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Physical Mapping of Herpes Simplex Virus Type 1 Mutations by Marker Rescue

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A generally applicable technique which permits the rescue of selected genetic markers from fragments of herpes simplex virus DNA is described. Baby hamster kidney cells infected at the nonpermissive temperature with intact DNA from temperature-sensitive mutants or with fragmented wild-type DNA produce no, or little, infectious progeny. Coinfection results in an increased yield of virus, demonstrating the rescue of genetic information from the DNA fragments. This progeny virus consists of both wild-type and temperature-sensitive virus, demonstrating that both recombination and complementation can occur in coinfected cells. Rescue experiments using isolated fragments produced with various restriction endonucleases have enabled us to locate five temperature-sensitive mutations on the herpes simplex virus type 1 physical map. An adaptation of the technique has allowed the physical mapping of a mutation which affects the herpes simplex virus type 1 pyrimidine deoxyribonucleoside kinase gene. Comparison of the genetic and physical maps for these mutants reveals several anomalies which are discussed.

The genome of herpes simplex virus (HSV) is a molecule of linear double-stranded DNA of approximately 100×10^6 daltons (1, 15). Sheldrick and Berthelot (29) reported that DNA molecules isolated from virions were composed of long (U_L) and short (U_S) unique regions, each flanked by inverted repeated sequences, the sequences flanking U_L being designated TR_L and IR_L, and those flanking U_S designated TR_S and IR_S (see Fig. 2). In addition, they predicted that inversions of the S and L regions could occur to generate four possible genome arrangements. Subsequently, other workers have used partial denaturation mapping and restriction enzyme analysis to demonstrate the presence of these four genome arrangements in approximately equimolar amounts (4, 7, 12, 30).

The use of HSV mutants clearly constitutes a powerful approach to the understanding of the functional organization of these genomes and the role of certain products in virus replication, latency, and oncogenic transformation (25, 33). Temperature-sensitive (*ts*) mutants of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) have been isolated in a number of laboratories and in several cases have been arranged into series of nonoverlapping complementation groups (3, 8, 24, 34). Genetic maps have been constructed by analyzing the progeny of two- and three-factor crosses between these mutants (2, 3, 27, 35). Although the genetic maps indicate the linear order and relative map distances between the mutants, it is not possible to deduce from them the positions of the *ts* loci on the viral genome.

Physical maps of HSV-1 and HSV-2 DNAs have now been constructed for several restriction endonucleases (5, 30, 40; R. Cortini and N. M. Wilkie, J. Gen. Virol., in press), but the only function so far located on the genome is the viral thymidine kinase (19, 38). Marker rescue experiments (16) have been successfully used to map *ts* mutants of a number of DNA viruses (9, 16, 17, 20, 22, 37), and a similar approach for HSV-1 has already been outlined (41). Wilkie et al. (41) showed that shearing abolished the ability of wild-type (WT) HSV-1 DNA to give rise to infectious progeny in susceptible baby hamster kidney (BHK-21 C13) cells at the nonpermissive temperature. After superinfection with *ts* mutant virus, however, the recovery of WT recombinant virus was detected, demonstrating the rescue of the selected ts^+ marker from the randomly sheared DNA.

We have now developed an improved technique for demonstrating marker rescue from fragmented HSV-1 DNA, which is based upon the use of only DNA to infect cells. We have screened separated restriction endonuclease fragments of WT DNA for their ability to rescue progeny virus from cells mixedly infected at nonpermissive temperature with intact DNA ex-

tracted from five HSV-1 *ts* mutants. The fragments able to rescue efficiently were identified, and a knowledge of the physical maps of the HSV DNA enabled the *ts* mutations to be located on the viral genome. A similar marker rescue approach has also enabled us to physically map a mutation in the pyrimidine deoxyribonucleoside kinase gene.

MATERIALS AND METHODS

Cells. BHK-21 C13 cells were grown in Eagle medium supplemented with 10% tryptose phosphate broth and 10% calf serum (ETC 10) as previously described (18). Cell monolayers (2×10^6 cells) in plastic petri dishes (diameter, 50 mm; Flow Laboratories, Inc.) were used throughout for the DNA infections.

Virus DNA preparation. Cells were infected with WT HSV-1 (Glasgow strain 17, ts^+ syn^+), and DNA was prepared from cell-associated and cell-released virus as described by Wilkie (39). Cells infected with *ts* mutants of HSV-1 (3, 21; I. K. Crombie, Ph.D. thesis, University of Glasgow, Glasgow, Scotland, 1975) were incubated at 31°C, and viral DNA was similarly prepared from cell-associated and cell-released virus. The pyrimidine deoxyribonucleoside kinase-deficient mutant of HSV-1 strain 17 ($dPyK^{-7}$) was isolated by A. T. Jamieson, and $dPyK^{-7}$ DNA was prepared as described above.

Restriction endonuclease cleavage of DNA and purification of DNA fragments. The preparation of restriction endonucleases *Hin*dIII, *Xba* I, and *Bgl* II and the conditions for the cleavage of DNA were as previously described (4, 40, 43). The DNA fragments produced by these enzymes were separated by electrophoresis on cylindrical gels of 0.25% agarose (internal diameter, 1.5 cm; length, 18 cm) in the presence of 0.5 μg of ethidium bromide per ml, 1 to 2 μg of cleaved DNA being applied to each gel. DNA bands were visualized by irradiation with long-wavelength (366 nm) UV light and excised from the gels. Gel slices were dissolved in $NaClO_4$, and the DNA was purified by fractionation on hydroxylapatite columns (42) followed by extensive dialysis against 0.01 M Tris–0.001 M EDTA (pH 7.4). Recovery was monitored by the inclusion of trace amounts of ^{32}P-labeled DNA. Several experiments utilized unseparated restriction enzyme fragments or DNA which had been sheared by six passages through a 26G syringe needle (Yale Microlance).

Marker rescue of *ts* mutants. The method used was based upon the calcium phosphate DNA infectivity technique of Graham and van der Eb (10), as modified by Stow and Wilkie (31). Intact DNA extracted from *ts* mutant virus was mixed at a concentration of 0.5 to 1.0 μg/ml with 10 μg of calf thymus carrier DNA and WT DNA fragments per ml of HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid)-buffered saline (HEBS). HEBS consisted of (grams per liter): NaCl, 8.0; KCl, 0.37; $Na_2HPO_4 \cdot 2H_2O$, 0.125; dextrose, 1.0; and HEPES, 5.0. In experiments using purified restriction endonuclease fragments, the amount of fragment present per milliliter was that generated from 1 μg of intact HSV-1 DNA (see below). $CaCl_2$ was added to a final concentration of 130 mM, and 0.4 ml of the resulting fine precipitate was added to BHK cell monolayers from which the growth medium had been removed. The plates were incubated at 38.5°C (nonpermissive temperature) from the time of infection. After 45 min, EC5 (Eagle medium containing 5% calf serum) was added, and at 4 h postinfection the cells were treated for 4 min with 25% DMSO in HEBS as described by Stow and Wilkie (31). Incubation was continued for 3 days at 38.5°C in EC5 (unless otherwise stated). The cells were then harvested with the growth medium and disrupted by sonic treatment, and the virus yields were titrated on BHK cell monolayers at 38.5 and 31°C (the permissive temperature). Plates which were to be stained with Giemsa were incubated in EHu5 (Eagle medium containing 5% human serum) to prevent secondary plaque formation.

Rescue of defective pyrimidine deoxyribonucleoside kinase gene from $dPyK^{-7}$ fragments. The procedure was similar to that described above, except that WT DNA was co-precipitated with $dPyK^{-7}$ DNA fragments. Incubation was for 48 h at 36°C in EC5 containing 100 μg of 5-bromo-2'-deoxycytidine (BCdR; Sigma Chemical Co.) per ml. Virus yields were titrated in the presence (100 μg/ml) and absence of BCdR.

RESULTS

Marker rescue with unseparated WT DNA fragments. In an attempt to improve the efficiency of our HSV-1 marker rescue system (41), we investigated the possibility of using mixed infections with viral DNAs. BHK cell monolayers were infected as described above with co-precipitated intact *ts* DNA and fragmented WT DNA, produced either by shearing or by restriction endonuclease digestion. After 3 days, the plates were either harvested and titrated, or stained with Giemsa. Table 1 demonstrates that, although the DNA extracted from two mutants, *ts* D and *ts* F, is infectious at 31°C, no virus progeny can be detected after incubation at 38.5°C. The infectivity of WT DNA was abolished either by digestion with the restriction endonucleases *Hin*dIII, *Xba* I, or *Hpa* I, or by shearing. The average molecular weight of the sheared DNA was determined to be 6×10^6 to 10×10^6 by agarose gel electrophoresis. After mixed infections with mutant DNA and sheared or restricted WT DNA, phenotypically WT virus was detected upon titration of the plate harvests. This demonstrates the rescue of the WT markers corresponding to the *ts* D and *ts* F lesions from the fragmented WT DNA. Cytotoxic effects were not observed when cells were infected with *ts* mutant DNA at 38.5°C and, hence, plaques could be scored on the original infected plates. In contrast, when *ts* mutant virus particles were used, the cytopathic effect was

TABLE 1. *Rescue of ts D and ts F with unseparated WT DNA fragments*[a]

DNA I (fragmented)	DNA II (intact)	Incubation temp (°C)	Plaques/plate	38.5°C titer (PFU/plate)	31°C titer (PFU/plate)
—	*ts* D	31	250	1.0×10^3	7.2×10^5
—	*ts* D	38.5	0	<20	<20
—	*ts* F	31	450	<20	2.0×10^5
—	*ts* F	38.5	0	<20	<20
—	ts^+	38.5	>400[b]	7.6×10^8	7.6×10^8
Sheared ts^+	—	38.5	0	<20	<20
Sheared ts^+	*ts* D	38.5	26	6.4×10^5	1.3×10^6
Sheared ts^+	*ts* F	38.5	10	4.4×10^5	6.0×10^5
*Hin*dIII-digested ts^+[c]	—	38.5	0	<20	<20
*Hin*dIII-digested ts^+	*ts* D	38.5	46	1.6×10^6	2.6×10^6
*Hin*dIII-digested ts^+	*ts* F	38.5	14	1.1×10^6	1.6×10^6
Hpa I-digested ts^+[c]	—	38.5	0	<20	<20
Hpa I-digested ts^+	*ts* D	38.5	54	1.4×10^6	2.2×10^6
Hpa I-digested ts^+	*ts* F	38.5	14	2.0×10^6	3.4×10^6
Xba I-digested ts^+[c]	—	38.5	0	<20	<20
Xba I-digested ts^+	*ts* D	38.5	317	1.0×10^7	2.2×10^7
Xba I-digested ts^+	*ts* F	38.5	18	4.0×10^6	4.4×10^6

[a] Confluent monolayers of BHK-21 C13 cells were coinfected with 0.2 μg of intact *ts* DNA and 0.2 μg of fragmented WT DNA as described in the text. After 3 days, the cell monolayers were either stained and the plaques were counted, or they were harvested by scraping into the culture medium and disrupted by sonic treatment and the progeny virus was titrated at 38.5 and 31°C. —, DNA I or DNA II omitted.

[b] Plaques were counted after 2 days.

[c] Limit digests, as determined by agarose gel electrophoresis, were produced by each enzyme.

frequently too severe to allow detection of plaques. The use of DNA, as opposed to virus particles, also reduced the background due to revertants and to leakiness, which often interferes with the detection of recombinants (41). For these reasons, the mixed DNA technique was adopted for all the mapping experiments described in this paper.

Figure 1a shows a dose-response curve for marker rescue using increasing amounts of sheared DNA mixed with 0.2 μg of intact *ts* D DNA per plate. High concentrations of sheared DNA tend to reduce the efficiency of rescue, but optimum plaque numbers were obtained over the range of approximately 0.1 to 0.4 μg of sheared DNA per plate. When isolated restriction enzyme-generated fragments were used, each plate was therefore infected with the amount of DNA fragment equivalent to that obtained from 0.4 μg of undigested DNA. Thus, the classes of fragments present in 0.5 or 0.25 M amounts (4, 12) were at a sufficient concentration to allow efficient rescue. Between 0.2 and 0.4 μg of intact *ts* DNA per plate was found to be optimal for rescue under these conditions (Fig. 1b).

Progeny tests on well-isolated plaques picked from infected plates showed that WT recombinant virus could be recovered from all the plaques tested. *ts* virus was also present, indicating that complementation had also occurred (Table 2).

Rescue of *ts* mutants with specific restriction endonuclease fragments. Figure 2 shows the digest patterns of strain 17 DNA cleaved with *Hin*dIII, *Bgl* II, *Xba* I, and *Hin*dIII + *Xba* I, as well as the physical maps for these enzymes.

Table 3 shows the rescue of *ts* A with isolated fragments from three different restriction endonuclease digests. Control infections with each of the isolated fragments alone (not shown) or with *ts* A DNA alone (Table 3) yielded no detectable progeny. The rescue of the WT information was observed from only one DNA band in each of the digests, namely, *Xba* f, *Hin*d/*Xba* 4 + 4′, and *Bgl* II i. The *Hin*d/*Xba* 4 + 4′ band consists of two unresolved fragments, but only one of these (fragment 4) contains sequences in common with *Xba* f and *Bgl* II i. *ts* A is therefore rescued only by sequences contained within the *Bgl* II i region of the genome, and consequently the site of the *ts* lesion must lie within this region.

Table 4 shows that *ts* F is rescued only by the same set of fragments as *ts* A, and its location must be within the same region of the physical map.

Table 5 shows the data for marker rescue experiments using *ts* K. WT progeny were detected after coinfection with six of the bands of each digest, namely, *Hin*dIII/*Xba* 1, 2, 4 + 4′, 5, 6, 11 + 11′, and *Bgl* II a, b, c, e, g + h, and l. The only DNA sequences common to these bands are from the repetition bounding the short

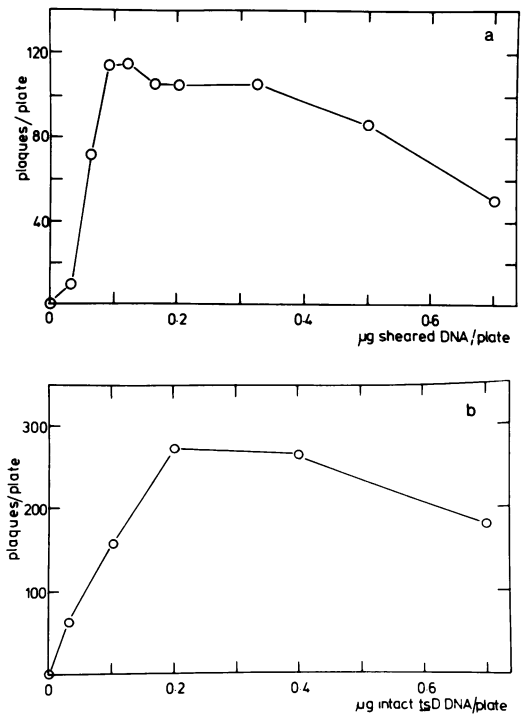

FIG. 1. *(a) Effect of increasing amounts of sheared WT DNA on the rescue of ts D. BHK cell monolayers were coinfected at 38.5°C with 0.2 μg of intact ts D DNA and increasing amounts of sheared WT DNA as described in the text. The plates were stained after 3 days, and plaques were counted. Each point represents the average of 4 replicate plates. (b) Effect of increasing amounts of intact ts D DNA on rescue of the WT marker from sheared DNA. BHK cell monolayers were coinfected with 0.4 μg of sheared DNA and increasing amounts of intact ts D DNA and treated as described above and in the text. It should be noted that the two experiments (a) and (b) were performed on different occasions.*

unique region, and these are the only bands which contain these reiterated sequences. It can therefore be concluded that the mutation in *ts* K maps in both the IR_S and TR_S regions of HSV-1 DNA. Rescue of *ts* D was observed with the same *Hin*dIII/*Xba* I fragments as *ts* K (Table 5), and by the same reasoning the *ts* D lesion must also be located within the IR_S and TR_S sequences.

Table 6 shows the rescue of *ts* S with *Bgl* II and *Hin*dIII/*Xba* I fragments. In this case, intact *ts* S DNA showed some degree of leakiness and, in addition, a high background level of rescue was observed with most of the WT DNA fragments. However, one fragment from each digest, namely *Bgl* II *k* and *Hin*dIII/*Xba* I 7, gave rise to virus yields which were respectively 16- and 70-fold greater than any of the remainder. As can be seen from Fig. 2, these fragments overlap on the physical map, and we conclude that *ts* S is located in this region of the genome.

Use of *ts* D DNA fragments in marker rescue experiments. *ts* D has been shown to map in the repeated sequences bounding the short unique region. Since it is possible that only one of these sequences may be expressed, we tested whether potentially functional WT sequences capable of rescuing *ts* D were present within the *ts* D genome itself. Table 7 shows that *ts* J (used as a control) was rescued efficiently with unseparated *Bgl* II fragments of both WT and *ts* D DNA. In the case of *ts* D, no progeny could be detected when the *ts* D DNA fragments were used, but efficient rescue was obtained with WT DNA fragments. Although sequences capable of rescuing *ts* D are present in WT DNA fragments containing either IR_S or TR_S, our result suggests that such sequences are absent from *ts* D DNA itself. The simplest explanation is that the sequence in which the *ts* D lesion occurs is repeated and present in both IR_S and TR_S. However, it is conceivable that a unique sequence, capable of transfer from one side of the S region to the other, is affected.

Mapping of the dPyK^{-7} mutation by marker rescue. By using the marker rescue approach, it should be possible to map any HSV mutation for which a suitable selection system can be found. Cooper (6) has reported that BCdR is phosphorylated in HSV-infected but not in uninfected cells. Consequently, BCdR inhibits the replication of WT HSV but is nontoxic to BHK cells (28). Moreover, Brown and Jamieson (S. M. Brown and A. T. Jamieson, *In Oncogenesis and Herpesvirus III*, in press) have shown that virus mutants deficient in the induction of the HSV-specified pyrimidine deoxyribonucleoside kinase (14) are not inhibited by BCdR. We have used one such mutant, dPyK^{-7} (isolated by A. T. Jamieson) in marker rescue experiments. The mutant appears to be deficient in viral thymidine and deoxycytidine kinase (TK and dCK) activities because of a mutation in the pyrimidine deoxyribonucleoside kinase structural gene (A. T. Jamieson and J. H. Subak-Sharpe, Virology, in press). Figure 3 shows that the yield of progeny virus from BHK cells infected with WT DNA is reduced by a factor of between 10^5 and 10^7 when BCdR is present in the culture medium at concentrations of 20 to 100 μg/ml. The yield from cells infected with dPyK^{-7} DNA is virtually unaffected. It was therefore tested whether an increase in infectious progeny could be observed in cells coinfected with intact WT DNA and fragmented dPyK^{-7} DNA in the presence of BCdR.

Table 8 shows that, although the infectivity of

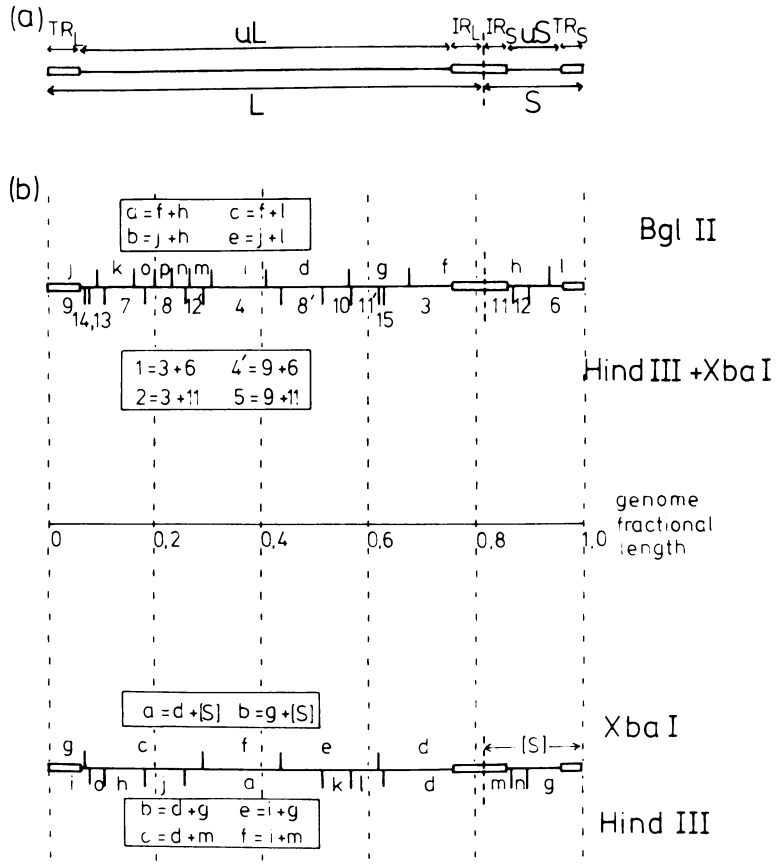

FIG. 2. *Digest patterns and physical maps for HSV-1 strain 17 DNA cleaved with restriction endonucleases Bgl II, HindIII + Xba I, Xba I, and HindIII. (a) Molecular model for the HSV genome, taken from Sheldrick and Berthelot (29) and using the nomenclature suggested at the Cold Spring Harbor herpesviruses meeting, September 1976. (b) Restriction enzyme maps taken from the data of Wilkie et al. (41, 43) and unpublished observations. The genome arrangement used throughout this communication is the same as that used and discussed by Wilkie et al. (Oncogenesis and Herpesvirus III, in press). Fragments which span the joint can be considered as the "fusion products" of two terminal fragments and are shown in boxes for each map. (c) Autoradiograms of ^{32}P-PO$_4$-labeled HSV-1 DNA digested with the four restriction endonuclease combinations and separated by electrophoresis on 0.3 or 0.4% agarose slab gels as described by Preston et al. (submitted for publication).*

WT DNA is inhibited in the presence of BCdR, the addition of *Hin*dIII or *Xba* I fragments of dPyK^{-7} DNA to the intact WT DNA resulted in a greater than 100-fold increase in the yield of virus. The yield of progeny virus after coinfection with isolated dPyK^{-7} *Xba* fragments is shown in Fig. 4a. Only *Xba* f gave a marked increase in virus yield. The results of a similar experiment with *Hin*dIII fragments of dPyK^{-7} DNA (Fig. 4b) demonstrate that a marked increase in virus yield was obtained with *Hin*dIII a + b, and of these fragments only *Hin*dIII a overlaps *Xba* f (Fig. 2; 40). Provided that the lesion in dPyK^{-7} is in the structural gene, the results locate that part of the HSV dPyK gene to within *Xba* f. Progeny virus was plaque-purified from five separated plates where marker rescue had occurred. When tested, 24 of 25 clones were able to replicate in the presence of BCdR and had therefore rescued the mutant gene from the dPyK^{-7} fragments.

DISCUSSION

This paper describes an efficient new system for detecting the rescue of selected genetic markers from fragmented HSV-1 DNA by mixed infection with intact DNA molecules. In contrast to the heteroduplex technique of marker rescue successfully employed with ϕX174, simian virus 40, and polyoma (9, 13, 16, 17, 20, 22, 37), the

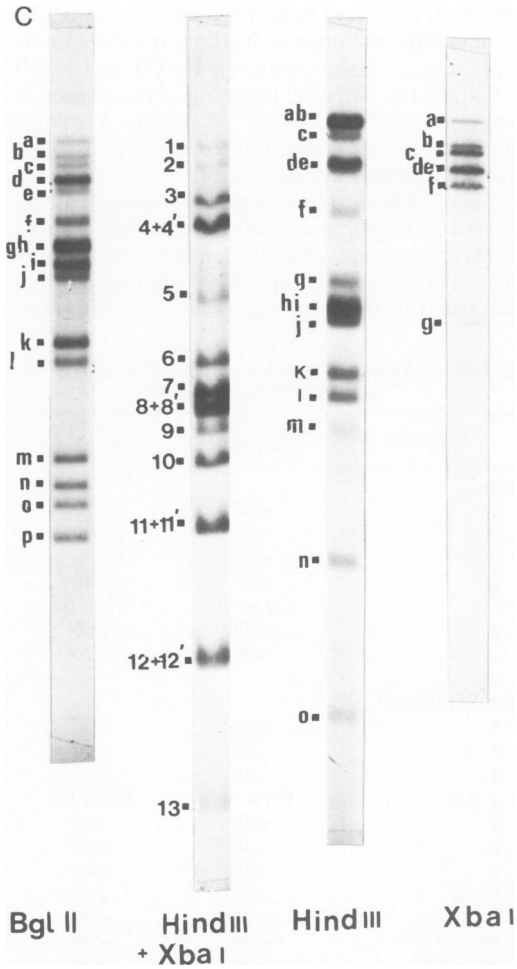

FIG. 2. *Continued*

TABLE 2. *Tests on rescued progeny virus*[a]

Plaque no.	31°C titer (PFU)	38.5°C titer (PFU)	EOP[b]	Phenotype
(i)				
1	1.6×10^4	1.6×10^4	1.0	
2	1.5×10^4	4.9×10^3	0.32	
3	3.7×10^3	3.1×10^3	0.84	
4	2.5×10^3	2.4×10^3	0.96	
5	9.1×10^2	6.7×10^2	0.74	
6	8.5×10^3	3.2×10^3	0.38	
7	1.8×10^4	1.5×10^4	0.83	
8	3.7×10^3	2.4×10^3	0.65	
9	3.5×10^3	1.4×10^3	0.40	
10	4.8×10^4	4.3×10^4	0.90	
11	1.8×10^3	1.6×10^3	0.89	
12	1.0×10^4	6.5×10^3	0.65	
13	1.2×10^5	1.1×10^5	0.92	
14	2.1×10^5	1.7×10^5	0.81	
(ii)				
2/1	3.0×10^2	<10		*ts*
2/2	3.9×10^2	<10		*ts*
2/3	2.9×10^2	<10		*ts*
2/4	1.4×10^3	<10		*ts*
2/5	1.3×10^3	7.6×10^2		ts^+
2/6	5.2×10^2	3.2×10^2		ts^+
2/7	5.8×10^2	5.6×10^2		ts^+
2/8	3.2×10^4	3.1×10^4		ts^+
2/9	2.7×10^3	3.2×10^3		ts^+
2/10	3.0×10^2	<10		*ts*

[a] Cells were coinfected with intact *ts* J DNA and sheared WT DNA as described in the text. The monolayers were incubated for 3 days at 38.5°C in EHu5 and washed, and 14 well-isolated plaques were picked. The progeny virus was titrated at 38.5 and 31°C (section i). Ten well-isolated plaques were picked from the progeny of plaque number 2 at 31°C and titrated at 38.5 and 31°C (section ii).

[b] EOP, Efficiency of plating; values indicate ratio of 38.5°C titer to 31°C titer.

interacting genomes in our system are present in the form of double-stranded DNA. The generation of progeny which carry markers derived from the fragmented DNA must therefore be a consequence of recombination between the fragments and intact DNA molecules. In the experiments using *ts* mutants, both ts^+ recombinant progeny virus and complemented *ts* virus were detected, but we cannot yet say whether, or in what way, complementation and recombination are interdependent.

By using isolated restriction endonuclease fragments of ts^+ DNA, five *ts* mutations have been located on the viral genome. Consistent mapping results were obtained by using different preparations of intact *ts* DNA and WT DNA fragments, but several points concerning the method should be noted. The efficiency of rescue of a particular marker from unseparated DNA fragments was usually somewhat higher than from the isolated fragment(s) which contained the rescuing sequences, and this probably reflects a loss in the biological activity of the DNA fragment occurring during the isolation procedure. The yields in some of the experiments (Tables 4 and 5) were very low, suggesting that only a very small number of rescue events were occurring per plate. Nevertheless, under these conditions reproducible results were obtained, and spurious rescue due to the presence of contaminating sequences in the fragment preparations was not observed. In contrast, in experiments with *ts* S (Table 6) and some experiments with *ts* D (data not shown), the efficiency of rescue was much greater, and large numbers of plaques were seen on infected plates where marker rescue had occurred. Upon titration of the virus yields, high background levels of rescue with many of the fragments were observed, and

TABLE 3. *Rescue of ts A with Xba I, HindIII/Xba I, and Bgl II fragments*[a]

Fragment	38.5°C titer (PFU/plate)	31°C titer (PFU/plate)
Xba I		
a	<20	<20
b	<20	<20
c	<20	<20
de	<20	<20
f	2.9×10^6	3.4×10^6
g	<20	<20
—	<20	<20
*Hin*dIII/*Xba* I		
1	<40	<40
2	<40	<40
3	<40	<40
4 + 4′	1.8×10^4	4.0×10^4
5	<40	<40
6	<40	<40
7	<40	<40
8 + 8′	<40	<40
9	<40	<40
10	<40	<40
11 + 11′	<40	<40
12 + 12′	<40	<40
13	<40	<40
14	<40	<40
15	<40	<40
—	<40	<40
Bgl II		
a	<40	<40
b	<40	<40
c	<40	<40
d	<40	<40
e	<40	<40
f	<40	<40
gh	<40	<40
i	3.2×10^4	5.6×10^4
j	<40	<40
k	<40	<40
l	<40	<40
m	<40	<40
n	<40	<40
o	<40	<40
p	<40	<40
—	<40	<40

[a] BHK cells were infected at 38.5°C with 0.4 μg of intact *ts* A DNA and amounts of isolated WT restriction endonuclease fragments equivalent to 0.4 μg of intact DNA. After 3 days, progeny virus was titrated at 38.5 and 31°C. —, *ts* A DNA alone.

this appears to be due to sequence contamination of these fragments. This problem was only apparent in experiments in which the overall efficiency of rescue was relatively high, and the presence of contaminating fragments was not detected upon re-electrophoresis of the isolated fragments. Although the data presented in Table 6 for *ts* S are not as clear as for the other *ts* mutants, the physical map location for *ts* S (and also *ts* A, *ts* D, and *ts* K) has been confirmed by using a different approach which involves intertypic marker rescue between HSV-1 and HSV-2, followed by physical mapping of the crossover points (N. D. Stow and N. M. Wilkie, Virology, in press). In this communication, suitable selective conditions were also found which enabled a dPyK mutation to be mapped by marker rescue. Recently, Wigler et al. (38) showed that a small *Bam*HI fragment of HSV-1 DNA could transform TK^- L cells to the TK^+ phenotype, and that the transformants expressed viral TK activ-

TABLE 4. *Rescue of ts F with Xba I, HindIII/XbaI, and Bgl II fragments*[a]

Fragment	38.5°C titer (PFU/plate)	31°C titer (PFU/plate)
Xba I		
a	<20	<20
b	<20	<20
c	<20	<20
de	<20	<20
f	1.0×10^3	2.7×10^3
g	<20	<20
—	<20	<20
*Hin*dIII/*Xba* I		
1	<40	<40
2	<40	<40
3	<40	<40
4 + 4′	2.6×10^3	8.4×10^3
5	<40	<40
6	<40	<40
7	<40	<40
8 + 8′	<40	<40
9	<40	<40
10	<40	<40
11 + 11′	<40	<40
12 + 12′	<40	<40
13	<40	<40
14	<40	<40
15	<40	<40
—	<40	<40
Bgl II		
a	<40	<40
b	<40	<40
c	<40	<40
d	<40	<40
e	<40	<40
f	<40	<40
gh	<40	<40
i	6.8×10^2	3.7×10^3
j	<40	<40
k	<40	<40
l	<40	<40
m	<40	<40
n	<40	<40
o	<40	<40
p	<40	<40
—	<40	<40

[a] Conditions were as described in Table 3. —, *ts* F DNA alone.

TABLE 5. *Rescue of ts K with HindIII/Xba I and Bgl II fragments and rescue of ts D with HindIII/Xba I fragments*[a]

Fragment	38.5°C titer (PFU/plate)	31°C titer (PFU/plate)
*Hin*dIII/*Xba* I[b]		
1	3.6×10^2	5.2×10^3
2	8.0×10^2	3.2×10^3
3	<40	<40
4 + 4′	3.2×10^2	1.9×10^3
5	1.2×10^2	2.8×10^2
6	2.2×10^3	2.2×10^4
7	<40	<40
8 + 8′	<40	<40
9	<40	<40
10	<40	<40
11 + 11′	4.8×10^2	1.1×10^4
12 + 12′	<40	<40
13	<40	<40
14	<40	<40
15	<40	<40
—	<40	<40
Bgl II[b]		
a	8.4×10^2	1.4×10^3
b	3.2×10^2	6.4×10^2
c	2.1×10^3	1.5×10^4
d	<40	<40
e	9.6×10^2	5.6×10^3
f	<40	<40
gh	1.2×10^2	1.4×10^3
i	<40	<40
j	<40	<40
k	<40	<40
l	5.2×10^2	3.4×10^3
m	<40	<40
n	<40	<40
o	<40	<40
p	<40	<40
—	<40	<40
*Hin*dIII/*Xba* I[c]		
1	1.6×10^3	4.0×10^3
2	2.4×10^2	1.6×10^3
3	<40	<40
4 + 4′	5.6×10^3	8.4×10^3
5	6.0×10^3	9.2×10^3
6	8.8×10^3	1.2×10^4
7	<40	<40
8 + 8′	<40	<40
9	<40	<40
10	<40	<40
11 + 11′	5.6×10^3	1.7×10^4
12 + 12′	<40	<40
13	<40	<40
14	<40	<40
15	<40	<40
—	<40	<40

[a] Conditions were as described in Table 3. —, *ts* DNA alone.
[b] Rescue of *ts* K.
[c] Rescue of *ts* D.

TABLE 6. *Rescue of ts S with HindIII/Xba and Bgl II fragments*[a]

Fragment	38.5°C titer (PFU/plate)	31°C titer (PFU/plate)
*Hin*dIII/*Xba* I		
1	2.4×10^2	2.1×10^3
2	2.7×10^3	1.1×10^4
3	<40	1.9×10^3
4 + 4′	3.2×10^2	1.5×10^3
5	6.4×10^3	6.8×10^3
6	<40	5.6×10^2
7	2.4×10^7	2.7×10^7
8 + 8′	2.6×10^5	3.8×10^5
9	4.8×10^3	1.6×10^4
10	4.4×10^2	5.6×10^2
11 + 11′	<40	7.8×10^2
12 + 12′	<40	8.8×10^2
13	<40	1.6×10^2
14	80	5.6×10^2
15	<40	1.1×10^3
—	<40	8.8×10^2
Bgl II		
a	1.2×10^5	1.8×10^5
b	4.0×10^3	6.4×10^3
c	<40	1.2×10^3
d	1.5×10^5	1.7×10^5
e	3.4×10^4	4.8×10^4
f	1.8×10^3	3.6×10^3
gh	2.7×10^4	7.2×10^4
i	7.8×10^4	1.5×10^5
j	1.1×10^5	9.6×10^4
k	2.5×10^6	4.4×10^6
l	5.6×10^3	1.7×10^4
m	8.4×10^3	2.4×10^4
n	6.0×10^3	1.3×10^4
o	3.2×10^2	4.0×10^4
p	4.8×10^2	2.6×10^3
—	<40	8.8×10^2

[a] Conditions were as described in Table 3. —, *ts* S DNA alone.

ity. Physical mapping studies have located this fragment near the left-hand end of *Xba* f as indicated in Fig. 5. Given that the dPyK locus corresponds with the TK structural gene, our location agrees, within the present limits of resolution, with that of Wigler et al. (38). The results, and that of Wigler et al. (38) are summarized in Fig. 5, which also shows the genetic map for these mutants (3; Brown and Jamieson, *Oncogenesis and Herpesvirus III*, in press; D. Dargan and J. H. Subak-Sharpe, unpublished data).

The physical map locations of the *ts* markers have already been compared with the HSV-1 transcript and polypeptide maps (N. M. Wilkie, N. D. Stow, H. S. Marsden, V. Preston, R. Cortini, M. C. Timbury, and J. H. Subak-Sharpe, *In Oncogenesis and Herpesvirus III*, in press). Here we would also like to note that two of the mutants (*ts* A and *ts* F) synthesize viral DNA at the nonpermissive temperature, whereas the other three (*ts* S, *ts* K, and *ts* D) do not (21). Although *ts* A and *ts* F map in the same region

TABLE 7. *Rescue of ts D and ts J with WT and ts D unseparated Bgl II fragments*[a]

DNA I (intact)	DNA II (fragmented)	Incubation temp (°C)	Plaques/plate	38.5°C titer (PFU/plate)	31°C titer (PFU/plate)
ts J	—	38.5	0	<40	<40
ts D	—	38.5	0	<40	<40
—	*Bgl* II-digested ts^+	38.5	0	<40	<40
—	*Bgl* II-digested *ts* D	38.5	0	<40	<40
ts J	*Bgl* II-digested ts^+	38.5	40	3.2×10^6	3.2×10^6
ts J	*Bgl* II-digested *ts* D	38.5	32	2.7×10^6	3.2×10^6
ts D	*Bgl* II-digested ts^+	38.5	62	2.4×10^6	2.9×10^6
ts D	*Bgl* II-digested *ts* D	38.5	0	<40	<40

[a] The experimental procedure was as described in Table 1. —, DNA I or DNA II omitted.

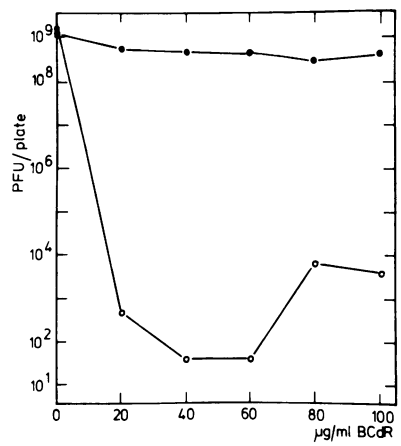

FIG. 3. *Effect of BCdR on the yield of progeny virus from cells infected with WT or dPyK^{-7} DNA. BHK cells were infected with 0.2 μg of WT or dPyK^{-7} DNA at 36°C in the presence of various concentrations of BCdR in the culture medium. After 48 h, the cells were harvested by being scraped into the growth medium, and progeny virus was titrated in the absence of BCdR. Symbols: ●, dPyK^{-7} DNA; ○, WT DNA.*

of the genome, we have also located a DNA negative mutant (*ts* H) very close to *ts* A by intertypic marker rescue (Stow and Wilkie, Virology, in press). It is therefore clear that those functions required for the synthesis of viral DNA which we have already mapped are widely dispersed throughout the HSV-1 genome.

Comparison of the genetic map for the various markers with their physical map positions (Fig. 5) reveals several interesting anomalies.

(i) The recombination frequencies do not reflect physical map distances accurately. Thus, for example, although *ts* F and *ts* A are located further apart on the genetic map than *ts* A and *ts* D, they are considerably closer on the physical map. It is, however, too soon to say whether this observation indicates the presence of definable "hot spots" for recombination on the HSV genome.

(ii) As described earlier, DNA isolated from HSV virions occurs in four arrangements related by inversions of the L and S segments (4, 7, 12, 30, 36). Based on the implied assumption that all four arrangements were equally infectious and able to enter into recombination in infected cells, Clements et al. (4) predicted that all genetic markers in the long unique region should map equidistantly from any marker in the short unique region, and similar considerations can clearly be applied to markers in the repeats flanking S. The genetic map (Fig. 5), however, enables three of the markers located in the long unique region (dPyK, *ts* F, and *ts* A) to be ordered unambiguously with respect to the two markers which map in the sequences flanking the short unique region (*ts* D and *ts* K). This raises the question as to whether all four arrangements of the HSV genome are equally able to initiate infection, replicate, and recombine.

(iii) Although three markers in L (dPyK, A, and F) were not resolved in these experiments, the data of Wigler et al. (38) place TK (and hence dPyK) to the left of *ts* F and *ts* A. The orientation of the L region indicated in Fig. 5 therefore gives an order dPyK (F, A) (D, K) which is consistent with the genetic map of Brown et al. (3) and Brown and Jamieson (*Oncogenesis and Herpesvirus III*, in press), whereas the alternative orientation places the dPyK locus internally. However, since *ts* S appears to map genetically between *ts* A and *ts* D (Dargan and Subak-Sharpe, unpublished data), the physical order of the four markers located within the L region [i.e., *ts* S, dPyK, (*ts* F, *ts* A)] conflicts with their order on the genetic map (dPyK, F, A, and S). On the basis of the available data, it is therefore not possible to orient the genetic and physical maps.

Thus, the data presented here do not yet allow us to form conclusions as to whether all four genome arrangements could be equally infectious and able to replicate and to recombine, or whether one or more of these properties is restricted to only one (or perhaps two) arrange-

TABLE 8. *Rescue of dPyK gene from unseparated dPyK^{-7} DNA fragments*[a]

DNA I (intact)	DNA II (fragmented)	Conditions of incubation	Titer in absence of BCdR (PFU/plate)	Titer in presence of BCdR (PFU/plate)
—	*Xba* I-digested dPyK^{-7}	−BCdR	<40	<40
—	*Hin*dIII-digested dPyK^{-7}	−BCdR	<40	<40
dPyK^{-7}	—	−BCdR	1.2×10^9	8.4×10^8
dPyK^{-7}	—	+BCdR	7.6×10^8	4.4×10^8
WT	—	−BCdR	7.2×10^8	1.3×10^6[b]
WT	—	+BCdR	5.6×10^2	40
WT	Xba I-digested dPyK^{-7}	+BCdR	8.4×10^4	2.6×10^4
WT	*Hin*dIII-digested dPyK^{-7}	+BCdR	1.1×10^5	5.2×10^4

[a] BHK cells were infected with 0.2 μg of intact DNA and 0.2 μg of fragmented dPyK^{-7} DNA, in the absence or presence (100 μg/ml) of BCdR. Plates were incubated for 2 days at 36°C and harvested and titrated in the presence and absence of BCdR.

[b] Mostly minute "leak" plaques.

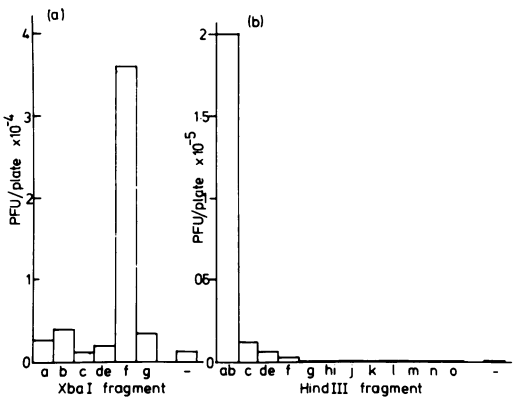

FIG. 4. *Rescue of the dPyK^{-7} lesion from Xba I and HindIII dPyK^{-7} DNA fragments. BHK cells were coinfected with 0.2 μg of intact WT DNA and amounts of dPyK^{-7} fragments equivalent to 0.4 μg of intact DNA. Incubation was for 48 h in the presence of BCdR at 100 μg/ml. Virus yields were titrated in the absence of BCdR.*

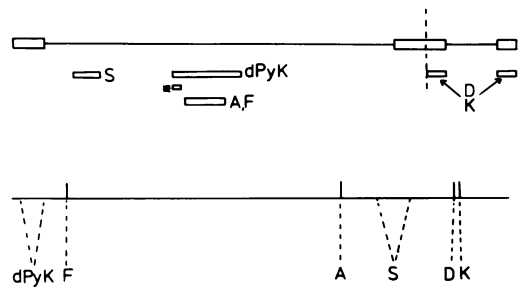

FIG. 5. *Summary of the mapping data described in this paper. The genetic map is taken from the data of Brown et al. (3), Brown and Jamieson (Oncogenesis and Herpesvirus III, in press), and Dargan and Subak-Sharpe (unpublished data). (*) Indicates the location of the HSV-1 TK gene based on the work of Wigler et al. (38) and the physical mapping of Davison and Wilkie (unpublished data).*

ments (23; L. Morse, L. Pereira, B. Roizman, and P. A. Schaffer, *In Oncogenesis and Herpesvirus III*, in press; Wilkie et al., *In Oncogenesis and Herpesvirus III*, in press). We have identified certain anomalies between the physical and genetic maps, but at the present time are unable to provide a reasonable explanation for these. The accurate location of further markers on both the genetic and physical maps should resolve some of these questions and enable the interactions between loci in the various regions of the genome to be better understood.

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