HSV-2 (*ts*6 *paa*^{s})		2.8×10^8	$<10^{-5}$	1.1×10^{-4}
HSV-1 (*ts*$^+$ *paa*^{r}-1)		8.6×10^8	1.1	0.8

[a] DNA Cross: HSV-1 fragments (*ts*$^+$ *paa*^{r}-1) × HSV-2 (*ts*6 *paa*^{s}); selection: *ts*$^+$ (ability to grow at 38.5°C).
[b] EOP in the presence (100 μg/ml) and absence of PAA at 31°C; similar results at 38.5°C.

TABLE 2. *Intertypic recombinants: RH series*[a]

Recombinant	*ts*$^+$ DNA fragments produced by:	Titer at 31°C (PFU/ml)	Relative EOP 38.5°C/31°C	Relative EOP ± drug[b]
RH-11	*Hin*dIII	3.2×10^8	5.0	0.4
RH-12	*Hin*dIII	2.5×10^8	1.8	0.5
RH-13	*Hin*dIII	2.7×10^8	3.3	0.4
RH-14	*Hin*dIII	4.2×10^8	2.4	0.8
RH-21	*Hpa*I	5.2×10^8	2.5	0.2
RH-22	*Hpa*I	1.2×10^8	3.9	0.2
RH-23	*Hpa*I	4.9×10^7	2.2	0.4
RH-24	*Hpa*I	9.6×10^7	2.5	0.2
RH-31	*Eco*RI	1.6×10^7	3.8	0.3
RH-32	*Eco*RI	9.7×10^6	3.3	0.3
HSV-1 (*ts*H *paa*^{s})		1.6×10^7	1.3×10^{-4}	$<10^{-4}$
HSV-2 (*ts*$^+$ *paa*^{r}-2)		2.0×10^5	5	0.5

[a] DNA cross: HSV-1 (*ts*H *paa*^{s}) × HSV-2 fragments (*ts*$^+$ *paa*^{r}-2); selection: *ts*$^+$ (ability to grow at 38.5°C).
[b] EOP in the presence (100 μg/ml) and absence of PAA at 31°C; similar results at 38.5°C.

Figures 2, 3, 4, and 5 show the restriction endonuclease fragment patterns of recombinants in the RH and R6 series. The RH series was isolated after the rescue of HSV-1 *ts*H DNA with DNA fragments of HSV-2 (*ts*$^+$ *paa*^{r}-2), and the R6 series was isolated after the rescue of HSV-2 *ts*6 DNA with DNA fragments of HSV-1 (*ts*$^+$ *paa*^{r}-1). In the interest of brevity, only those key recombinants which define the limits of mapping are shown. The analysis is presented by discussing the restriction enzyme maps of the R6 and RH groups enzyme by enzyme, to show that the same limited region of the genome is affected in each case. Changes in restriction enzyme sites outside this region will not be discussed in detail. To aid the analysis, open circles indicate the loss of fragments compared with the *ts* parents, and solid squares indicate the appearance of new fragments not present in the *ts* parents. Restriction sites will be designated by the two letters that identify the fragment on either side (Fig. 1).

From the data shown in Fig. 2, it can be deduced that R6-9, R6-14, and R6-30 have lost the HSV-2 *Hin*dIII *h-e* site. This produces a fusion fragment which migrates slightly slower than HSV-2 *Hin*dIII *a*. R6-26 shows no change

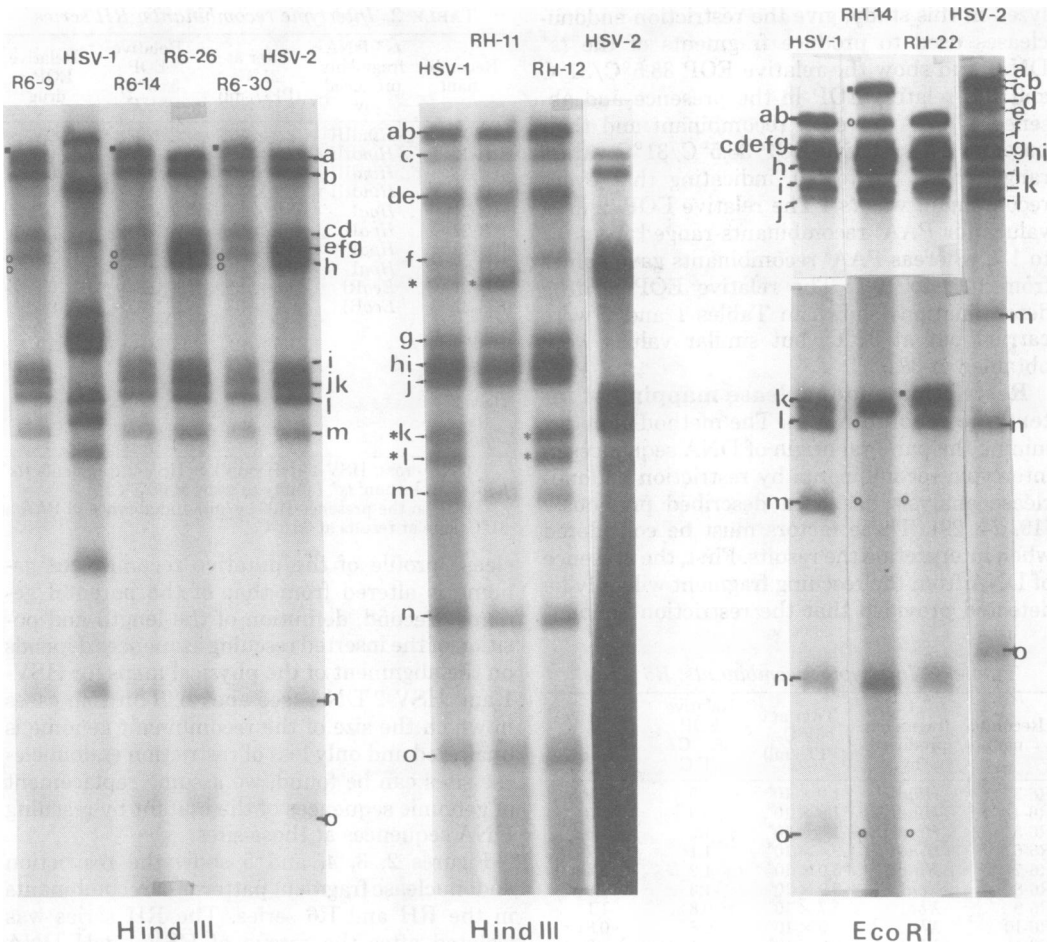

FIG. 2. *Autoradiographs of ^{32}P-labeled DNA of intertypic recombinants and their parents digested with HindIII and EcoRI. The EcoRI gel is a composite of different exposures of the same gel. The parental patterns are from infections with the actual parents used to generate the recombinants, i.e., HSV-1 (ts$^+$ paa^r-1) and HSV-2 (ts6 paa^s) for the R6 series and HSV-2 (ts$^+$ paa^r-2) and HSV-1 (tsH paa^s) for the RH series. DNA fragments are lettered as previously (24). Designations: ■, new fragments found in recombinants which are not present in the ts parent; ○, fragments found in ts parents which are missing in recombinants; *, fragments due to segregation of mixtures present in the parental stock of HSV-1 tsH.*

compared with HSV-2 *ts*6. The stock of HSV-1 *ts*H used consists of a mixed population in which approximately half the molecules have lost the *Hin*dIII *k-l* site to generate a fusion band which migrates faster than *Hin*dIII *f* (bands designated with an asterisk). Cloned recombinants have segregated into one or the other of these forms, and RH-11 and RH-12 show no other change in their *Hin*dIII pattern compared with the HSV-1 *ts*H parent. The *Eco*RI analyses of RH-14 and RH-22 show the loss of the HSV-1 *Eco*RI *m-o* site, giving rise to larger fragments.

Figure 3 shows the analysis with *Bgl*II. R6-9, R6-14, and R6-26 share the loss of the HSV-2 *Bgl*II *o* fragment. R6-14 has also lost the HSV-2 *Bgl*II *j* fragment, but this is not the case for R6-9 and R6-26. It therefore follows that R6-9 and R6-26 have lost the HSV-2 *Bgl*II *o-c* site. R6-30 has a profile similar to R6-9 (data not shown). RH-11 and RH-12 show the reverse situation, having acquired the HSV-2 *Bgl*II *o-c* site and consequently a smaller fragment replacing the normal HSV-1 *Bgl*II *d* fragment. Although not clear on the gel shown, RH-14 has the same *Bgl*II restriction profile as RH-11 and RH-12. RH-22 has lost the HSV-1 *Bgl*II *i* fragment and has acquired two new fragments with the mobility of HSV-2 *Bgl*II *j* and *o*.

The *Kpn*I analysis in Fig. 4 shows that R6-9, R6-14, and R6-30 have all acquired a new fragment with the mobility of HSV-1 *Kpn*I *v*. These recombinants have also acquired a fragment

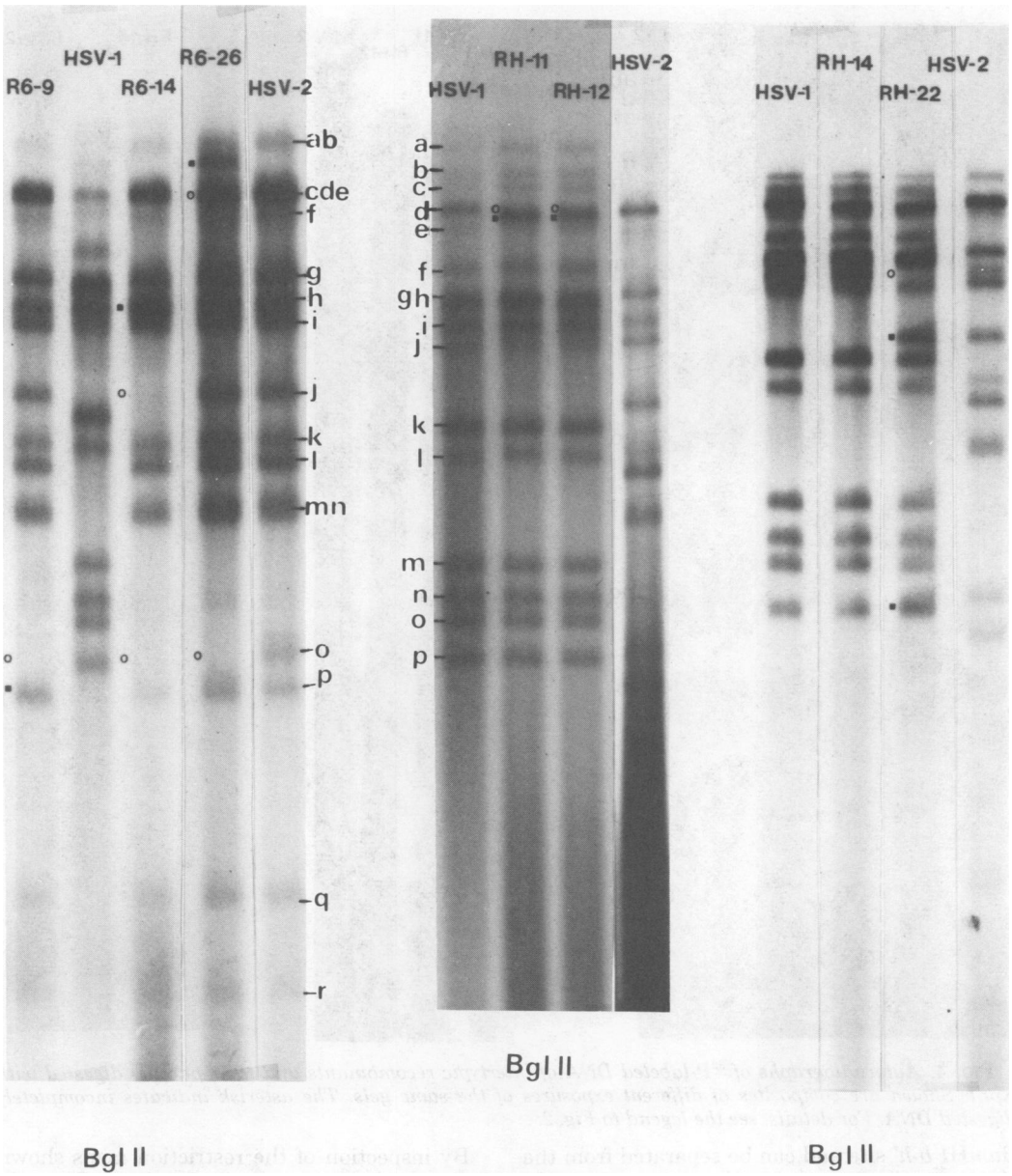

FIG. 3. *Autoradiographs of* ^{32}*P-labeled DNA of intertypic recombinants and their parents digested with BglII. For details, see the legend to Fig. 2.*

which has the mobility of HSV-1 *Kpn*I *p* in the case of R6-14 and a slightly lower mobility in the cases of R6-9 and R6-30. This is because all three recombinants have acquired the HSV-1 *Kpn*I *p-v* site, but only R6-14 has also acquired the HSV-1 *Kpn*I *n-p* site. R6-26, RH-11, and RH-12 show no change compared with their *ts* parents. The important feature in RH-14 and RH-22 is the acquisition of a new fragment which migrates slower than HSV-1 *Kpn*I *abcd* due to the loss of the HSV-1 *Kpn*I *x-c* site.

The analysis with *Bam*HI is shown in Fig. 5; in this case the structure of the recombinant which carries the nonselected *paa*^{r}-1 marker (R6-9) and those which are sensitive to PAA (R6-14, R6-26, and R6-30) can be compared. All of these recombinants have lost HSV-2 *Bam*HI *h'*, but only R6-9 has also lost the adjoining *j'* fragment. The three PAA^s recombinants have therefore retained the HSV-2 *Bam*HI *h'-j'* site but lost the *b-h'* site. It follows that the *paa*^{r}-1 mutation must lie to the right of the HSV-2

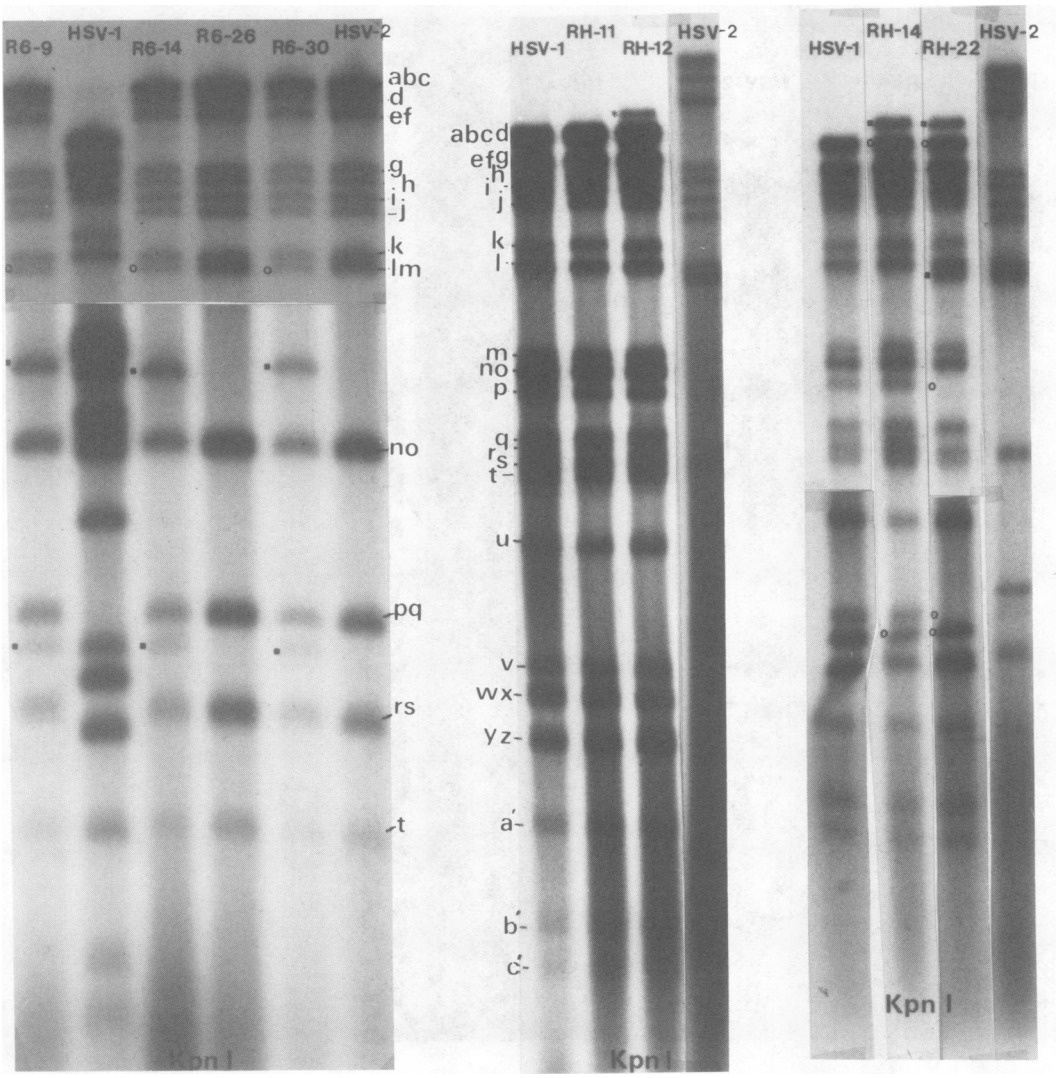

FIG. 4. *Autoradiographs of ^{32}P-labeled DNA of intertypic recombinants and their parents digested with KpnI. Shown are composites of different exposures of the same gels. The asterisk indicates incompletely digested DNA. For details, see the legend to Fig. 2.*

*Bam*HI *b-h'* site and can be separated from the HSV-1 *ts*$^+$ counterpart of the *ts*6 mutation. The same region is involved in the RH recombinants. RH-11 and RH-12 both have lost HSV-1 *Bam*HI *r* (difficult to see since *r* comigrates with *q* in this HSV-1 stock), and a new band which migrates slower than HSV-1 *Bam*HI *b'* has appeared due to the acquisition of the HSV-2 *Bam*HI *b-h'* site. Furthermore, RH-12 contains a new fragment which comigrates with HSV-2 *Bam*HI *h'*, and the insertion of HSV-2 DNA in RH-12 therefore includes the HSV-2 *Bam*HI *h'-j'* site. RH-14 and RH-22 show changes in the same region, but in addition have acquired longer segments of the HSV-2 genome.

By inspection of the restriction maps shown in Fig. 1, it can be concluded that all of the changes in restriction sites found in the genomes of R6-9, R6-14, R6-26, R6-30, RH-11, RH-12, RH-14, and RH-22 are totally consistent with the genome structures shown in Fig. 6, 7, and 8. The genome structures of the other recombinants were deduced in the same way.

Analysis of intertypic recombinants. The genome structures indicating the parental origin of the restriction endonuclease sites for seven enzymes for 35 recombinants are shown in Fig. 6, 7, and 8. Figure 6 shows recombinants isolated from cells coinfected with intact HSV-2 (*ts*6 *paa*^{s}) and unseparated *Hin*dII, *Xba*I, or *Hpa*I

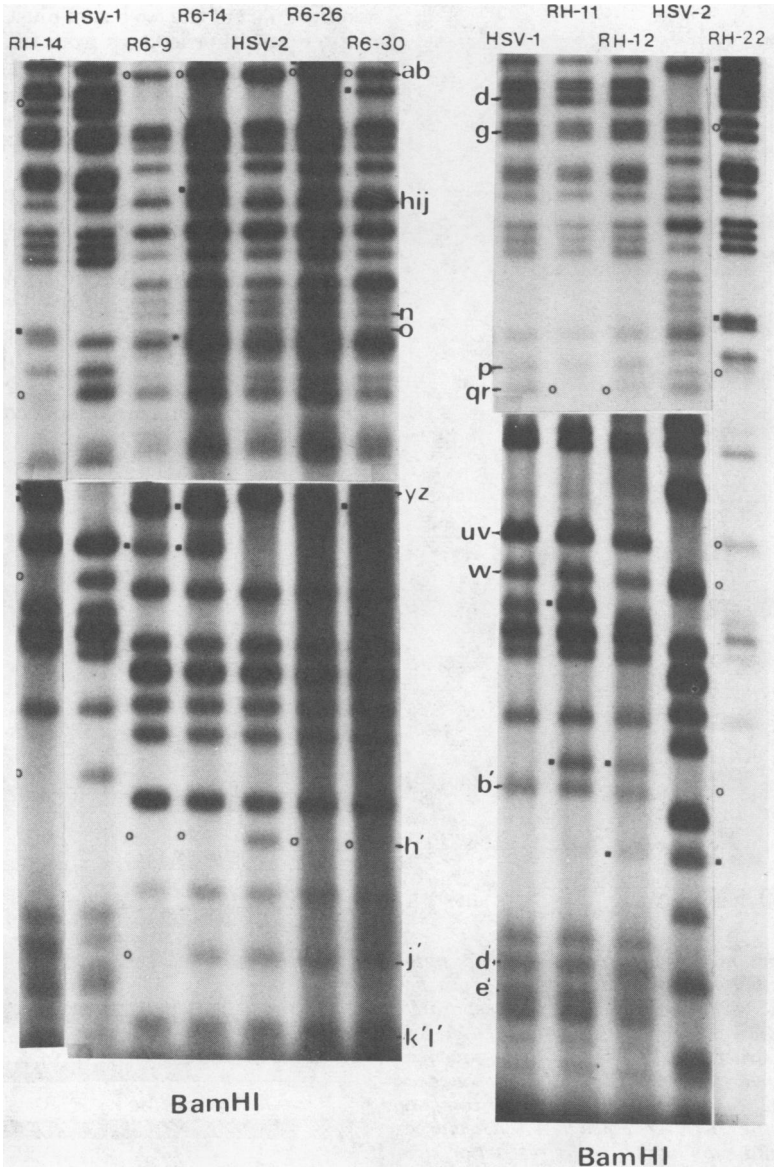

FIG. 5. *Autoradiographs of* ^{32}*P-labeled DNA of intertypic recombinants and their parents digested with BamHI. Shown are composites of different gels. DNA fragments identified by A. Davison (personal communication). For details, see the legend to Fig. 2.*

fragments of HSV-1 (*ts*$^+$ *paa*^{r}-1) DNA. All of the recombinants contain HSV-1 DNA sequences in the region between map units 38 and 41. Eleven of the 12 recombinants are phenotypically PAA^r, indicating strong linkage between *paa*^{r}-1 and the *ts*$^+$ (HSV-1) counterpart of *ts*6 (HSV-2). Recombinant R6-14 is PAA^s and, assuming that it is not simply a PAA^s revertant of the *paa*^{r}-1 (HSV-1) mutation, in this recombinant *paa*^{r}-1 and the *ts*$^+$ (HSV-1) counterpart of *ts*6 (HSV-2) have been split. When the genome structure of R6-14 is compared with those of the other recombinants shown in Fig. 6, it can be concluded that the *paa*^{r}-1 lesion must lie to the right of the *ts*$^+$ (HSV-1) counterpart of *ts*6 (HSV-2).

In an attempt to obtain further recombinants which split these sites, a second marker rescue experiment was carried out using either *Eco*RI or *Bgl*II fragments of HSV-1 (*ts*$^+$ paa^r-1) DNA. *Eco*RI and *Bgl*II have sites within the region of HSV-1 DNA between map units 38 and 41 (enzymes 3 and 4, respectively, in Fig. 1). The

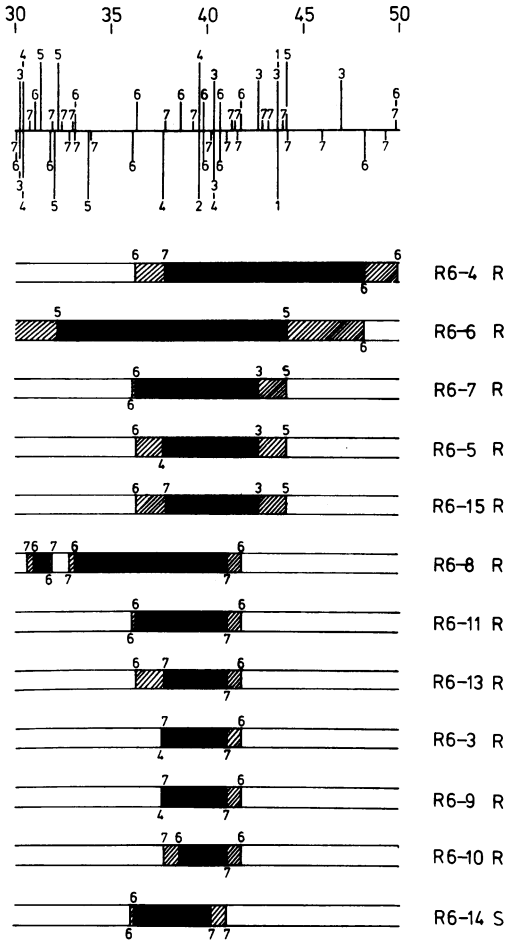

FIG. 6. *Summary of the genome structure of independent recombinants isolated from the cross between intact HSV-2 (ts6 paa^s) DNA and unseparated restriction fragments of HSV-1 (ts$^+$ paa^r-1) DNA. For each genome structure, the HSV-2 DNA sequences are shown in white and the HSV-1 DNA sequences are shown in black, with the region of uncertainty of the position of the crossover shown as a hatched area. The numbers indicate the delimiting restriction sites (above for type 1 and below for type 2). The code for these numbers is the same as in Fig. 1. R indicates PAA^r, and S indicates PAA^s.*

genome structures of recombinants obtained from this experiment are shown in Fig. 7. Recombinants R6-18, R6-19, R6-20, and R6-24 were rescued with HSV-1 *Eco*RI fragments and are all PAA^r. However, in each case the inserted HSV-1 DNA sequences span the HSV-1 *Eco*RI site at approximately map unit 40. The results indicate a strong selection for recombination events involving HSV-1 DNA fragments which retained this *Eco*RI site ("partials" or annealed ends) and further suggest that the ts$^+$ (HSV-1) counterpart of the *ts6* lesion must map near that site. Four other isolates from this cross (R6-17, R6-21, R6-22 and R6-23 [Table 1]) were phenotypically ts$^+$ and PAA^s but contained only HSV-2 restriction endonuclease sites with the seven enzymes used. In this case they were probably ts$^+$ revertants of HSV-2 *ts6*, or they contained undetected insertions of HSV-1 DNA.

Recombinants R6-26, R6-27, R6-28, R6-29, R6-30, R6-31, R6-32, R6-33, R6-34, and R6-35 were rescued with HSV-1 *Bgl*II fragments (Fig. 7). Only one isolate (R6-27, Table 1) had neither detectable HSV-1 restriction sites present nor evidence of HSV-2 sites missing; all of the others contained overlapping HSV-1 DNA sequences. Seven of the nine recombinants contained HSV-

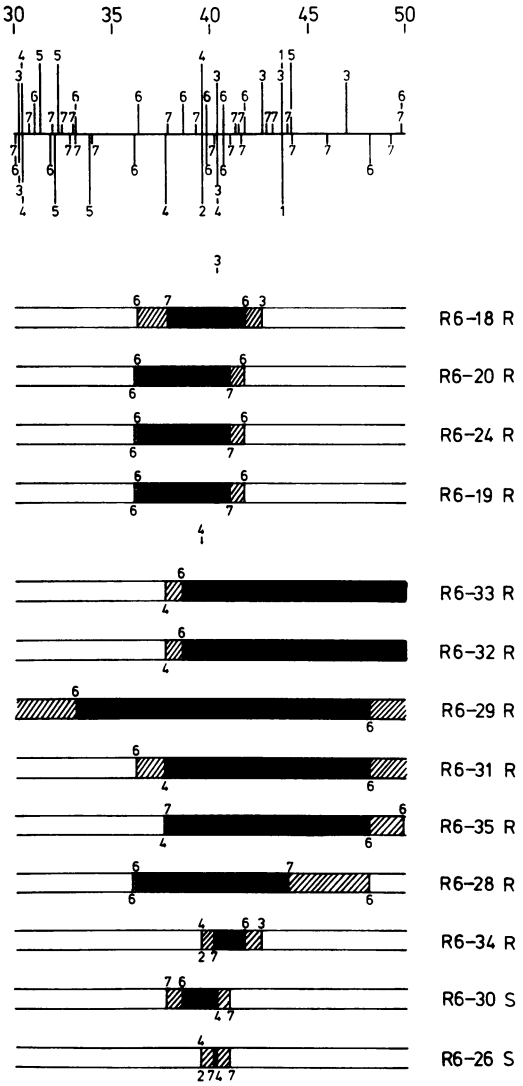

FIG. 7. *See legend to Fig. 6.*

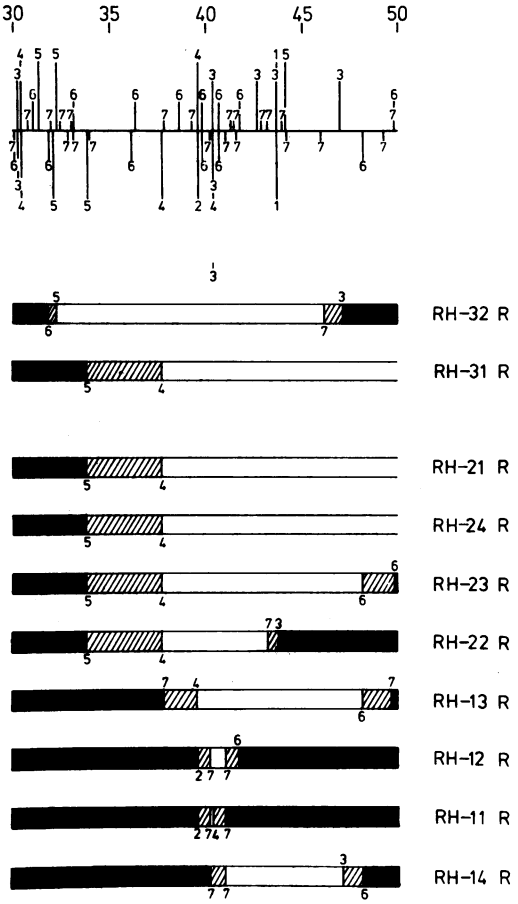

FIG. 8. *Summary of the genome structure of independent recombinants isolated from the cross between intact HSV-1 (tsH paa^s) DNA and unseparated restriction fragments of HSV-2 (ts$^+$ paa^r-2) DNA. For details, see the legend to Fig. 6.*

1 DNA sequences which spanned the *Bgl*II site near map unit 40, which suggests that the *ts*$^+$ (HSV-1) counterpart of the *ts*6 lesion also maps near this site. Two recombinants (R6-26 and R6-34) and HSV-1 DNA sequences which did not contain this site, showing that the *ts*$^+$ (HSV-1) counterpart of the *ts*6 lesion must map to the right of it. Two recombinants (R6-26 and R6-30) were phenotypically PAA^s and, unless reversion of the *paa*^{r}-1 mutation is in each case the explanation, these recombinants have separated the *paa*^{r}-1 site and the *ts*$^+$ (HSV-1) counterpart of the *ts*6 lesion. The genome structures of these PAA^s recombinants compared with the PAA^r recombinants show that *paa*^{r}-1 must lie to the right, at a similar position obtained by comparing R6-14 (PAA^s) with the other PAA^r recombinants.

The reciprocal experiment was then carried out using the mutant HSV-1 *ts*H (HSV-1 *ts*H does not complement HSV-2 *ts*6; L. I. Messer, Ph.D. thesis, University of Glasgow, Glasgow, Scotland, 1978). Recombinants were isolated from cells coinfected with intact DNA from HSV-1 (*ts*H *paa*^{s}) and restriction endonuclease fragments of HSV-2 (*ts*$^+$ *paa*^{r}-2) DNA. The genome structures of these recombinants are shown in Fig. 8. All of the recombinants are PAA^r, indicating strong linkage between *paa*^{r}-2 and the *ts*$^+$ (HSV-2) counterpart of the *ts*H lesion. Two recombinants (RH-31 and RH-32) were rescued with HSV-2 *Eco*RI fragments but nevertheless contained the HSV-2 *Eco*RI site near map unit 40, suggesting that the *ts*$^+$ (HSV-2) counterpart of *ts*H maps near this site. When the uncertainty in the location of the crossover points is taken into account, all of the recombinants of the RH series had HSV-2 DNA sequences in common within the HSV-2 *Bam*HI *h'* fragment.

Summary of analysis. In the analysis shown in Fig. 6, 7, and 8, we have mapped the HSV-1 DNA sequences (or HSV-2 DNA sequences) which correct the *ts* lesion in HSV-2 *ts*6 (or HSV-1 *ts*H). We assume that the correction mechanism usually involves the replacement of defective sequences in the *ts* parent by the *ts*$^+$ counterpart from the other serotype. Other explanations are possible, but if the assumption is correct, the map location of the *ts*6 mutation is defined by the HSV-1 DNA sequences which the R6 series of recombinants have in common. Inspection of the data shown in Fig. 6 and 7 indicates that this region is defined on the left by the HSV-2 *Hin*dIII *h-e* site or the HSV-1 *Bgl*II *i-d* site (R6-26 and R6-34) and on the right by the HSV-2 *Bam*HI *h'*-j' site (R6-14, R6-26, and R6-30; see Fig. 6, 7, and 1). The region of the genome containing the *ts*H lesion can be derived in the same way by inspection of the data for the RH series (Fig. 8) and is defined by the HSV-2 *Bam*HI *h'* fragment, which is a fragment of 1.3 kilobase pairs. These data are summarized in Fig. 9; note that the limits shown take into account the uncertainty in the location of the crossover points.

Figure 9 also summarizes the maximum map coordinates for the HSV-1 (*paa*^{r}-1) and the HSV-2 (*paa*^{r}-2) *paa*^{r} mutations. All of the RH series of recombinants acquired PAA^r and presumably contained the *paa*^{r}-2 mutation. Thus, the map coordinates which define the *paa*^{r}-2 mutation are the same as those for the *ts*H lesion. Three R6 recombinants were PAA^s, and therefore the *paa*^{r}-1 mutation was split from the *ts*$^+$ (HSV-1) counterpart of *ts*6. Comparison of the genome structure of R6-14, R6-26, and R6-

30 with the remaining recombinants in the R6 series shows that *paa*^{r}-1 must map at the right of *ts*6 between the coordinates shown in Fig. 9. These are defined on the left by the HSV-2 *Bgl*II *o-c* site and on the right by the HSV-1 *Kpn*I *x-c* site (see Fig. 6, 7, and 1).

DISCUSSION

This communication describes the detailed physical mapping of mutations (*ts*H and *ts*6) present in *ts* mutants of HSV-1 and HSV-2 which induce thermolabile DNA polymerase activity (5; Crombie, Ph.D. thesis) and those (*paa*^{r}-1 and *paa*^{r}-2) present in two mutants resistant to PAA. Both phenotypes are consistent with lesions in genes which affect viral DNA polymerase activity in some way. The method of mapping involved genetic analysis of intertypic recombinants combined with restriction endonuclease mapping of their DNA. The rationale behind such an approach has been stated previously (19, 24; this paper), but it is worthwhile to consider in more detail what is actually being mapped in such experiments. The interpretation of mapping results for *ts* lesions is based on the assumption that rescuing DNA sequences contain the *ts*$^+$ counterpart of the *ts* mutation and that they replace sequences at the site of the mutation. This interpretation would be invalid if "second site" rearrangements resulting in inter- or intragenic suppression were commonly responsible for the observed phenotypes of the recombinants. The recombinants isolated in this study show a wide range in the length of rescuing DNA sequences, which argues against crossovers which result in intragenic suppression of the mutant phenotype. Another general drawback to the mapping rationale would be in cases in which heterozygosity is possible. In this case a *ts*$^+$ phenotype could be acquired without loss of the gene coding for the *ts* phenotype. In the present work, the reciprocal mapping of HSV-1 and HSV-2 mutations which lie in the same complementation group (*ts*H and *ts*6 [Messer, Ph.D. thesis]) puts both mutations in a region of DNA smaller than that thought necessary to code for the expected protein (DNA polymerase [see below]). Since intratypic marker rescue experiments using isogenic material also put both mutations into the same region (P. Chartrand, N. M. Wilkie, and M. C. Timbury, manuscript in preparation; Stow, Ph.D. thesis), this reduces the likelihood that heterozygosity is a problem for this locus. Interpretation of the mapping of *paa*^{r} (the nonselected marker) depends only on the assumption that inserted DNA sequences contain the *paa*^{r} mutations.

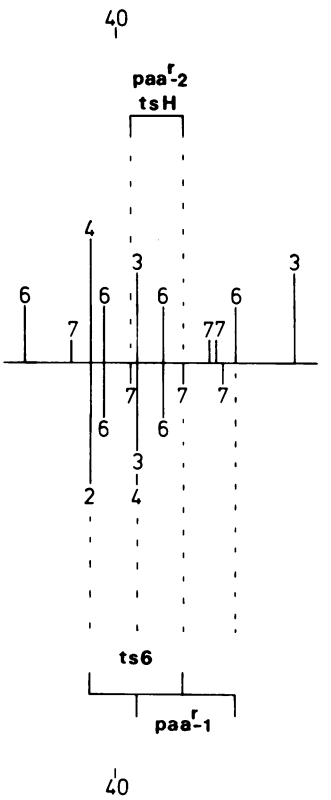

FIG. 9. *Summary of the mapping data for tsH, ts6, paa*^{r}*-1, and paa*^{r}*-2. The region of the genome shown is an enlargement of the 39- to 43-map unit region shown in Fig. 1.*

Given that the assumptions are correct, the mapping studies with *ts*H and *ts*6 delimit the location for both the *ts* lesions. The clones which contain inserted HSV-1 DNA sequences allow mapping of the *paa*^{r}-1 (HSV-1) mutation, and those with HSV-2 DNA insertions allow the mapping of the *paa*^{r}-2 (HSV-2) mutation. From Fig. 9 it can be seen that all four mutations map in one region of HSV DNA, which spans approximately 2×10^6 daltons (3.3 kilobase pairs). When intratypic marker rescue (isogenic system) was used, the mapping of *ts*H and *ts*6 mutations gave very similar results (Chartrand et al., manuscript in preparation; Stow, Ph.D. thesis).

This is the first report of the simultaneous and independent mapping of an identifiable function of HSV-1 and HSV-2 (PAA^r), and the results strongly suggest closely similar map locations. The uncertainty in the fine alignment of the HSV-1 and HSV-2 physical maps may obviously affect the interpretation, but it should be

noted that the map locations for the mutations *ts*H, *ts*6, and *paa*^{r}-2 are defined by restriction endonuclease sites in the HSV-2 physical map.

Recent studies utilizing HSV-1/HSV-2 intertypic recombinants have permitted the mapping of several HSV-induced polypeptides (14, 16). The order of polypeptides in the region under study (14) was found to be V_{mw} 117 (glycoprotein), V_{mw} 136'(130) (DNA binding protein), and V_{mw} 28. In the Marsden et al. (14) study, five intertypic recombinants isolated after rescue of HSV-1 *ts*H were analyzed. The only polypeptide which never segregated from the rescuing DNA sequences was the V_{mw} 136'(130) DNA binding protein. This probably excludes the genes for the other polypeptides from containing the site for the *ts*H mutation, but it does not prove that the gene for the V_{mw} 136'(130) protein does contain the site for the *ts*H lesion. Purified viral DNA polymerase has been reported to have a molecular weight of 150,000 (18), but it has not yet been assigned a map locus.

Since mutants carrying the *ts*H, *ts*6, *paa*^{r}-1, or *paa*^{r}-2 mutation show phenotypic properties compatible with lesions in virus-induced DNA polymerase activity (5, 6; Crombie, Ph.D. thesis), the question arises whether the four lesions are in the same gene. *ts*H and *ts*6 do not complement each other (Messer, Ph.D. thesis) and, provided unforeseen complications do not arise in intertypic complementation tests, these mutations are likely to reside in the same gene. The *paa*^{r}-1 and *paa*^{r}-2 mutations lie very near the *ts*H and *ts*6 lesions. The coding capacity needed for the 150,000-molecular-weight polypeptide which Powell and Purifoy (18) found in a purified preparation of HSV DNA polymerase is approximately 4.6 kilobase pairs. The maximum mapping limits (3.3 kilobase pairs; Fig. 9) are therefore consistent with all four mutations lying in the structural gene of the viral DNA polymerase, but do not prove that hypothesis. A *paa*^{r} mutation has recently been mapped (9), using intratypic marker rescue, and reported to lie to the right of the HSV-1 *Hpa*I *b-h* site (map unit 45; see Fig. 1). This is at least 3.8 kilobase pairs to the right of the *paa*^{r} lesions reported in this study. This raises the possibility that two independent genes can carry mutations which result in a PAA^r phenotype. If the PAA^r phenotype is the result of modifications in the viral DNA polymerase, this may imply two loci which are involved in DNA polymerase activity. In this respect it is interesting to note that two *ts* mutations (*ts*C4 and *ts*D9) which lie in two different HSV-1 complementation groups (22) induce a viral DNA polymerase activity which, when extensively purified, is thermolabile in vitro (20; K. L. Powell and D. J. M. Purifoy, personal communication). The *ts*D9 mutation, which also results in a PAA^r phenotype (see above), has been found to map between 0.30 and 0.45 (fractional length) (D. S. Parris and P. A. Schaffer, personal communication). Although this is consistent with our mapping data, more precise physical mapping and further complementation tests will be required to resolve the precise relationship of the different *paa*^{r} mutations and the *ts*H, *ts*6, *ts*D9, and *ts*C4 lesions.

ACKNOWLEDGMENTS

We thank M. Murphy and C. Adair for cheerful and expert assistance, A. Davison for the preparation of restriction endonucleases, and J. Hay for providing seed stocks of the PAA^r mutants. We also thank K. L. Powell, D. J. M. Purifoy, D. S. Parris, and P. A. Schaffer for the personal communication of unpublished data. We are grateful to J. H. Subak-Sharpe for his interest throughout this study and for valuable criticism of the manuscript.

P.C. is the recipient of a fellowship from the Canadian Medical Research Council.

LITERATURE CITED

1. **Aron, G. M., D. J. M. Purifoy, and P. A. Schaffer.** 1975. DNA synthesis and DNA polymerase activity of herpes simplex virus type 1 temperature-sensitive mutants. J. Virol. **16**:498–507.
2. **Clements, J. B., R. Cortini, and N. M. Wilkie.** 1976. Analysis of herpesvirus DNA substructure by means of restriction endonucleases. J. Gen. Virol. **30**:243–256.
3. **Cortini, R., and N. M. Wilkie.** 1978. Physical maps for HSV type 2 DNA with five restriction endonucleases. J. Gen. Virol. **39**:259–280.
4. **Graham, F. L., and A. I. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
5. **Hay, J., H. Moss, A. T. Jamieson, and M. C. Timbury.** 1976. Herpesvirus proteins: DNA polymerase and pyrimidine deoxynucleoside kinase activities in temperature-sensitive mutants of herpes simplex virus type 2. J. Gen. Virol. **31**:65–73.
6. **Hay, J., and J. H. Subak-Sharpe.** 1976. Mutants of herpes simplex virus type 1 and 2 that are resistant to phosphonoacetic acid induce altered DNA polymerase activities in infected cells. J. Gen. Virol. **31**:145–148.
7. **Honess, R. W., and D. H. Watson.** 1977. Herpes simplex virus resistance and sensitivity to phosphonoacetic acid. J. Virol. **21**:584–600.
8. **Joffre, J. T., P. A. Schaffer, and D. S. Parris.** 1977. Genetics of resistance to phosphonoacetic acid in strain KOS of herpes simplex virus type 1. J. Virol. **23**:833–836.
9. **Knipe, D. M., W. T. Ruyechan, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. III. Fine mapping of a genetic locus determining resistance to phosphonoacetate by two methods of marker transfer. J. Virol. **29**:698–704.
10. **Knipe, D. M., W. T. Ruyechan, B. Roizman, and I. W. Halliburton.** 1978. Molecular genetics of herpes simplex virus. Demonstration of regions of obligatory and non-obligatory identity within diploid regions of the genome. Proc. Natl. Acad. Sci. U.S.A. **75**:3896–3900.
11. **Macpherson, I., and M. Stoker.** 1962. Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. Virology **16**: 147–151.

12. **Mao, J. C. H., E. E. Robishaw, and L. R. Overby.** 1975. Inhibition of DNA polymerase activity from herpes simplex virus-infected W-38 cells by phosphonoacetic acid. J. Virol. **15**:1281–1283.
13. **Marsden, H. S., I. K. Crombie, and J. H. Subak-Sharpe.** 1976. Control of protein synthesis in herpesvirus-infected cells: analysis of the polypeptide induced by wild type and sixteen temperature-sensitive mutants of HSV strain 17. J. Gen. Virol. **31**:347–372.
14. **Marsden, H. S., N. D. Stow, V. G. Preston, M. C. Timbury, and N. M. Wilkie.** 1978. Physical mapping of herpes simplex virus-induced polypeptides. J. Virol. **28**:624–655.
15. **Morse, L. S., T. G. Buchman, B. Roizman, and P. A. Schaffer.** 1977. Anatomy of herpes simplex virus DNA. IX. Apparent exclusion of some parental DNA arrangements in the generation of intertypic (HSV-1 × HSV-2) recombinants. J. Virol. **24**:231–248.
16. **Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer.** 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 × HSV-2 recombinants. J. Virol. **26**:389–410.
17. **Overby, L. R., E. E. Robishaw, J. B. Schleicher, A. Ruter, N. L. Shipkowitz, and J. C. H. Mao.** 1974. Phosphonoacetic acid: inhibitor of herpes simplex virus. Antimicrob. Agents Chemother. **6**:360–365.
18. **Powell, K. L., and D. J. M. Purifoy.** 1977. Nonstructural proteins of herpes simplex virus. I. Purification of the induced DNA polymerase. J. Virol. **24**:618–626.
19. **Preston, V. G., A. J. Davison, H. S. Marsden, M. C. Timbury, J. H. Subak-Sharpe, and N. M. Wilkie.** 1978. Recombinants between herpes simplex virus types 1 and 2: analysis of genome structures and expression of immediate early polypeptides. J. Virol. **28**:499–517.
20. **Purifoy, D. J. M., R. B. Lewis, and K. L. Powell.** 1977. Identification of the herpes simplex virus DNA polymerase gene. Nature (London) **269**:621–623.
21. **Purifoy, D. J. M., and K. L. Powell.** 1977. Herpes simplex virus DNA polymerase as the site of phosphonoacetate sensitivity: temperature-sensitive mutants. J. Virol. **29**:470–477.
22. **Schaffer, P. A., G. M. Aron, N. Biswal, and M. Benyesh-Melnick.** 1973. Temperature-sensitive mutants of herpes simplex virus type 1: isolation, complementation and partial characterization. Virology **52**:57–71.
23. **Stow, N. D., and N. M. Wilkie.** 1976. An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. J. Gen. Virol. **33**: 447–458.
24. **Stow, N. D., and N. M. Wilkie.** 1978. Physical mapping of temperature-sensitive mutations of herpes simplex virus type 1 by intertypic marker rescue. Virology **90**: 1–11.
25. **Timbury, M. C.** 1971. Temperature-sensitive mutants of herpes simplex virus type 2. J. Gen. Virol. **13**:373–376.
26. **Timbury, M. C., and L. Calder.** 1976. Temperature-sensitive mutants of herpes simplex virus type 2: a provisional linkage map based on recombination analysis. J. Gen. Virol. **30**:179–186.
27. **Wilkie, N. M.** 1973. The synthesis and substructure of herpesvirus DNA: the distribution of alkali-labile single strand interruptions in HSV-1 DNA. J. Gen. Virol. **21**: 453–467.
28. **Wilkie, N. M.** 1976. Physical maps for herpes simplex virus type 1 DNA for restriction endonucleases *Hin*d III, *Hpa*-1 and *X. bad.* J. Virol. **20**:222–233.
29. **Wilkie, N. M., N. D. Stow, H. S. Marsden, V. Preston, R. Cortini, M. C. Timbury, and J. H. Subak-Sharpe.** 1977. Physical mapping of herpes simplex virus coded functions and polypeptides by marker rescue and analysis of HSV-1/HSV-2 intertypic recombinants, p. 11–31. *In* G. de The, W. Henle, and F. Rapp (ed.), Proceedings of the Symposium on Herpesviruses and Oncogenesis. III, part 1. International Agency for Research Against Cancer, Lyon, France.