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Inversion Events in the HSV-1 Genome Are Directly Mediated by the Viral DNA Replication Machinery and Lack Sequence Specificity

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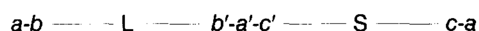
Summary

The bacterial transposable element Tn5 was observed to undergo high-frequency sequence inversion when integrated into the herpes simplex virus type 1 (HSV-1) genome. Deletion analysis of the IS50 elements through which this recombination event occurred demonstrated the absence of *cis*-acting signals involved in the inversion process. Several observations suggested an intimate association of the recombination mechanism with HSV-1 DNA replication, including the ability of the seven viral genes that are essential for HSV-1 DNA synthesis to mediate Tn5 inversion in the absence of any other viral functions. Comparable results were obtained by using duplicate copies of the L-S junction of the HSV-1 genome. Thus inversion of the L and S components of the HSV-1 genome during productive infection does not appear to be a site-specific process, but rather is the result of generalized recombination mediated by the complex of gene products that replicate the viral DNA.

Introduction

The genome of herpes simplex virus type 1 (HSV-1) is a 152 kb linear double-stranded DNA molecule that consists of a 108 kb long (L) and a 13 kb short (S) component. The L and S components are each flanked by the inverted repeat sequences *b* (9 kb) and *c* (6.5 kb), respectively, and are both bracketed by the inverted repeat sequence *a* (250–500 bp), as shown in the following arrangement:



(Sheldrick and Berthelot, 1974; Wadsworth et al., 1975; Hayward et al., 1975).

The L and S components of the HSV-1 genome have been demonstrated to invert relative to each other, resulting in the formation of four equimolar isomeric populations of viral DNA (Hayward et al., 1975; Delius and Clements, 1976). It has been suggested that these inversions are due to homologous recombination between the inverted repeats flanking the L and S components (Sheldrick and Berthelot, 1974; Smiley et al., 1981). Later studies using engineered duplications of viral restriction fragments have implicated the *a* sequence as the *cis*-acting element through which a site-specific recombination event occurs (Mocarski et al., 1980; Mocarski and Roizman, 1981, 1982b; Chou and Roizman, 1985). The existence of a virally encoded, *trans*-acting function that mediates the inversion process was inferred by the inability of plasmid-borne *a* sequences to undergo recombination without HSV-1 superinfection (Mocarski and Roizman, 1982a). However, little is known about the molecular mechanism of this recombination event or the putative viral functions involved.

The bacterial transposable element Tn5, which consists of two inverted 1.5 kb IS50 insertion elements flanking a 2.8 kb central unique region containing a kanamycin/neomycin resistance determinant, has been widely used for insertion mutagenesis in prokaryotes (Berg and Berg, 1983; deBruijn and Lupski, 1984). Recently, we have developed a rapid means of identifying HSV-1 genes that are nonessential for replication in cell culture, using this transposon. Cloned HSV-1 restriction fragments containing Tn5 insertions in three S component genes were recombined into the viral genome without impairment of virus production in cell culture (Weber et al., 1987). In this report, we show that copies of Tn5 inserted into the HSV-1 genome undergo sequence inversion via high-frequency recombination between the duplicated IS50 elements of the transposon. The *cis*- and *trans*-acting functions required for Tn5 inversion were systematically analyzed in transient expression assays. The results of these studies indicate that the HSV-1 DNA replication machinery itself directs recombination between duplicated segments within the viral genome in a manner that is independent of specific DNA sequences. These findings strongly suggest that a similar mechanism underlies L and S component inversion in the HSV-1 genome, and have led to the reinterpretation of results from earlier studies concerning the role of the *a* sequence in genome isomerization.

Results

Tn5 Undergoes Sequence Inversion when Inserted into the HSV-1 Genome

HSV-1 (strain KOS) viruses containing Tn5 insertions within the US2, US4, and US5 genes were constructed in a previous study (Weber et al., 1987). During the characterization of these mutant viral genomes by restriction endonuclease and Southern blot analyses, it was observed that certain restriction digests yielded unexpected fragments. The analysis of one of these genomes, US2::Tn5, is presented in Figure 1. Restriction enzymes that cleave within the inverted IS50 repeats of Tn5 (PstI or HindIII) yielded the expected restriction fragments, while enzymes that cleave asymmetrically within the central unique region of the transposon (BamHI or SalI) generated not only the predicted restriction fragments, but an equivalent

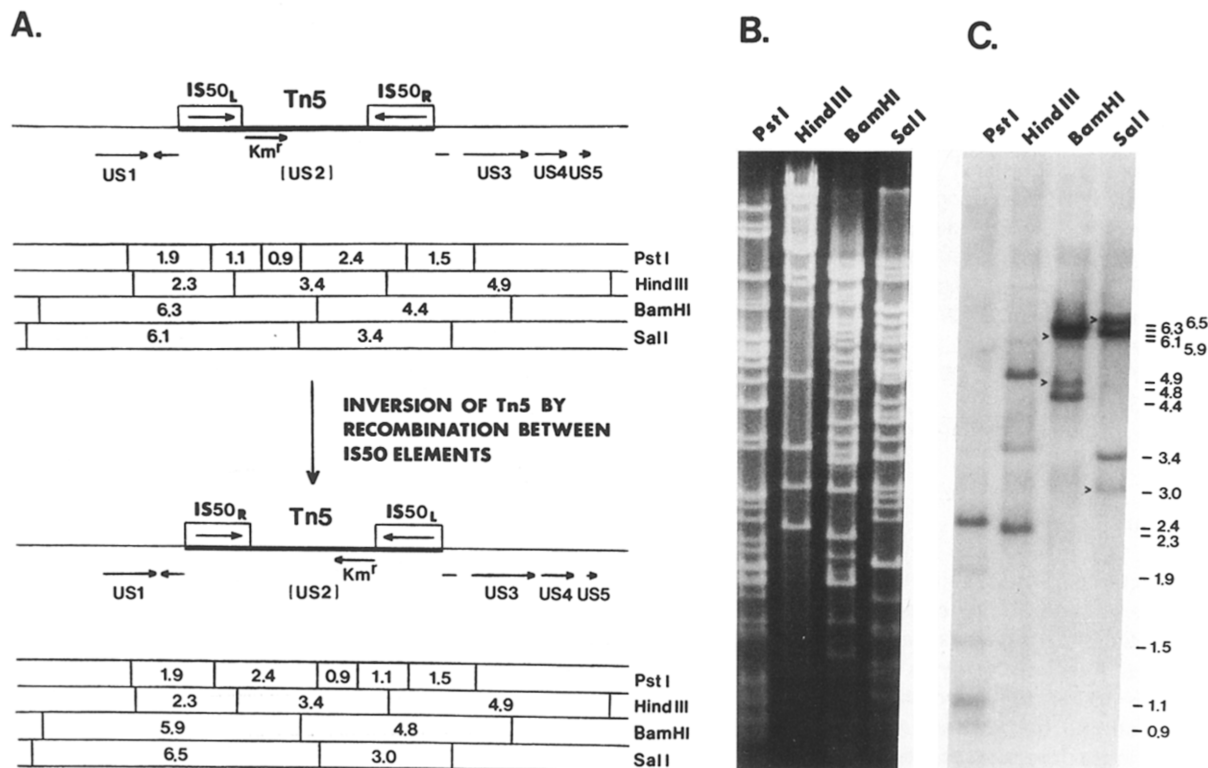


Figure 1. Inversion of Tn5 within the HSV-1 Genome

(A) Restriction map of HSV-1(KOS) US2::Tn5 DNA. The Tn5 transposon was inserted into the US2 open reading frame of the unique region (U_S) of the S component of the viral genome (Weber et al., 1987), thereby creating 3 novel PstI, 2 HindIII, 1 BamHI, and 1 SalI restriction sites. The upper panel shows the Tn5 insertion in its native orientation (i.e., the original orientation of Tn5 in the cloned HSV-1 restriction fragment before marker transfer into the viral genome), and the lower panel shows the Tn5 insertion after recombination between the $IS50_L$ and $IS50_R$ sequences has inverted the central unique region. Note that only BamHI and SalI, which cleave asymmetrically within the central unique region and not within the IS50 repeats, generate novel restriction fragments as a result of Tn5 inversion. Km^r = kanamycin phosphotransferase gene. (B) Agarose gel of restriction digests of US2::Tn5 genomic DNA. (C) Southern blot of gel in (B), hybridized with pUC19::Tn5 DNA radiolabeled with ^{32}P by nick translation. The novel restriction fragments resulting from inversion of Tn5 sequences are indicated by arrows.

number of novel fragments as well (Figures 1B and 1C). For example, digestion of US2::Tn5 DNA produced two new SalI fragments (6.5 and 3.0 kb) in addition to the expected 6.1 and 3.4 kb fragments. These novel restriction fragments could be explained only if the central unique region of Tn5 were able to invert as a result of recombination between its flanking IS50 elements, thereby repositioning the BamHI and SalI restriction sites of the transposon (Figure 1A). An identical phenomenon was also observed for the US4::Tn5 and US5::Tn5 mutant viruses (data not shown), suggesting that the Tn5 inversion process can occur equally well in different locations in the HSV-1 genome. This high-frequency inversion event creates two essentially equimolar isomeric forms of the mutant viral DNA, an outcome that is analogous to the generation of four equimolar isomers of HSV-1 DNA by the inversion of the L and S genomic components (Hayward et al., 1975; Delius and Clements, 1976). However, this inversion does not alter the actual insertion site of the transposon within the viral genome. These observations suggested that further characterization of the *cis*- and *trans*-acting functions involved in Tn5 inversion would help elucidate the little understood mechanism of HSV-1 genome isomerization.

Dependence of Tn5 Inversion on an HSV-1 Origin of DNA Replication in *Cis* and on Viral Gene Products in Trans

In order to analyze better the requirements for Tn5 inversion in the HSV-1 genome, the transposon was jumped onto the pUC19 derivative pMC110 (Figure 2A). This plasmid contains a functional origin of HSV-1 DNA replication (ori_S) and replicates only in the presence of active HSV-1 infection (Challberg, 1986). Restriction enzyme analysis of pMC110::Tn5 DNA from transfected Vero cells revealed novel fragments resulting from Tn5 inversion only if superinfecting HSV-1 was present; the plasmid was also clearly amplified by the viral DNA replication machinery (Figure 2C). These results suggest that Tn5 inversion requires a *trans*-acting function(s) encoded by HSV-1 and, possibly, replication of the transposon DNA.

To investigate the role of DNA synthesis in Tn5 inversion, the transposon was jumped onto a pUC19 plasmid that lacked a viral origin of replication (Figure 2B). Tn5 inversion was not observed in this plasmid after transfection and HSV-1 superinfection, even if the plasmid was digested with EcoRI before transfection to simulate linear viral DNA (Figure 2C). These results indicated that the

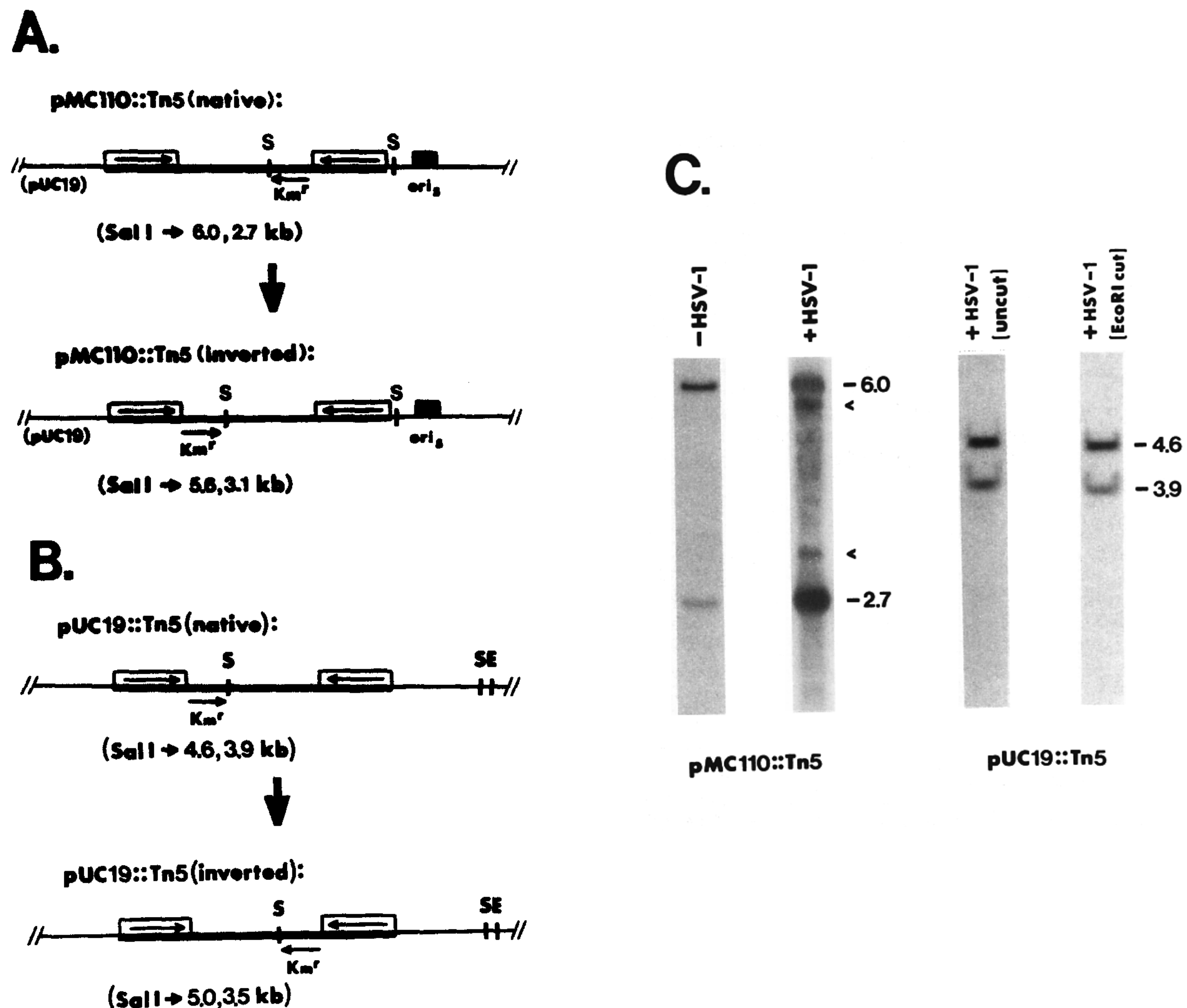


Figure 2. Requirements for Tn5 Inversion in HSV-1 Shuttle Plasmids

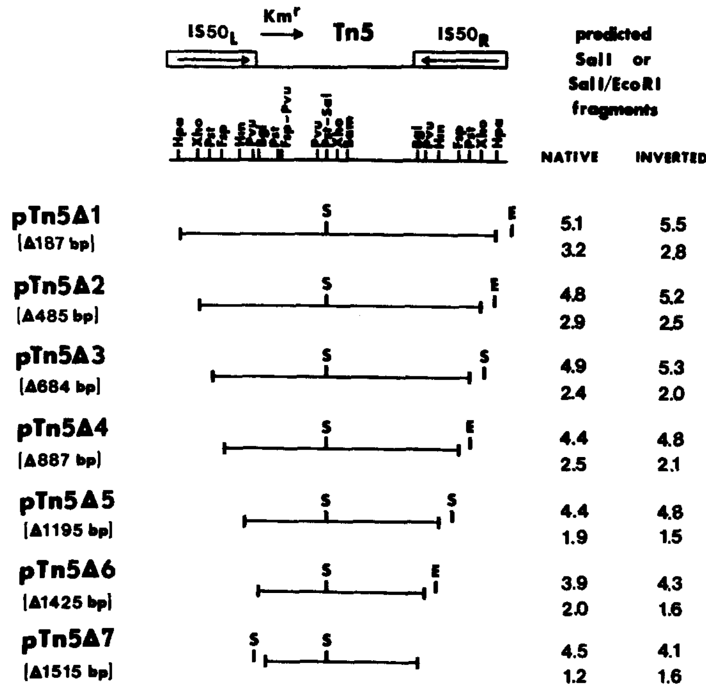
Tn5 was jumped onto the pUC19–ori$_S$ vector pMC110 (A) and onto pUC19 (B) as described in Experimental Procedures. The molecular weights of the SalI (S) restriction fragments of both plasmids in their native and inverted orientations are indicated. In one experiment, pUC19::Tn5 DNA was first linearized by cleavage with EcoRI at the site shown (E) prior to transfection. (C) Southern blot analysis of SalI-digested pMC110::Tn5 and pUC19::Tn5 DNA isolated after transfection and subsequent superinfection with HSV-1 where indicated. Restriction fragments corresponding to inversion products are identified by arrows. All autoradiographs were derived from identical exposure times.

recombination function(s) that mediates Tn5 inversion needs an actively replicating DNA template. The requirement for this DNA synthesis to be initiated at an HSV-1 origin was demonstrated in experiments described below.

Tn5 Inversion Is Mediated by Homologous Rather than Site-Specific Recombination between the IS50 Elements of the Transposon

It was unclear whether the high frequency of Tn5 inversion observed in the HSV-1 genome was due to generalized recombination between the IS50 repeats of the transposon or to site-specific recombination. The latter possibility seemed likely since not all duplicated sequences in HSV-1 mediate inversion events, and a site-specific recombination system has been postulated to mediate inversion of the L and S genomic components of the virus (Mocarski et al., 1980; Mocarski and Roizman, 1982b). To address this question, use was made of the ability of Tn5 to invert in the vector pMC110 (Figure 2). In addition to an HSV-1 origin of DNA replication, pMC110 contained all of the cloning sites of pUC19, into which deletion derivatives of Tn5 could be inserted. The use of this shuttle vector in transfections eliminated the need to recombine the Tn5 deletions back into the viral genome by marker transfer.

Initially, seven sequential deletions of 200–300 bp in the two IS50 elements of Tn5 were constructed (plasmids pTnΔ1 through pTn5Δ7, shown in Figure 3A). Since these deletions extended inward from the termini of the transposon, the distance between the recombination sites remained constant, so that the frequency of recombination would not be affected. These constructions were transfected into Vero cells and superinfected with HSV-1 prior to isolation and restriction analysis of DNA. As shown in Figure 3B, novel bands resulting from Tn5 inversion were visible in pTn5Δ1 through pTn5Δ3, but not in pTn5Δ4 through pTn5Δ7. Thus recombination between IS50 ele-



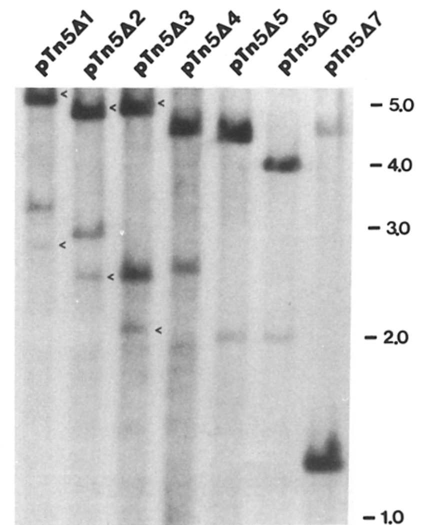


Figure 3. Determination of the IS50 Sequences That Mediate Tn5 Inversion

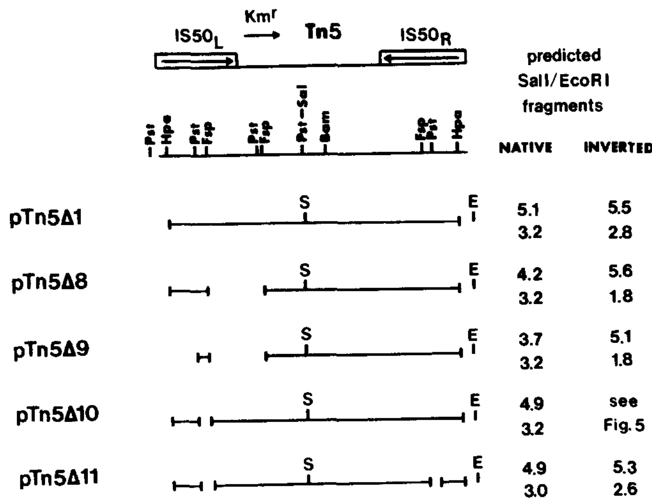
(A) Construction of Tn5 deletion derivatives in pMC110. Identical deletions of 200–300 bp in both IS50 elements of Tn5 were generated by cloning the denoted Tn5 restriction fragments into apropriate sites in pMC110 (see Experimental Procedures). The sizes of the deletions in the 1534 bp IS50 elements are indicated below each plasmid name. Two restriction sites on each construction were used to visualize Tn5 inversion: the unique SalI site in Tn5, and either the unique SalI site (for pTn5Δ3, pTn5Δ5, and pTn5Δ7, or the unique EcoRI site (for pTn5Δ1, pTn5Δ2, pTn5Δ4, and pTn5Δ6, all of which lost the unique SalI–HincII site during cloning) in the pMC110 vector. The molecular weights of the SalI or SalI–EcoRI restriction fragments for the native and inverted orientatins of each plasmid are indicated on the right. (B) Southern blot analysis of SalI- or SalI–EcoRI-digested plasmid DNA isolated after transfection and subsequent superinfection with HSV-1. Restriction fragments corresponding to inversion products are indicated by arrows.

ments could be readily observed until sequences 684–887 bp from the end of the transposon (i.e., between the PstI and FspI sites of IS50) and inward were deleted, suggesting that a target sequence for site-specific recombination may exist within this 204 bp span of DNA. Alternatively, these results could be interpreted to suggest that Tn5 inversion is mediated by a generalized recombination process that requires a critical length of homology between IS50 repeats to be detected by Southern blot analysis.

To distinguish between these two possibilities, a second set of constructions was prepared by creating deletions in the inverting Tn5 derivative pTn5Δ1 (Figure 4A). Elimination of IS50 sequences internal to the FspI site at 887 bp (plasmid pTn5Δ8) did not abolish Tn5 inversion (Figure 4B), which was consistent with the hypothesis that a signal for site-specific recombination was present 684–887 bp from the end of Tn5. This construction also demonstrated that the distance between the IS50 repeats could be varied without adversely affecting the ability of Tn5 to undergo sequence inversion. However, deletion of all IS50 sequences internal to the FspI site at 887 bp as well as external to the PstI site at 684 bp (plasmid pTn5Δ9) resulted in a Tn5 derivative that failed to invert (Figure 4B). This indicated that the segment of IS50 684–887 bp from the end of Tn5 did not contain a target sequence for site-specific recombination. In agreement with this conclusion, the converse construction, in which only this 204 bp sequence was deleted from both IS50 elements (plasmid pTn5Δ11), was unimparied in its ability to invert (Figure 4B). Thus removal of the putative site-specific recombination signal had no effect on the inversion process.

Taken together, the results of this deletion analysis demonstrated that Tn5 inversion was the result of homologous recombination between the two IS50 elements of the transposon, and that no specific sequences within the IS50 repeats were recognized by the HSV-1 recombination system. Tn5 inversion also appeared to require greater than 600 bp of IS50 homology for detectable recombination to occur. Inversion was not observed for the Tn5 deletion derivatives pTn5Δ4, pTn5Δ5, pTn5Δ6, pTn5Δ7, and pTn5Δ9, each of which had 600 bp of IS50 homology or less, while all other constructions tested had 700 bp of IS50 homology or more and underwent detectable inversion.

A final demonstration of the lack of sequence specificity required for Tn5 inversion was provided by the analysis of pTn5Δ10, a construction that contains the 204 bp deletion



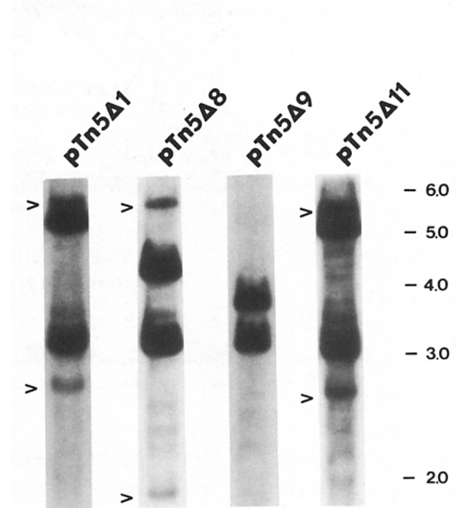
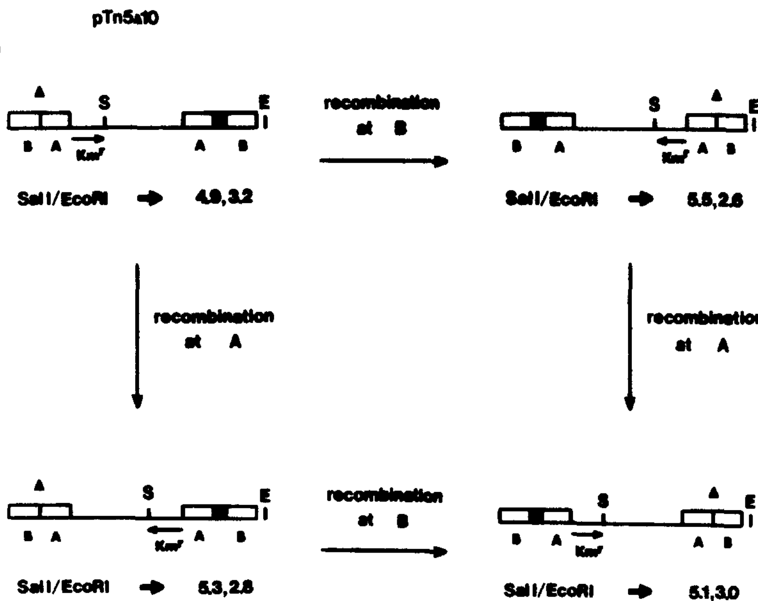


Figure 4. Determination of the IS50 Sequences That Mediate Tn5 Inversion

(A) Construction of deletion derivatives of pTn5Δ1. Deletions were generated in the inverting Tn5 derivative pTn5Δ1 by using PstI and FspI sites within the transposon (see Experimental Procedures). The unique SalI site in Tn5 and the unique EcoRI site in the pMC110 vector were used to visualize Tn5 inversion. The molecular weights of the SalI–EcoRI restriction fragments for the native and inverted orientations of each plasmid are indicated on the right. (B) Southern blot analysis of SalI–EcoRI-digested plasmid DNA isolated after transfection and subsequent superinfection with HSV-1. Restriction fragments corresponding to inversion products are indicated by arrows.

of pTn5Δ11 in only one of the two IS50 repeats (Figure 4A). Since the sequences flanking the 204 bp deletion in IS50$_L$ of pTn5Δ10 are similar in size, they should have equivalent recombinogenic potential. If equal frequencies of recombination between these two sequences (termed region A and region B) and their homologs in IS50$_R$ do occur, then three novel forms of pTn5Δ10 should arise after inversion (Figure 5A). Restriction fragments corresponding to each of the four predicted structures of pTn5Δ10 were indeed detected when transfected plasmid



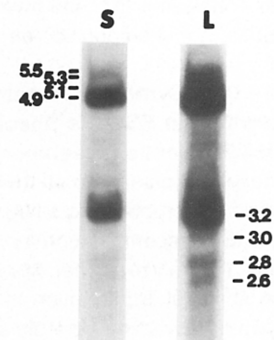


Figure 5. Inversion Products of pTn5Δ10

(A) Generation of three distinct inversion products from the native pTn5Δ10 plasmid following recombination at sequences either internal (region A) or external (region B) to the 204 bp deletion (Δ) present in IS50$_L$. The unique SalI site in Tn5 and the unique EcoRI site in the pMC110 vector were used to visualize Tn5 inversion. The molecular weights of the SalI–EcoRI restriction fragments of each pTn5Δ10 derivative are indicated. (B) Southern blot analysis of SalI–EcoRI-digested plasmid DNA isolated after transfection and superinfection with HSV-1. Restrictions fragments corresponding to those predicted in (A) are identified by molecular weight on short (S) and long (L) exposures of the autoradiograph.

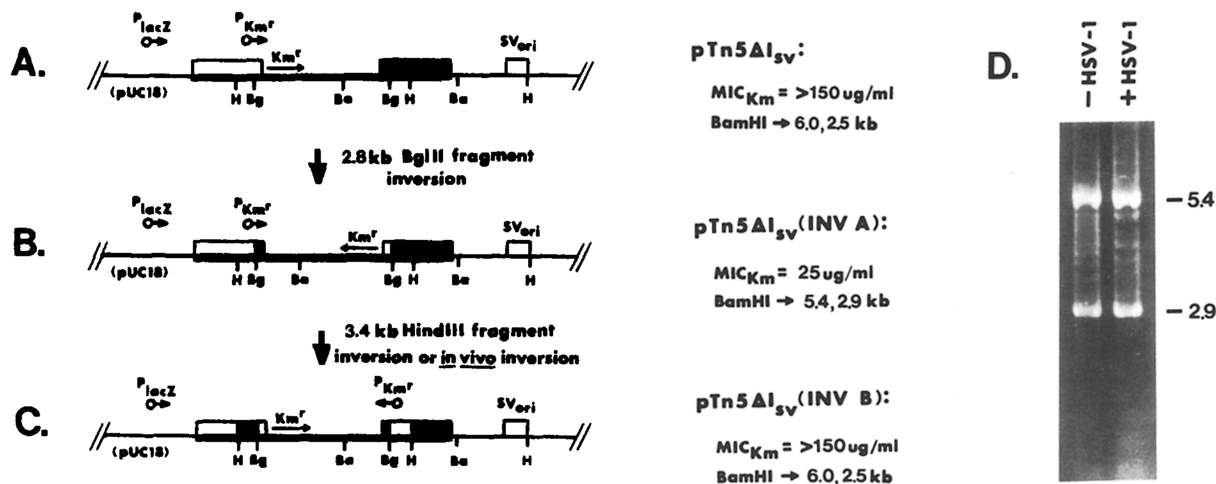


Figure 6. Construction of an SV40 Shuttle Plasmid for the Rapid Quantitation of Tn5 Inversion Events in COS7 Cells

(A) Construction of pTn5Δ1$_{sv}$. The 5.4 kb HpaI fragment of Tn5 (the same fragment cloned into pMC110 to yield the inverting Tn5 derivative pTn5Δ1) was cloned into the SmaI site of pCW522 in the orientation shown to yield pTn5Δ1$_{sv}$. The pCW522 vector was constructed by cloning a 0.4 kb PstI-HindIII fragment containing one of the two SV40 origins of replication in pgD-DHFR (Berman et al., 1983) into pUC18. The resulting plasmid was a shuttle vector that had retained the cloning sites of pUC18 and lost the "poison sequences" of pBR322 that inhibit SV40 DNA replicatiion (Lusky and Botchan, 1981). The 5.4 kb HpaI fragment of Tn5 lacked the three promoters that transcribe the transposase gene as well as a cruciform DNA structure that terminates transcription originating outside of Tn5 (Krebs and Reznikoff, 1986). As a result, the kanamycin phosphotransferase gene (Km^r) of pTn5Δ1$_{sv}$ could be transcribed either by its natural promoter in IS50 (P Km^r) or by the *lacZ* gene promoter of pUC18 immediately adjacent to the HpaI fragment (P lacZ). (B) Construction of pTn5Δ1$_{sv}$(INV A). The central 2.8 kb BglII fragment of pTn5Δ$_{sv}$ was inverted to yield pTn5Δ$_{sv}$(INV A). This forced the Km^r gene to be transcribed by the extremely weak Km^r gene promoter in IS50$_R$ (Rothstein and Reznikoff, 1981), resulting in greatly reduced host cell resistance to kanamycin (minimum inhibitory concentration >25 μg/ml). (C) Construction of pTn5Δ1$_{sv}$(INV B). The central 3.4 kb HindIII fragment of pTn5Δ1$_{sv}$(INV A) was inverted to yield pTn5Δ1$_{sv}$(INV B). As a result, the Km^r gene was transcribed by the *lacZ* promoter of pUC18. Since this promoter operates at constitutive levels in E. coli strain DH1, pTn5Δ1$_{sv}$(INV B) conferred normal levels of kanamycin resistance on its host cell. This in vitro construction mimics what would happen if the Tn5 sequences of pTn5Δ1$_{sv}$(INV A) inverted in vivo in COS7 cells. Thus, by rescuing plasmid DNA from COS7 cells in E. coli DH1 (a *recA* strain that "freezes" the Tn5 orientation) and comparing the number of transformants resistant to 100 μg/ml of kanamycin with the total number of ampicillin-resistant transformants, an accurate assessment of the ability of Tn5 to invert in an SV40 minichromosome can be obtained. However, no kanamycin-resistant transformants were observed after rescue of pTn5Δ1$_{sv}$(INV A) DNA from COS7 cells (see Results). The ampicillin-resistant transformants were then pooled and their plasmid DNA analyzed. As indicated in (B) and (C), the native and inverted orientations of Tn5 in pTn5Δ1$_{sv}$(INV A) have characteristic BamHI restriction fragments. BamHI digestion of plasmid DNA from the pooled transformants (D) revealed the absence of the inverted form of pTn5Δ1$_{sv}$(INV A), which confirmed the kanamycin-sensitive phenotype. The presence or absence of HSV-1 superinfection after transfection of plasmid DNA is indicated above the lanes.

DNA was analyzed (Figure 5B). These results clearly demonstrate that recombination could readily occur through at least two distinct regions of IS50, which is consistent with the conclusion that Tn5 inversion is mediated by homologous, not site-specific, recombination.

The DNA Synthesis Requirement of Sequence Inversion in HSV-1 Is Specific for the HSV-1 DNA Replication Machinery

The results presented above (Figure 2) demonstrated that an actively replicating DNA template was necessary for the HSV-1 recombination function(s) to mediate Tn5 inversion. To analyze further this DNA synthesis requirement, the ability of Tn5 to invert in an SV40 shuttle vector was examined by using a simple and highly sensitive recombination assay. An SV40 shuttle plasmid, pTn5Δ1$_{sv}$(INV A), was constructed in which the kanamycin phosphotransferase gene of Tn5 is activated after the transposon undergoes sequence inversion (Figure 6). Upon rescue of DpnI-resistant Hirt extract DNA from transfected COS7 cells in E coli, the degree of Tn5 inversion in pTn5Δ1$_{sv}$(INV A) can be rapidly and accurately assessed by calculating the percent of kanamycin-resistant transformants.

In duplicate experiments, 1100 and 3800 transformants per microgram of Hirt extract DNA were obtained after transfection of pTn5Δ1$_{sv}$(INV A), with and without HSV-1 superinfection, respectively. In contrast to this high level of rescue, fewer than 5 transformants per microgram of Hirt extract DNA were observed after transfection of pTn5Δ1, which lacks an SV40 origin of replication. This demonstrated that the DpnI digestion step was successful in eliminating unreplicated plasmid DNA. However, none of the rescued pTn5Δ1$_{sv}$(INV A) transformants were found to be resistant to kanamycin. This lack of Tn5 inversion, even if superinfecting HSV-1 was present after transfection, was confirmed by restriction endonuclease analysis of plasmid DNA isolated from pooled pTn5Δ1$_{sv}$(INV A) transformants (Figure 6D).

These results demonstrated that the HSV-1 gene product(s) that acts in *trans* to mediate Tn5 inversion does not function if the transposon DNA is replicated through a non-HSV-1 origin of DNA synthesis. The ability of Tn5 to invert under these conditions was not due to differences in host cell backgrounds, since Tn5 inversion in a replicating pMC110 plasmid occured at similar frequencies in both Vero and COS7 cell lines (data not shown). In addi-

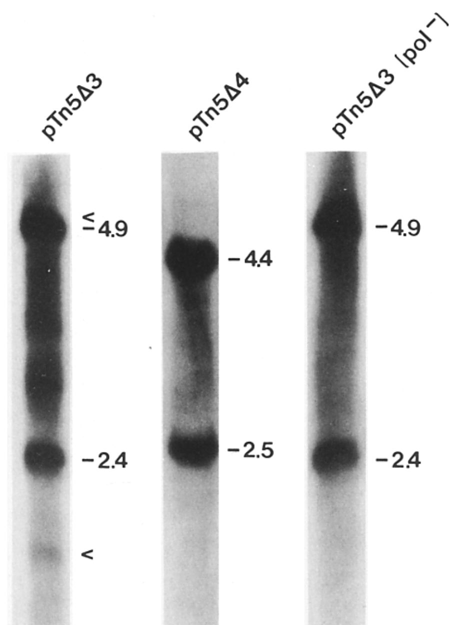


Figure 7. Tn5 Inversion in a Transient Replication Assay Containing Only the Seven HSV-1 Genes Required for DNA Synthesis

The Tn5 derivatives pTn5Δ3 and pTn5Δ4 were replicated in transient assays (Challberg, 1986; Wu et al., 1988) and subjected to Souther blot analysis as described in Experimental Procedures. The construction and the predicted inversion products of pTn5Δ3 and pTn5Δ4 are illustrated in Figure 3. In the final lane, the HSV-1 DNA polymerase gene was omitted from the transfection mix. Restriction fragments corresponding to inversion products are indicated by arrows.

tion, the supercoiled chromatin-like structure of the SV40 minichromosome was not refractory to the action of recombination proteins, since a dimer form of the shuttle vector could be resolved into its monomeric components after replication in COS7 cells (Weber, unpublished observations). This indicated that the pTn5Δ1_{sv}(INV A) plasmid used in these experiments was indeed a suitable template for recombination.

The Seven HSV-1 Genes that Direct DNA Synthesis in Transient Assays Can Also Promote Tn5 Inversion

Recently, the minimum complement of HSV-1 genes that can mediate DNA synthesis in a transient replication assay has been determined (Challberg, 1986; Wu et al., 1988). These seven genes encode the viral DNA polymerase, the major DNA-binding protein (ICP8), and five other proteins with unknown roles in DNA replication. The results obtained using SV40 shuttle plasmids indicated that the HSV-1 gene product(s) that mediates recombination is intimately associated with this viral DNA replication machinery. Thus, cloned HSV-1 restriction fragments were added to the mixture of plasmids required for transient DNA replication in an attempt to map the gene(s) that encodes the viral recombination function. A pMC110 vector carrying Tn5 sequences was used as the recombination target.

The results of these assays revealed that the seven HSV-1 DNA replication enzymes alone mediated Tn5 inversion; no additional viral gene products were required for detectable recombination to occur. Novel restriction fragments resulting from sequence inversion were observed in pTn5Δ3 but not in pTn5Δ4 (Figure 7), indicating that recombination in the transient replication assay still required greater than 600 bp of IS50 homology to occur at detectable levels. In addition, Tn5 inversion was abolished if any of the seven replication genes (in the example shown, the polymerase gene) were omitted from the transfection mix (Figure 7), verifying the absolute requirement for DNA synthesis in HSV-1 recombination.

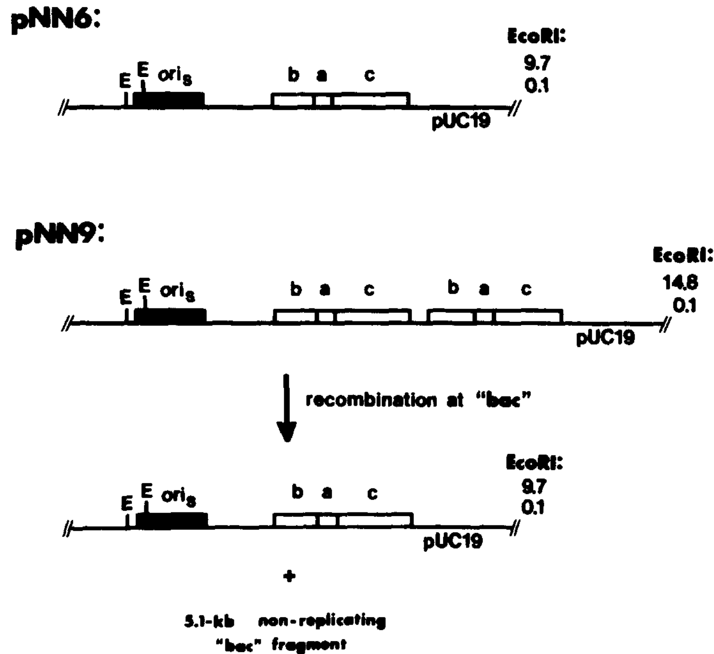
Similar results were obtained by using a plasmid containing duplicate copies of the *b-a-c* junction, the region of the HSV-1 genome through which L and S component inversion occurs (Mocarski et al., 1980; Smiley et al., 1981). A plasmid carrying a single *b-a-c* sequence, pNN6, was propagated normally by the seven viral DNA replication genes and yielded a single 9.7 kb fragment after digestion with EcoRI (Figure 8). However, a plasmid containing two direct repeats of the *b-a-c* sequence, pNN9, was found to undergo a deletion event mediated by recombination across the duplicated segments. This resulted in the appearance of a novel 9.7 kb fragment in addition to the predicted 14.8 kb fragment in EcoRI-digested pNN9 DNA isolated from transient replication assays (Figure 8). Thus recombination in HSV-1 is mediated by a function associated with the HSV-1 DNA replication machinery, not by a separate viral gene product, and this mechanism is very likely to be responsible for all sequence inversions observed in the HSV-1 genome.

Discussion

In an earlier work, it was shown that the HSV-1 genome could stably accommodate insertions of the bacterial transposon Tn5 (Weber et al., 1987). A closer inspection of these mutant virus genomes in this study led to the surprising finding that Tn5 undergoes sequence inversion via a high-frequency recombination event between its duplicated IS50 elements. This phenomenon is in marked contrast to the behavior of the transposon in its natural bacterial host, where Tn5 inversion occurs at such a low frequency (approximately 10^{-3} in recombination-proficient strains) that specialized systems are required to detect it (Yagil et al., 1980; Weber, Levine, and Glorioso, submitted). A similar high level of sequence inversion was observed when Tn5 was inserted into the 2μm plasmid of yeast (Jayaram and Broach, 1983); however, this was later found to be due to a gene conversion–like event, rather than site-specific recombination, mediated by the plasmid-encoded FLP protein (Jayaram, 1986). In contrast, the results of our study show that Tn5 inversion in the HSV-1 genome is due to a true recombination event. To our knowledge, this is the first demonstration of sequence inversion in HSV-1 mediated through non-HSV-1 DNA and, more generally, the only known recognition of prokaryotic DNA sequences by a eukaryotic recombination system.

The inversion of Tn5 sequences was entirely analogous to the L and S component inversion that occurs in the HSV-1 genome. Like the genomic inversions, which gener-

A.



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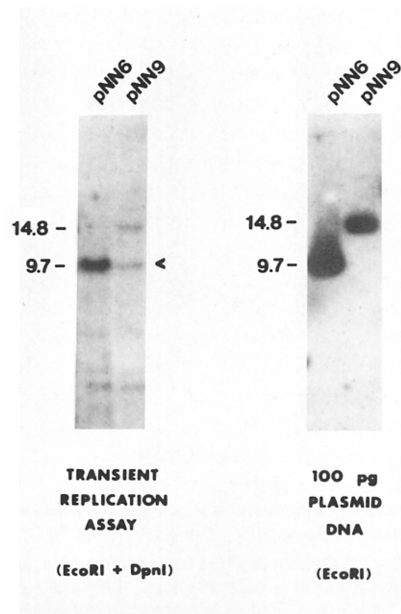

Figure 8. Recombination between HSV-1 *b-a-c* Sequences in Transient Replication Assay Containing Only the Seven HSV-1 Genes Required for DNA Synthesis

(A) Construction of plasmids containing *b-a-c* sequences. pNN6 was generated by inserting the 2.0 kb BamHI X fragment (which contains an origin of replication ori_S) and the 5.1 kb BamHI SQ fragment (which contains the *b-a-c* junction) of HSV-1 into the SmaI and HindIII sites, respectively, of pUC19. pNN9 was constructed by inserting a second BamHI SQ fragment into the BamHI site of pNN6 in direct orientation to the resident BamHI SQ fragment. Recombination between the tandem *b-a-c* sequences of pNN9 is predicted to generate a plasmid that is essentially identical to pNN6 plus a nonreplicating fragment that contains a *b-a-c* sequence. The molecular weights of the EcoRI fragments for all plasmids are listed on the right, and sequences corresponding to the BamHI X and SQ fragments are indicated by black and white boxes, respectively, in the diagrams. (B) Southern blot analysis of pNN6 and pNN9 after replication by the minimum complement of HSV-1 DNA replication genes in transient assays (Challberg, 1986; Wu et al., 1988). Restriction fragments corresponding to recombination products are indicated by arrows, and EcoRI digests of untransfected plasmid DNAs (at right) are included as controls. ^{32}P-labeled pUC19 DNA was used as the probe in blot hybridizations.

ate four equimolar isomeric forms of viral DNA (Hayward et al., 1975; Delius and Clements, 1976), Tn5 inversion created two additional equimolar isomers of viral DNA (Figure 1). The fortuitous observation that Tn5 inverts within the viral genome was the basis for the development of a useful model system for studying the mechanisms underlying HSV-1 genome isomerization and viral recombination in general. Through the use of shuttle plasmids carrying both prokaryotic and viral origins of replication and recently developed transient DNA replication assays (Challberg, 1986), the *cis-* and *trans-*acting requirements for Tn5 inversion could be readily analyzed. These experiments were performed out of the context of the HSV-1 genome, thereby eliminating the need for complex genetic manipulations of the virus itself. The results of this study indicate that recombination in HSV-1 can no longer be viewed simply as a viral gene product acting upon a specific DNA sequence, in the manner of well-characterized prokaryotic and yeast recombination systems (Sternberg and Hamilton, 1981; Broach, 1982). Rather, the inversion of DNA sequences in HSV-1 is caused by an apparently nonspecific event between two duplicated segments and is directly mediated by the viral gene products that replicate the genomic DNA.

DNA Sequences That Mediate Recombination in HSV-1

Initial restriction enzyme site mapping of HSV-1::Tn5 genomes (Figure 1) indicated that Tn5 inversion was the result of recombination between the duplicated IS50 elements of the transposon. Deletion analysis of the IS50 repeats (Figures 3, 4, and 5) demonstrated that this phenomenon was not mediated by site-specific recombination occurring at a particular sequence of the transposon. Rather, the inversion was the result of a generalized recombination process that required greater than 600 bp of IS50 homology to be detected by Southern blot analysis. Thus the machinery that mediates inversion of the L and S components of the HSV-1 genome can also readily promote sequence inversion in an unrelated prokaryotic DNA by nonspecific recombination mechanisms. As discussed above, this is not a trivial result in that high-frequency recombination between the two IS50 elements has not been reported in other biological systems.

These obervations suggest that the large inverted duplications flanking the L and S components (i.e., the *b* and *c* sequences, respectively) should be adequate regions of homology for generating the genomic inversions of HSV-1. This hypothesis was first proposed in the original structural characterizations of the HSV-1 genome (Sheldrick and Berthelot, 1974) as well as in later studies (Smiley et al., 1981). However, the demonstration that duplication of the *b-a-c*, *b-a*, and *a-c* sequences could cause additional genomic isomerization led to the interpretation that the *a* sequence was the *cis*-acting signal through which a site-specific recombination event occurred (Mocarski et al., 1980; Mocarski and Roizman, 1982b). This was subsequently supported by the ability of the *a* sequence to mediate L component inversion in the presence of heterotypic HSV-1 and HSV-2 *b* sequences (Davison and Wilkie, 1983) and in the absence of one copy of the *b* sequence (Davison et al., 1981). These studies convincingly demonstrate that the *a* sequence is sufficient for HSV-1 genome inversion; however, they do not in any way prove that the *a* sequence is required for isomerization. In fact, three lines of evidence support the conclusion of this report that the *a* sequence is not a target sequence recognized by an HSV-1 site-specific recombination system.

First, the deletion of the complete *b-a-c* sequence of the L-S junction has been shown to result in the formation of noninverting or "frozen" HSV-1 genomes (Poffenberger et al., 1983; Jenkins et all., 1985; Jenkins and Roizman, 1986). Longnecker and Roizman have recently characterized a virus in which the entire *b-a-c* junction is deleted except for one third of the *b* sequence. Although the S component was frozen, the L component of this mutant was shown to undergo considerable inversion by recombination across the remaining *b* sequences (Longnecker and Roizman, 1986). This demonstration of *a* sequence–independent isomerizaiton indicates that the inversion process can be mediated by homologous rather than site-specific recombination, which is consistent with the results presented in this report.

Second, a number of HSV-1 sequences other than the *a* sequence have been shown to promote recombination events when duplicated in the viral genome. These include the BamHI L fragment (Pogue-Geile et al., 1985), the glycoprotein C gene (Pogue-Geile et al., 1985), a subfragment of the BamHI N fragment containing the ICP4 promoter (Poffenberger et al., 1983; Jenkins et al., 1985; Jenkins and Roizman, 1986), and a portion of the *c* sequence (Vermuza and Smiley, 1985). This clearly shows that recombination can occur between any two regions of homology in the HSV-1 genome. However, not all sequences duplicated in the viral genome have been observed to mediate inversion events (Mocarski et al., 1980; Post and Roizman, 1981; Lee et al., 1982; Gibson and Spear, 1983). This is not surprising, since many recombination events within the HSV-1 genome can apparently escape detection by Southern blot analysis: the frequency of inversion in Tn5 derivatives that had less than 600 bp of IS50 homology was low enough to evade detection completely (Figures 3 and 4). In addition, some of these predicted inversion events may be lethal for the virus. For example, inversion of a segment flanked by two duplicated sequences may result in the formation of defective virus genomes (Smiley et al., 1981) or in the inactivation of an essential virus gene by displacement of its promoter or polyadenylation signals.

Finally, systematic dissection of the *a* sequence itself has led to inconclusive results concerning the *cis*-acting signal recognized by the putative site-specific recombination system. Chou and Roizman's suggestion that the 37 bp DR4 repeats of the HSV-1 strain F *a* sequence are the critical signal involved in inversion (Chou and Roizman, 1985) is weakened by the fact that all other characterized *a* sequences, including those of strains 17 and USA-8 (Davison and Wilkie, 1981), Justin (Mocarski et al., 1985), and KOS (Varmuza and Smiley, 1985), lack DR4 repeats. In contrast, Varmuza and Smiley found in similar studies that none of the subfragments of the HSV-1 strain KOS *a* sequence promoted high levels of the predicted genomic isomerization; however, low levels of recombination were detected for two SmaI fragments of the *b-a-c* junction (Varmuza and Smiley, 1985). One of these fragments contained the ten 11 bp DR2 repeats of the *a* sequence, and the other included the complex of reiterations 3′ of the ICP4 gene in the *c* sequence (Mocarski and Roizman, 1981; McGeoch et al., 1986). These authors suggested that L and S component inversion results not from a specific signal within the *a* sequence, but from the summed recombinogenic activity of sequences dispersed throughout the *b-a-c* junction. The results of Chou and Roizman are in fact consistent with this hypothesis: duplication of the DR2 repeats alone gave only low levels of inversion, but employing both the DR2 and the DR4 repeats enhanced this effect (Chou and Roizman, 1985).

Computer-based homology searches in the IS50 elements of Tn5 (Weber, unpublished observations) and the BamHI L fragment (D. McGeoch, personal communication), both of which promote high levels of sequence inversion, have revealed no significant homology to the reiterations of the *a* sequence, thus lending further support to the conclusion that these repeats do not mediate a site-specific recombination event. In addition, neither IS50 (this report) nor the BamHI L fragment (Pogue-Geile et al., 1985) undergoes cross-reactive recombination with the *a* sequence, which would be predicted if a site-specific recombination system existed in HSV-1.

Thus the *a* sequence does not appear to be a specific signal for mediating genome isomerization in HSV-1. However, the observation that even small subfragments of the *a* sequence can direct sequence inversions (Chou and Roizman, 1985; Varmuza and Smiley, 1985), and that one of the two cleavage-packaging signals present in the *a* sequence may promote illegitimate recombination events (Deiss et al., 1986), suggests that the *a* sequence may possess recombinogenic properties not found in other HSV-1 sequences. Although this possibility cannot be ruled out by the evidence presented in this report, it is clear from our studies on Tn5 inversion that sequence homology alone is sufficient for high-frequency recombination events to occur in the HSV-1 genome.

Alternatively, the GC-rich reiterations of the *a* sequence,

and of the *b-a-c* junction in general, may be inherently recombinogenic. At least eight different sets of tandem reiterations throughout the *b-a-c* junction have been described (summarized in Rixon et al., 1984). These sequences range in size from 11 bp to 37 bp, are repeated from a few to several dozen times, and are predominantly G-rich on one strand and C-rich on the other. The DR2 repeats, which are a typical example of this type of reiteration, have been shown to form a novel secondary structure (termed "anisomorphic" DNA) in which the complementary strands each assume different conformations because of their unusual base composition. The resulting supercoiling in an inherently inflexible sequence leads to stress and cracking in the overall DNA structure, which has been postulated to play a role in the biological activity of this region of the HSV-1 genome (Wohlrab et al., 1987). This observation, together with the results of Varmuza and Smiley (1985), suggests that these reiterations scattered throughout the *b-a-c* junction are the recombinational "hot spots" through which HSV-1 genome inversions are likely to be mediated. It is also worth noting that many illegitimate recombination events in HSV-1 have been shown to involve similar GC-rich sequences (Umene, 1986; Umene and Enquist, 1985; Mocarski et al., 1985; Knopf, 1987), and that one of the GC-rich reiterations of the *c* sequence may be related to an immunoglobulin class-switch signal (Gomez-Marquez et al., 1985).

Viral Functions That Mediate Recombination in HSV-1

Studies using shuttle plasmids demonstrated that Tn5 inversion could be detected only if viral gene products from an active infection were supplied in *trans* and if an HSV-1 origin of DNA replication was present in *cis* (Figure 2). The latter observaton indicated that the putative viral function that mediates Tn5 inversion had an absolute requirement for DNA synthesis. However, the failure of Tn5 to invert in an SV40 shuttle plasmid, even in the presence of superinfecting virus, demonstrated that this phenomenon was specific for HSV-1 DNA synthesis (Figure 6). Taken together, these results suggested that the HSV-1 function(s) that mediates sequence inversion is intimately associated with the viral DNA replication machinery. Direct evidence for this came from transient DNA replication assays, in which the seven HSV-1 genes that comprise the minimum complement of DNA synthesis enzymes (Wu et al., 1988) were shown to mediate detectable Tn5 inversion (Figure 7). Like Tn5, the *b-a-c* junction was able to promote recombination events when an HSV-1 origin (Mocarski and Roizman, 1982a), but not an SV40 origin (Weber, unpublished observations) was linked in *cis* during active HSV-1 infection. The minimal HSV-1 replication enzymes were also able to direct recombination across a duplicated *b-a-c* junction (Figure 8). Thus, sequence inversion in HSV-1 appears to be mediated entirely by the complex of proteins that replicate the viral DNA, not by an independent recombinase function. This supports the observation that discrete sequences (which would act as recognition sites for the putative site-specific recombinase) are not required for inversion events in the HSV-1 genome.

The fact that seven viral gene products can not only replicate HSV-1 DNA but also mediate recombination events does not preclude the possibility that host cell functions are involved in these processes. Indeed, additional host cell enzymes are almost certainly required for the replication of viral DNA (Wu et al., 1988). However, it seems unlikely that a host cell protein(s) mediates sequence inversions in HSV-1, since such a function would have to be ubiquitous in the multitude of permissive cell types in which HSV-1 undergoes genome isomerization, and would have had to evolve in a manner that allowed it to interact specifically with only the HSV-1 DNA replication machinery. A more logical hypothesis is that the process of HSV-1 DNA replication per se is recombinogenic. It is possible, for example, that HSV-1 DNA replication depends on recombination, as has been suggested for bacteriophage T4 (Mosig, 1983; Formosa and Alberts, 1986). In such a case, one or more of the seven viral gene products required for DNA synthesis may act directly to catalyze recombination. Alternatively, HSV-1 DNA replication intermediates may contain structures that are in themselves recombinogenic. Examples of these include single-stranded DNA produced by leading strand synthesis that is not tightly coupled to lagging strand synthesis, and double-strand breaks, which have been shown to act as substrates for homologous recombination in a number of systems (Szostak et al., 1983).

Support for the double-strand break mechanism comes from the Red recombination system of bacteriophage λ, which mediates recombination events at normal frequencies only if DNA synthesis is unimpaired (Stahl et al., 1972). Red-mediated recombination has been postulated to occur by repair of randomly distributed double-chain breaks generated at the tips of rolling circle replication forms (Stahl et al., 1985; Thaler et al., 1987a, 1987b). Like bacteriophage λ, HSV-1 replicates its DNA by a rolling circle mechanism (Jacob et al., 1979), and may generate similar double-chain breaks during DNA synthesis. These same cleavages could also serve as initiation sites for the gene conversion–like repair of heterozygous diploid sequences in the HSV-1 genome (Varmuza and Smiley, 1984; Pogue-Geile and Spear, 1986).

The results of our study suggest that not only does site-specific recombination not exist in HSV-1 in the classical sense (i.e., a specialized protein acting at a distinct sequence present on two pieces of DNA), but that the characteristic inversion of the L and S genomic components may simply be a consequence of DNA replication across the two diploid regions of the HSV-1 genome. This view of the nonessential nature of genome isomerization is supported by the observations that noninverting HSV-1 genomes are completely viable (Poffenberger et al., 1983; Jenkins et al., 1985; Jenkins and Roizman, 1986), no temperature-sensitive mutants for genome inversion (but not DNA synthesis) have ever been isolated, and numerous other members of the herpesvirus family lack inverting L and S components. Further elucidation of the mechanism of generalized recombination in HSV-1 will almost certainly require the development of an in vitro DNA replication system using purified viral components.

Experimental Procedures

Cells and Viruses

HSV-1(KOS) was the HSV-1 strain used throughout this work. The Tn5 mutant viruses US2::Tn5, US4::Tn5, and US5::Tn5 were constructed and characterized previously (Weber et al., 1987). Vero and COS7 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum.

Plasmid Constructions

The construction of pMC110 was described previously (Challberg, 1986). pUC19::Tn5 and pMC110::Tn5 were obtained by Tn5 mutagenesis of plasmid DNA using the defective phage carrier λ467::Tn5 (DeBruijn and Lupski, 1984). The Tn5 insertions were mapped to the 2300 bp and 350 bp coordinates of pUC19 and pMC110, respectively. pUC19::Tn5 was used as the source of Tn5 DNA in all subsequent constructions.

The Tn5 deletion derivatives illustrated in Figure 3 were constructed by cloning restriction fragments of Tn5 generated by seven different enzymes into the cloning sites of pMC110 in the orientation shown. The 5.4 kb Hpal fragment was inserted into the Hincll site to yield pTn5Δ1; the 4.8 kb Xhol partial digest fragment was inserted into the Sall site to yield pTn5Δ2; the 4.4 kb Pstl partial digest fragment was inserted into the Pstl site to yield pTn5Δ3; the 4.0 kb Fspl partial fragment was inserted into the Hincll site to yield pTn5Δ4; the 3.4 kb Hindlll fragment was inserted into the Hindlll site to yield pTn5Δ5; the 3.0 kb Pvull partial digest fragment was inserted into the Hincll site to yield pTn5Δ6; and the 2.8 kb Bglll fragment was inserted into the BamHI site to yield pTn5Δ7. All seven plasmids retained the kanamycin phosphotransferase gene of Tn5 and therefore conferred resistance to kanamycin upon their hosts, which aided in their construction.

The Tn5 deletion derivatives illustrated in Figure 4 were constructed by manipulation of the Tn5 sequences in pTn5Δ1. pTn5Δ1. pTn5Δ8 was obtained from pTn5Δ1 by elimination of the 0.9 kb Fspl fragment spanning $IS50_L$ and the kanamycin phosphotransferase gene, using partial digestion and religation of the appropriate restriction fragment. pTn5Δ9 was then generated from pTn5Δ8 by deletion of the 0.5 kb Pstl fragment spanning $IS50_L$ and the adjacent pUC19 sequences, using similar methods. pTn5Δ10 was constructed by first inserting a Pstl linker into the Fspl site of $IS50_L$ and then deleting the 0.2 kb sequence between this new Pstl site and the natural Pstl site of $IS50_L$ by partial Pstl digestion and religation. pTn5Δ11 was derived from pTn5Δ10 by duplicating the 0.2 kb deletion of $IS50_L$ in $IS50_R$; this was accomplished by replacing the normal Xhol–Bglll fragment of $IS50_R$ with the mutant Xhol Bglll fragment of $IS50_L$.

The construction of the SV40 shuttle vector pCW522 and its three Tn5 derivatives, pTn5Δ1_{sv}, pTn5Δ1sv(INV A), and pTn5Δ1_{sv}(INV B), is described in detail in Figure 6. The construction of the two plasmids containing *b-a-c* sequences, pNN6 and pNN9, is described in Figure 8.

Inversion Assay for HSV-1 Shuttle Plasmids Carrying Tn5 Derivatives

Plasmids (5 μg of each) were transfected into Vero cells grown to confluency overnight by using calcium phosphate precipitation (Graham and van der Eb, 1973) and 25% dimethyl sulfoxide shock. After 18 hr, the medium was changed and the cells were superinfected with HSV-1(KOS) at a multiplicity of infection of 10. After 24 hr, the infected cell DNA was isolated by a modified procedure of F. Homa (unpublished). Briefly, the transfected cells were washed twice with warm PBS and lysed by the addition of 2 ml of TE buffer containing 0.5% SDS and 0.5% sarkosine. After incubations with RNAase A (0.5 mg/ml, 1 hr, 37°C) and pronase (2 mg/ml, 2 hr, 42°C), the lysate was gently extracted twice with TE-saturated phenol and thrice with chloroform. The DNA was then split equally into three Eppendorf tubes and precipitated overnight. The DNA pellet in one tube was then resuspended in 100 μl of restriction buffer and digested overnight with 30 units of Sall or Sall plus EcoRI. The restricted DNA was then reprecipitated, electrophoresed on a 0.8% agarose gel, transferred to GeneScreen Plus (NEN DuPont), and hybridized with pUC19::Tn5 DNA radiolabeled with ^{32}P by nick translation.

In transient DNA replication assays, the transfection mix contained 1 μg of target plasmid (pTn5Δ3, pTn5Δ4, pNN6, or pNN9); 1 μg each of pIGA15 (Gelman and Silverstein, 1985) and pSG1-EK1 (Quinlan and Knipe, 1985), which contain the HSV-1 immediate early genes ICP0 and ICP4, respectively, for transactivation of the cloned HSV-1 DNA replication genes; and 0.5 μg each of pNN1, pNN3, pNN4, pNN5, pMC160-1, pMC160-2, and pCW21 (Wu et al., 1988), which contain the seven HSV-1 DNA replication genes. The cells were shocked with 15% glycerol, and 18 hr later the transfected DNA was isolated and analyzed as described above.

Rescue of SV40 Shuttle Plasmids

Plasmids (5 μg of each) were transfected into COS7 cells grown to confluency overnight by using 25% dimethyl sulfoxide shock. After 18 hr, the medium was changed and the cells were mock infected or superinfected with HSV-1(KOS) at a multiplicity of infection of 10. After 24 hr, the transfected cells were washed twice with warm PBS and their DNA was isolated by the method of Hirt (1967). The Hirt supernatants were subsequently treated with RNAase A (100 μg/ml, 1 hr, 37°C) and proteinase K (100 μg/ml, 2 hr, 45°C), and were finally extracted twice each with phenol:chloroform (1:1) and chloroform. The DNA was split into two Eppendorf tubes, precipitated with ethanol overnight, and digested thoroughly with Dpnl (which degrades the unreplicated input DNA) before transformation into the E. coli *recA* strains DH1 or HB101. Plasmid DNA was isolated from pooled transformants by the method of Birnboim and Doly (1979).

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