Molecular genetics of herpes simplex virus: Demonstration of regions of obligatory and nonobligatory identity within diploid regions of the genome by sequence replacement and insertion

(reiterated sequences/marker rescue/recombination)

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ABSTRACT The DNAs of herpes simplex virus (HSV) 1 and 2 consist of two components, L and S, each composed of unique sequences bracketed by inverted repeats. In this study we have probed the structure of the reiterated regions of the S component in marker rescue experiments involving transfection of cells with mixtures of intact HSV-1 mutant viral DNA and individual DNA fragments generated by restriction endonuclease digestion of wild-type HSV-1 or HSV-2 DNAs. The results were as follows: (i) HSV is diploid for the wild-type sequences that rescue two temperature-sensitive (*ts*) mutants. DNA fragments from both reiterated regions of the S component of HSV-1(F) DNA can rescue *tsLB2* and *tsD* mutants. (ii) Identity of the entire reiterated sequence at both ends of S is not obligatory because only one end of the S component of wild phenotype virus HSV-1(1061) rescues *tsD* even though both ends rescue *tsLB2*. (iii) Genes in both reiterated sequences can be expressed. We produced, by marker rescue experiments, recombinants with heterotypic ends of the S component, and these specified corresponding polypeptides characteristic of both HSV-1 and HSV-2. (iv) The reiterated sequences of the S component may contain a region of obligatory identity. Thus, several recombinant clones produced by rescue with HSV-2 DNA contained identical HSV-2 DNA insertions within both reiterated regions of the HSV-1 S component. Consistent with this conclusion, the termini of the S component in the heterodiploids described in *iii* were identical by restriction enzyme analysis. (v) The observation that HSV DNA can be expanded by at least 5×10^6 by means of insertion in the S component suggests that it can be a vehicle for exogenous DNA.

Herpes simplex virus 1 (HSV-1) specifies approximately 50 polypeptides which form at least three groups, designated α , β , and γ , whose synthesis is coordinately regulated and sequentially ordered (1). Its DNA (97×10^6 molecular weight) consists of two covalently linked components designated L and S, comprising 82 and 18% of total DNA, respectively (Fig. 1A). Both L and S consist of unique sequences (U_L and U_S) bracketed by inverted repeats (2). The reiterated sequences of L, designated *ab* and *b'a'*, each consists of 6% of total DNA, whereas the reiterated sequences of S, designated *a'c'* and *ca*, each consists of 4.3% of total DNA (3). HSV-1 DNA extracted from virions consists of four equimolar populations differing in the orientation of the L and S components relative to each other (4, 5). The four populations have been designated P (prototype), I_L (inversion of L), I_S (inversion of S), and I_{SL} (inversion of both S and L) (Fig. 1A). One consequence of this DNA arrangement is that restriction endonucleases that do not cleave within the reiterated regions generate three classes of fragments. The first class, corresponding in concentration to the molarity of intact DNA, comprises fragments mapping between the first and last

cleavage within the unique L and unique S regions. The second class comprises the four terminal fragments of L and S, each present in 0.5 M concentration relative to the molarity of the intact DNA. The third class comprises 0.25 M fragments which arise through the joining of the 0.5 M L and S terminal fragments across the *b'a'a'c'* junction as a consequence of the inversions of the L and S components (Fig. 1).

A closely related virus, herpes simplex virus 2 (HSV-2), contains a DNA molecule that structurally resembles HSV-1 DNA and is colinear with it, but differs in the position of restriction endonuclease cleavage sites (6). Analysis of HSV-1 $\times$ HSV-2 recombinants established that the template for the α infected cell polypeptide (ICP) 4 maps near the ends of the S component (7). ICP 4 is processed to a higher molecular weight after synthesis and the HSV-2 ICP 4 forms migrate more slowly in sodium dodecyl sulfate/polyacrylamide gels than the HSV-1 counterparts (7). Evidence for the correspondence of polypeptides identified as HSV-1 and HSV-2 ICP 4 is given elsewhere (7).

This study concerns the reiterated *ca* sequence in the S component of HSV DNA. From experiments involving the rescue of two HSV-1 temperature-sensitive (*ts*) mutants with HSV-1 and HSV-2 restriction endonuclease fragments, we established that at least a portion of the templates for α ICP 4 maps within the reiterated *ca* sequence and that HSV DNA can be haploid, diploid, or at least partially heterodiploid with respect to the expression of this gene. From analysis of the DNA from intertypic recombinants produced by rescue of HSV-1 *ts* mutants with HSV-2 fragments, it emerged that there may be an obligatory identity of sequences in a portion of the *ca* sequences at both ends of the S component.

MATERIALS AND METHODS

Viruses and Cells. Virus strains used were: HSV-2(C) (ref. 8); HSV-1 (HFEM) *tsLB2*, a *ts* mutant derived from strain HSV-1 (HFEM) (ref. 9); HSV-1 (17) *tsD*, a *ts* mutant derived from HSV-1(17) (ref. 10), generously furnished to us by John H. Subak-Sharpe. The mutants *tsD* and *tsLB2* fail to complement and show no significant recombination (unpublished data). Construction of HSV-1 intratypic recombinant 1061 will be described elsewhere. Virus stocks were prepared and titrated on HEp-2 and Vero cells as described (6). Rabbit skin cells were originally obtained from J. McLaren.

Purification and Analysis of Viral DNA and Restriction Endonucleases. Intact viral DNA was purified from nucleocapsids (3) or from infected cells (11). Purification of *EcoRI*, *Hsu I*, *Bgl II*, and *Xba I* restriction endonucleases, ^{32}P labeling

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Abbreviations: HSV, herpes simplex virus; ICP, infected cell polypeptide; *ts*, temperature-sensitive.

FIG. 1. Sequence arrangement and restriction endonuclease cleavage maps of the HSV-1 and HSV-2 DNAs. (A) Schematic representation of unique (U_L and U_S) and reiterated regions (ab and ca) of HSV DNA and *Hsu* I maps of HSV-1 DNA shown for all four arrangements of the DNA. The number below each fragment indicates the molecular weight in millions. (B) Maps of the prototype arrangement of HSV-1(F) and HSV-1(1061) DNAs. (C) Maps of the prototype arrangement of HSV-2(G) DNA.

of DNA, conditions of digestion of DNA, electrophoresis of the DNA fragments in agarose gels, autoradiography of the electrophoretically separated fragments, and preparative purification of fragments were as described (4, 6, 12). *Kpn* I was obtained from New England Bio Labs, Beverly, MA.

Marker Rescue of *ts* Mutants. Rabbit skin cell monolayers

were transfected (13) with mixtures of 0.1 μ g of purified intact mutant viral DNA and 0.1–0.2 μ g of individual purified HSV-1 or HSV-2 DNA fragments. The cultures were incubated at 33.5° for 3–4 days. Progeny virus was then harvested as described (6) and titered at 33.5 and 39° on Vero cells.

Cloning and Designation of Recombinant Viruses. All

Table 1. Marker rescue of *ts*LB2 and *ts*D

Recipient HSV-1 DNA	Donor DNA	Enzyme	S component fragment*			With- out donor
		<i>Hsu</i> I	M	N	G	
			4.7	3.2	9.0	
<i>ts</i> LB2	F†		27	0.2	55	0.6
<i>ts</i> D	F		3	0.03	8	0.1
		<i>Hsu</i> I/ <i>Bgl</i> II	I	R	J	
			8.2	2.9	6.8	
<i>ts</i> LB2	1061†		430	0.7	680	0.5
<i>ts</i> D	1061		85	0.4	1.0	1.0
		<i>Eco</i> RI	K ₁	H	K ₂	
			3.5	9.8	3.5	
<i>ts</i> LB2	F†		22†	0.1	22†	0.06
<i>ts</i> D	F		20†	0.004	20†	0.5
		<i>Xba</i> I	H	J	I	
			8.4	4.4	5.5	
<i>ts</i> LB2	G§		0.05	2	12	0.1
<i>ts</i> D	G		2	8	68	0.01

* Italicized numbers refer to molecular weight in millions. Values below are plating efficiency (39°/33° titer $\times 10^4$) of progeny virus from transfected culture.

† HSV-1 strain.

‡ K₁ and K₂ were assayed as a mixture.

§ HSV-2 strain.

FIG. 2. Autoradiograms of ^{14}C -labeled infected cell polypeptides electrophoretically separated in 9% polyacrylamide gels containing sodium dodecyl sulfate. HEP-2 cultures infected with 20 plaque-forming units/cell, were maintained at 39° and labeled with [^{14}C]leucine, [^{14}C]isoleucine, and [^{14}C]valine 1–3 hr after infection. The two halves of the figure represent portions of autoradiograms of separate slab gels. Underlined and non-underlined numbers identify HSV-2 and HSV-1 polypeptides, respectively.

recombinants in this report were isolated by four cycles of cloning under soft agarose overlay medium at 39° as described (6). Virus clones resulting from rescue of HSV-1(HFEM) *ts*LB2 or HSV-1(17) *ts*D with HSV-2(G) DNA fragments were designated LG or DG, respectively, followed by a clone number.

Labeling and Analysis of Infected Cell Proteins. The procedures for labeling proteins synthesized in infected HEP-2 cells with [¹⁴C]leucine, [¹⁴C]isoleucine, and [¹⁴C]valine and their analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and autoradiography were as published elsewhere (7).

RESULTS

Rescue of HSV-1 Mutants *ts*LB2 and *ts*D with HSV-1 DNA Fragments. Three series of experiments were done. In the first, *ts*LB2 and *ts*D were rescued with *Hsu* I/*Bgl* II restriction endonuclease fragments of HSV-1(1061) DNA. As illustrated in Table 1, *ts*LB2 was rescued by fragments *Hsu* I/*Bgl* II-I and *Hsu* I/*Bgl* II-J, whereas *ts*D was rescued only by the fragment *Hsu* I/*Bgl* II-I generated from the left end of the S component. Furthermore, the 0.25 M fragments of HSV-1(1061) DNA encompassing the left end of the S component were also capable of rescue of *ts*D (data not shown). None of the fragments from the L component rescued these mutants (data not shown). In the second series, we repeated the rescue with *Hsu* I fragments of HSV-1(F) DNA arising from the S component. Both mutants were converted to wild-type phenotype with fragments *Hsu* I-G and *Hsu* I-M generated from both ends of the S component (Table 1). The third series of experiments involved the rescue of *ts*D and *ts*LB2 with *Eco*RI restriction endonuclease fragments of the S component of HSV-1(F) DNA. As shown in Table 1, the terminal 3.5 × 10⁶ molecular weight fragments, *Eco*RI-K_{1,2} rescued both *ts* mutants. We conclude the following: (i) The *ts* lesions in *ts*LB2 and *ts*D are in the reiterated sequences of the S component because the 3.5 × 10⁶ *Eco*RI-K_{1,2} fragments arise from within the *ca* and *a'**c'* sequences (Figure 1B). (ii) HSV-1(F) is diploid with respect to the sequences capable of converting both *ts* mutants to wild-type phenotype. (iii) HSV-1(1061) is diploid with respect to the mutated gene sequences of *ts*LB2 but haploid or heterodiploid with respect to the *ts*D sequence. (iv) Both *ts*D and *ts*LB2 must be either homodiploid in the sense that the mutation is present in both copies of the reiterated sequences or haploid in the sense that the gene sequences in one of the termini are not expressed. Failure of all attempts to rescue *ts*LB2 with its own fragments (data not shown) suggests that both *a'**c'* and *ca* are mutated in some way.

Rescue of *ts* Mutants with HSV-2 Restriction Endonuclease Fragments. In this series of experiments *ts*LB2 and *ts*D were rescued with *Xba* I restriction endonuclease fragments of HSV-2(G) DNA. As shown in Table 1, only the right fragment (*Xba* I-I) from the S component efficiently converted the *ts* mutants to wild-type phenotype.

Genotypic and Phenotypic Properties of *ts*⁺ Clones Obtained by Rescue of *ts*LB2 and *ts*D with *Xba* I-I of HSV-2 DNA. A total of 14 *ts*⁺ clones, 10 from the rescue of *ts*LB2 (LG clones) and 4 from the rescue of *ts*D (DG clones), were isolated and characterized with respect to the DNA sequence arrangement and the early polypeptides that they specified. Analyses of these clones showed that they form three groups.

The DNAs of the four clones in group 1 (e.g., LG7 and DG10) could not be differentiated from parental HSV-1 DNA by their restriction enzyme cleavage sites. These clones are the result of back-mutation or an undetectable recombinational event.

FIG. 3. (A) Restriction endonuclease cleavage maps of the S component of the DNAs of the recombinant DG1, the HSV-1 parental recipient (17) *ts*D, and the HSV-2(G) *Xba* I-I donor fragment. (B) Restriction endonuclease cleavage maps of the S component of the DNA of the recombinant LG3, the parental (HFEM) *ts*LB2 (HSV-1 recipient), and the HSV-2(G) *Xba* I-I donor fragment.

The second group, DG1 and DG9, specified polypeptides that comigrated with fully and partially processed forms of HSV-2(G) ICP 4 (see DG1 in Fig. 2). Restriction endonuclease analysis of the DNA of these clones, (detailed for DG1 in Figs. 3 and 4) indicate that 6 × 10⁶ in molecular weight of HSV-1 DNA were replaced by a total of 11 × 10⁶ in molecular weight of HSV-2 DNA, resulting in an increase of 5 × 10⁶ in the size of the genome. Moreover, the HSV-2 DNA sequences were inserted into two places, i.e., as much as 5 × 10⁶ in molecular weight of HSV-2 DNA was inserted into each of the left and right reiterated regions within the original HSV-1 map positions 0.83–0.865 and 0.965–1.0. In view of the small likelihood of such unusual identical insertions of HSV-2 sequences into both ends of HSV-1 S component DNA, it seemed reasonable to conclude that the insertion occurred at one end of the DNA and was duplicated in the other end.

The third group comprises eight *ts* clones, each of which specified two forms of ICP 4 and therefore appeared to be heterodiploid for the ICP 4 gene. As illustrated in Fig. 2, LG3 is an example of a clone specifying polypeptides that comigrated with both HSV-2(G) and *ts*LB2 ICP 4, whereas LG19 exemplifies a clone that specified a polypeptide that comigrated with the HSV-2(G) ICP 4 and a polypeptide with an electrophoretic mobility faster than that of the HSV-1 *ts* parent. Analyses of the DNAs with *Hsu* I, *Bgl* II, and *Eco*RI (detailed for clone LG3 in Figs. 3 and 4) indicated that in all clones the *Xba* I-I fragment of HSV-2 DNA replaced an identical amount of DNA in the right end of the S component between map position 0.945 to as far as position 1.0.

In light of the hypothesis that a portion of the reiterated sequences can be copied into the other end during replication, the *Eco*RI-K₁ and *Eco*RI-K₂ fragments of LG3 DNA were isolated and cleaved with *Kpn* I endonuclease. This enzyme cleaves within 1.2 × 10⁶ from the end of the reiterated *ac* sequence of HSV-2 (Fig. 1C) but not of the HSV-1 counterpart (unpublished data). The absence of a *Kpn* I cleavage site in both

FIG. 4. Autoradiograms of agarose gels containing electrophoretically separated restriction endonuclease fragments of the parental and recombinant viral DNAs. All gels were 0.45% agarose except those for the samples cleaved with *EcoRI/Bgl* II, which were 0.6% gels. For the recombinant clones DG1 and LG3, only those fragments altered from those of the parental *ts* mutant are labeled. The 0.25 M fragments are labeled with two letters representing the fused terminal fragments. The differences in the fragment patterns of the DNA of HSV-1 (HFEM) *tsLB2* and HSV-1 (17) *tsD* are a 2 million deletion between map units 0.70 and 0.83 in HFEM DNA, slight increase in size of the S component of HFEM DNA, and loss of the *Hsu* I cleavage site between fragments M and N in HFEM DNA. The mapping of LG3 DNA is based largely on the *EcoRI* pattern. Thus, digestion of LG3 DNA with *EcoRI* endonuclease revealed a new fragment, K_2 , which approximately comigrated with the HSV-2 *EcoRI*-M fragment. The HSV-2 *EcoRI*-M fragment is contained within the *Xba* I-I fragment used for the rescue, suggesting that this new fragment represented incorporated HSV-2 DNA sequences. The intensity of the $K_{1,2}$ band was reduced approximately one-half relative to the DNA of *tsLB2*, suggesting the replacement of one end of S by the HSV-2 DNA and a resulting loss of one of the two terminal K fragments (see Fig. 3B). The fragment *EcoRI*-H was reduced in size, also the result of the larger HSV-2 *EcoRI* fragment at one end of S. Because *EcoRI* cleaved only within the reiterated region, analyses with this enzyme did not allow a determination of which end of the S component had been replaced by HSV-2 DNA. To determine this location, we cleaved LG3 DNA simultaneously with *EcoRI* and *Bgl* II. LG3 DNA lacked one fragment, R, from the right end of S adjacent to the L_2 fragment, and a new smaller fragment R' was observed which was probably the result of replacement of the right end of S by the longer HSV-2 L_2 fragment (Fig. 3B). The H fragment, and therefore the left end of S, remained unaltered. Mapping of DG1 DNA is also based largely on the *EcoRI* pattern. Thus, both 0.5 M terminal fragments of the S component, K_1 and K_2 , were missing from the *EcoRI* digest. The presence of two new 0.5 M fragments, $K_{1,2}$, which migrated with the HSV-2 *EcoRI*-M fragment, suggested that the HSV-2 DNA was inserted at both ends and replaced the HSV-1 *EcoRI*- $K_{1,2}$ fragments. However, the HSV-1 *EcoRI*-H fragment from the middle of the S component was not altered in size; therefore, insertion of HSV-2 DNA must have occurred within the terminal HSV-1 *EcoRI*-K fragments. Although not visible, two new fragments of identical size, approximately 1.2×10^6 molecular weight, were present in the *EcoRI* digest of DG1 DNA. We conclude that these fragments represent the distance between the HSV-1 and HSV-2 *EcoRI* cleavage sites at each end of S in this DNA (see Fig. 3A). This predicted a total increase in size of 2.6 million in both ends of S. The increase in size of the terminal S fragments was also apparent from the size of the 0.25 M *EcoRI* fragments, EK_1 , EK_2 , JK_1 , and JK_2 . The increase in the size of the termini of S was also observed in the digestion pattern of DG1 DNA with *Hsu* I and *Bgl* II (Fig. 3).

EcoRI- K_1 and K'_2 fragments of LG3 DNA (Fig. 5) indicates that the HSV-2 sequences replaced HSV-1 sequences to the right of map position 0.945 but not extending beyond 0.988. Therefore, the termini of the reiterated sequences are identical even though a portion of the rest of the repeats are nonidentical.

DISCUSSION

Several significant conclusions can be drawn from the data presented in this paper. (i) HSV-1 can be partially diploid for gene coding sequences. This conclusion is based on the observations that *tsLB2* and *tsD* are rescued by fragments derived from both ends of the HSV-1(F) S component, implying that wild-type sequences are present in two copies. Furthermore, fragments derived entirely from the reiterated *ca* sequence also rescue these mutants, giving credence to that conclusion because that sequence would be present at both ends. Our results are consistent with the report by Wilkie *et al.* (14) that *tsD* is rescued by fragments from both ends of S.

(ii) Both sets of templates can be expressed. This conclusion

rests firmly on the observation that clones LG3 and LG7 with heterotypic S component ends produce ICPs characteristic of both HSV-1 and HSV-2 and are therefore phenotypically heterodiploid.

(iii) Diploidy of wild-type sequences is not obligatory for the expression of wild-type phenotype and by extension, a portion of *a'**c'* and *ca* sequences can be nonidentical. This conclusion rests on the observation that HSV-1 (6) *tsD* could be rescued by both ends of the S component of HSV-1(F) but only by the left end of the HSV-1(1061) S component, even though HSV-1(1061) is capable of wild-type growth. The observation that only the right end of the HSV-2(G) S component efficiently rescues these mutants reinforces this conclusion but should not be considered as reflecting a general property of HSV-2 strains because a recombinant specifying both HSV-1 and HSV-2 ICP 4 was found to contain HSV-2 sequences from the left end of the S component (7). This strain of HSV-2, HSV-2(186), may differ from HSV-2(G) in the location, number, or expression of ICP 4 templates.

(iv) Mutations in both ends of S are obligatory in the genes containing the *tsD* and *tsLB2* mutations. As cited in the text,

FIG. 5. Cleavage of isolated restriction endonuclease fragments with *Kpn* I. ³²P-Labeled DNA fragments listed below were isolated, cleaved with *Kpn* I, and subjected to electrophoresis on a 1% slab gel as described (8, 17). (Lane a) LG3 DNA cleaved with *Eco*RI; (lanes b and c) HFEM (*ts*LB2) *Eco*RI-*K*₁,*K*₂ fragment without and with *Kpn* I digestion, respectively; (lanes d and e) HSV-2(G) *Eco*RI-M fragment without and with *Kpn* I digestion, respectively; (lanes f and g) LG3 *Eco*RI-*K*₁ fragment without and with *Kpn* I digestion, respectively; (lanes h and i) LG3 *Eco*RI-*K*₂ fragment without and with *Kpn* I digestion, respectively.

this conclusion is reinforced by the fact that fragments from the same DNA do not rescue these mutants.

(v) The data are consistent with the hypothesis that *ts*LB2 and *ts*D are mutations in the gene for ICP 4 and consequently HSV can be at least partially diploid for this gene. These conclusions are based on several observations. The mutations map within the boundaries of the template of ICP 4 (7). Furthermore, the ICP 4 produced by the mutants at the nonpermissive temperature is not processed efficiently (9). However, it is not clear whether all of the coding sequences for ICP 4 map within the repeated *ca* sequence.

(vi) A portion of the *a'**c'* and *ca* sequences may contain region(s) of obligatory identity. More specifically, we postulate that a portion of the *a'**c'* sequence serves as a template for the obligatory repair of *ca* and that, in some instances, the entire *a'**c'* sequence acts as a template for the maintenance of the integrity of the *ca* region. The isolation of the recombinants DG1 and DG9 containing identical insertions in both ends of the S component is the strongest evidence that one region of the terminal repeats serves as a template for the other end. Two

such unusual, but identical, insertions in the same clone can only be explained by the postulate that the insertion occurred in one end of the S component and was copied into the other. It is also interesting to note that unique and repetitive HSV-2 DNA sequences have been inserted within the HSV-1 reiterated region and become part of an expanded reiterated region of the recombinant S component. In the heterodiploid clones LG3 and LG19, the region of identity of the reiterated regions is located near and perhaps extending within the *a* sequence of the *ca* region.

(vii) The observations of diploidy and autologous insertions—novel features for viruses infecting animal cells—raises several interesting predictions and possibilities. First, it is clear that correction of genetic lesions by recombination with a DNA restriction fragment will not invariably result in a simple replacement of the region carrying the defect by the wild-type DNA. This conclusion may be particularly significant in attempts to correct genetic defects in animal cells if reiterated sequences near the gene are located elsewhere in the genome. Second, because of the ability to tolerate insertions or expansion within the S component, wild-type HSV DNA could accommodate other viral genes or even heterologous DNA.

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