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Herpesvirus-dependent amplification and inversion of cell-associated viral thymidine kinase gene flanked by viral *a* sequences and linked to an origin of viral DNA replication

(herpes simplex virus/site-specific recombination/viral gene functions/*trans* assay system)

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ABSTRACT **The genome of herpes simplex virus 1 or 2 consists of two components, L and S, which invert relative to each other during infection. As a result, viral DNA consists of four equimolar populations of molecules differing solely in the relative orientations of the L and S components. Previous studies have shown that the *a* sequences, located in the same orientation at the genomic termini and in inverted orientation at the L–S junction, play a key role in the inversion of L and S components. In this report we describe a virus-dependent system designed to allow identification of the viral genes capable of acting in *trans* to invert DNA flanked by inverted copies of *a* sequences. In this system, cells are converted to the thymidine kinase-positive phenotype with a chimeric plasmid carrying the thymidine kinase gene flanked by inverted copies of the *a* sequence and linked to an origin of viral DNA replication derived from the S component. The DNA introduced into the cells is retained and propagated in its original sequence arrangement as head-to-tail concatemers. Infection of these cells with herpes simplex virus 1 or 2 results in as much as 100-fold amplification of the plasmid sequences and inversion of the DNA flanked by copies of the *a* sequence. In infected cells, the amplified resident DNA accumulates in head-to-tail concatemers and no rearrangement other than the inversions could be detected. These results suggest that the *a* sequence-dependent inversions require *trans*-acting viral gene products.**

Herpes simplex virus 1 (human herpesvirus 1, HSV-1) DNA, M_r of 96×10^6 (1, 2), consists of two components, L and S (3). Each component consists of unique sequences (U_L and U_S) bracketed by inverted repeats (3). The inverted repeats flanking the U_L sequences have been designated *ab* and *b'a'*, whereas those flanking the U_S sequences have been designated *a'c'* and *ca* (2). The terminal repeat, the *a* sequence (2, 4), is present in the same orientation at the ends of the molecule and in an inverted orientation at the L–S junction. In the course of infection, the L and S components invert relative to each other such that the progeny virus DNA consists of equimolar amounts of four isomers differing from each other solely in the relative orientations of the two components (5, 6).

The *a* sequence plays a key role in the inversion of L and S components. Previous reports have shown that a fragment spanning the junction between the L and S components and containing 156 base pairs (bp) of the *b* sequence, an entire *a* sequence (501 bp), and 618 bp of the *c* sequence caused additional inversions in HSV DNA when inserted into the middle of the L component (7). In similar experiments, additional inversions were also induced by fragments, derived from the genomic termini, consisting of the terminal *a* sequence linked to a portion of either the adjacent *b* sequence or the adjacent *c* sequence (unpublished data). The segments of the DNA that inverted were flanked by *a* sequences in inverted orientation. These findings lead to the conclusion that the *a* sequence is an inversion-specific sequence inasmuch as only the genomic DNA segments flanked by *a* sequences inverted during viral replication.

Essentially nothing is known about the viral gene products required for the inversion to occur. Part of the difficulty in identifying these products stems from the fact that the viral DNA in infected cells consists of all four isomers and it is impossible to differentiate between parental isomers and those inverted subsequent to infection.

To speed the development of systems for identification of the viral genes involved in the process of inversion, we have designed a system for the study of HSV DNA inversions in *trans*. In this system, a chimeric plasmid containing the HSV gene for thymidine kinase (TK) flanked by an inverted set of small fragments containing the *a* sequence and linked to an origin of viral DNA replication is used to convert TK^- cells to the TK^+ phenotype. The chimeric plasmid DNA is maintained in its original sequence arrangement in the TK^+ cells. When the converted TK^+ cells are infected with HSV-1 or HSV-2, the chimeric plasmid DNA is amplified, and the segment of DNA located between the two copies of the *a* sequence inverts.

MATERIALS AND METHODS

Cells and Viruses. HSV-1 RΔ305 is the designation of HSV-1(F) containing the TK gene with a 700-bp deletion (8). HSV-1 RΔ305 and HSV-2(G) were propagated in Vero cells as described (9).

Transformation of TK^- Cells. TK^- cells obtained from S. Kit were converted to TK^+ phenotype by the calcium phosphate precipitation (8) of 7.5 μg of undigested chimeric plasmid DNA onto 5×10^5 TK^- cells without carrier DNA. The TK^+ cells were selected in modified HAT medium (8) containing 8.0 μM thymidine, 0.1 mM hypoxanthine, and 0.44 μM methotrexate.

RESULTS

Virus-Dependent System for Detection of *trans*-Acting Viral Gene Products. We constructed two chimeric plasmids (Fig. 1). The first, pRB380, consisted of a *tk* gene flanked by inverted copies of the *a* sequence, linked to a fragment previously shown to contain an origin of HSV-1 DNA replication and carried in pRB10, a modified pBR322 vector. The second, pRB373, was identical to pRB380 except that it contained only one copy of the *a* sequence. TK^- cells were converted to the TK^+ phenotype with the chimeric plasmids and the resultant cell lines,

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Abbreviations: HSV, herpes simplex virus; TK, thymidine kinase; bp, base pair(s).

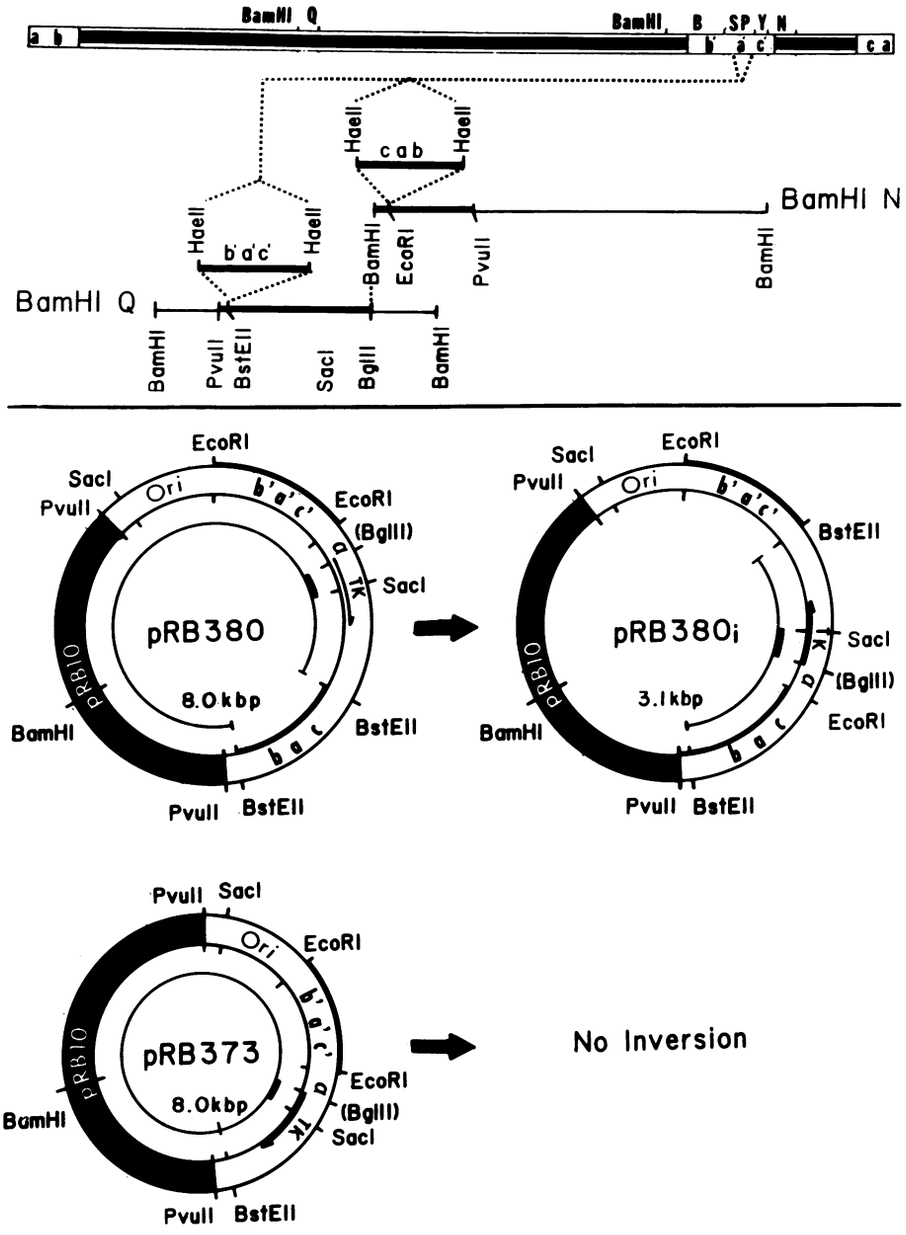

FIG. 1. Sequence arrangement in HSV-1 DNA and construction of the plasmids pRB380 and pRB373. (*Upper*) Idealized sequence arrangement of HSV-1 DNA showing the location of inverted repeats and selected restriction enzyme fragments. Chimeric plasmids were constructed as described (7, 10) and were based on the evidence (11) that origins of HSV DNA replication are contained in or near the *c* sequences of the S component. pRB370, a chimeric plasmid derived from pRB316 (8), was constructed by fusion of the *Pvu* II–*Bgl* II fragment of *Bam*HI fragment Q with the *Bam*HI–*Pvu* II fragment of HSV-1 *Bam*HI fragment N, both shown as heavy lines. In the process, the coding sequences of the natural *tk* gene were fused to the promoter-regulatory region of the α-4 viral gene (8). The *Pvu* II fragment carrying the chimeric *tk* gene was inserted into pRB10, a vector made by deletion of the DNA sequences between the *Pvu* I and *Eco*RI sites of pBR322. pRB373 was constructed by insertion of the *Hae* II fragment from pRB601 (7) carrying only one copy of the *a* sequence and portions of the *b* and *c* sequences into the *Eco*RI site of pRB370 as described (7). pRB380 was constructed by insertion of another copy of the *Hae* II fragment into the *Bst*EII site of pRB373. In both pRB373 and pRB380 constructions, the *Eco*RI and *Bst*EII sites were conserved. (*Lower*) Circular representation of pRB380 and its inverted form, pRB380$_i$, and of pRB373. The inner portions of circles indicate the fragments detected in *Bst*EII digests (thin line) by using the *Bgl* II–*Sac* I segment of the *tk* gene (thick line) as a probe. Because the *Bgl* II site is obliterated in the construction of the plasmids, its position is indicated in parentheses solely to show the region of homology to the *Bgl* II–*Sac* I *tk* probe. The location of the α promoter, the direction of transcription of the *tk* gene, and the location of the viral origin of DNA replication are as indicated.

L380 and L373, were analyzed to determine whether the resident HSV DNA sequences become inverted during infection of these cells with RΔ305, a TK$^-$ derivative of HSV-1(F). Electrophoretically separated fragments of DNA extracted from uninfected and RΔ305-infected L380 and L373 cells were examined for sequences homologous to the *Bgl* II–*Sac* I segment of *tk*, a region that was deleted from RΔ305 (8) but was present in the cells as part of the resident chimeric plasmid DNA. The results of hybridization of the *Bgl* II–*Sac* I *tk* probe to extracted L380 and L373 cell DNAs (Fig. 2) demonstrated the following three significant points.

1. *In uninfected and HSV-1-infected L380 or L373 cells, the chimeric plasmid sequences were arranged in head-to-tail concatemers.* Fig. 2 shows that, in undigested DNA extracted from uninfected and RΔ305-infected cells, sequences homologous to the *Bgl* II–*Sac* I *tk* probe migrated as high molecular weight

DNA (≥25 kbp) on electrophoresis in agarose gels. Digestion of L380 or L373 cell DNA with *Bgl* II, which does not cleave within the chimeric plasmid sequences, did not reduce the size of the sequence homologous to the *Bgl* II–*Sac* I *tk* probe (data not shown). In contrast, a single fragment was detected by hybridization of the *Bgl* II–*Sac* I *tk* probe to electrophoretically separated *Bam*HI digests of DNA from uninfected or from infected L380 and L373 DNA. The size of this fragment was precisely the size of the chimeric plasmid linearized by a restriction enzyme which cleaves the DNA only once (see Fig. 1).

2. *Infection of L380 or L373 cells with HSV-1 caused the amplification of resident chimeric plasmid sequences.* The number of copies of sequences homologous to the *Bgl* II–*Sac* I *tk* probe was greater in the DNA extracted from L380 cells 20 hr after infection with 20 plaque-forming units per cell than in DNA extracted from uninfected L380 cells. Based on the intensity of autoradiographic images, analogous to that shown in Fig. 2 for *Bst*EII-digested L380 cell DNA, the copy number of chimeric plasmid sequences was amplified 10- to 100-fold by viral infection.

3. *Infection of L380 cells with HSV-1 resulted in the inversion of DNA segments bracketed by inverted copies of the a sequence.* Electrophoretically separated digests of the infected L380 cell DNA contained new fragments homologous to the *Bgl* II–*Sac* I *tk* probe not present in uninfected cells (Fig. 2). The sizes of the new *Bst*EII, *Eco*RI, and *Sac* I fragments (Table 1; Fig. 2) containing sequences homologous to the probe were those predicted if the DNA segment flanked by the inverted *a* sequences were to invert as illustrated in Fig. 1. In similar experiments with L373 cells, hybridization analyses revealed no evidence of inversions in the resident chimeric plasmid DNAs amplified by HSV infection. As shown in Fig. 2, digests of DNA from infected or uninfected L373 cells had only a single *Bst*EII or *Sac* I fragment homologous to the *Bgl* II–*Sac* I *tk* probe.

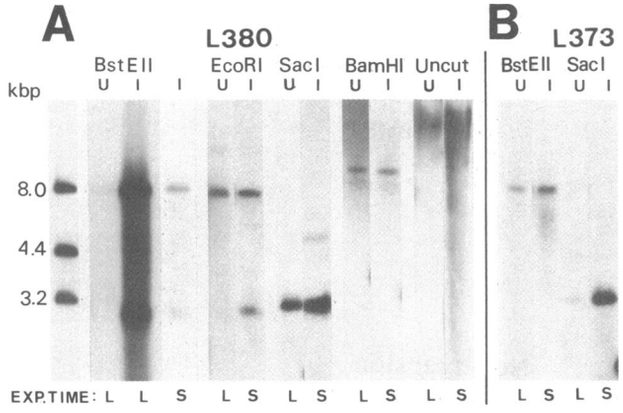

FIG. 2. Autoradiograms of electrophoretically separated restriction fragments homologous to HSV *Bgl* II–*Sac* I *tk* probe in L380 and L373 cell DNA. TK$^+$ cells, either uninfected or infected with 20 plaque-forming units of RΔ305 per cell for 20 hr, were suspended in 0.5 ml of 0.5% Nonidet P-40 in 1 mM EDTA/10 mM Tris·HCl, pH 7.5, and transferred to Eppendorf tubes with RNase A [Sigma, 10 mg/ml in 50 mM sodium acetate (pH 4.5), heated to 100°C for 5 min] at a final concentration of 25 μg/ml. After 5 min, proteinase K (Beckman, preincubated 1 hr at 37°C) and sodium dodecyl sulfate were added to 500 μg/ml and 1%, respectively. After incubation at 65°C for 2 hr the samples were phenol extracted and ethanol precipitated. Samples of cell DNA (3–10 μg) were limit digested with restriction enzymes. Digested and nondigested DNAs were subjected to electrophoresis in 0.75% agarose gels and transferred to nitrocellulose as described (9) except that transfer was done in 20× standard saline citrate (1× is 0.15 M NaCl/0.015 M sodium citrate, pH 7). The immobilized DNA was hybridized as described (9) except that 0.1 μg of *Bgl* II–*Sac* I *tk* fragment was radiolabeled (specific activity, 1–3 × 10^8 cpm/μg) with [α-^{32}P]dCTP by using a nick-translation kit (New England Nuclear). After hybridization, the nitrocellulose filters were washed five times as described (9). (*A*) *Bst*EII, *Eco*RI, *Sac* I, and *Bam*HI fragments from uninfected (U) or RΔ305-infected (I) L380 cell DNA homologous to the *Bgl* II–*Sac* I *tk* probe. The last set of lanes (Uncut) contained undigested cell DNA hybridized with the same probe. The relative intensities of the autoradiographic image before and after infection are directly comparable in the first set of lanes, containing *Bst*EII digests. In all other lanes the image of the uninfected cell DNA was obtained after a 10- to 50-fold longer exposure (L) than the image of the infected cell DNA (S, short exposure). (*B*) *Bst*EII and *Sac* I fragments from uninfected (U) or RΔ305-infected (I) L373 cell DNA homologous to the *Bgl* II–*Sac* I *tk* probe.

Infection of the L380 Cell Line with HSV-2. L380 cells were infected with HSV-2(G) as described in the legend to Fig. 3 to determine whether HSV-2 was capable of inducing the amplification and inversion of the resident plasmid sequences. The HSV-1 *Hae* II *cab* junction fragment was used as a probe for the resident chimeric plasmid sequences after HSV-2(G) infection. Inasmuch as this probe is specific for HSV-1 DNA in chimeric plasmids, the results (Fig. 3; Table 1) show that HSV-2(G) caused amplification of the L380 resident plasmid sequences and inversion of the DNA flanked by inverted copies of the *a* sequence.

Preliminary Mapping of an Origin of Viral DNA Replication. Previous studies have demonstrated that an origin of DNA synthesis is present in or near the reiterated sequences of the S component (11, 12). We took advantage of the availability, in connection with other studies (13), of L cell lines converted to TK$^+$ phenotype with chimeric *tk* fragments (shown schematically in Fig. 4) to map the location of the origin more precisely. In these studies, the cell lines were infected with RΔ305 and the relative amounts of the resident chimeric plasmid DNA amplified by the infection were determined by hybridization of the *Bgl* II–*Sac* I *tk* probe to electrophoretically separated fragments of DNA extracted from the infected cells and transferred to nitrocellulose (data not shown). Only the resident plasmids containing the sequence designated "ori" in Figs. 1 and 4 were amplified after RΔ305 infection. The origin of DNA replication maps within the *c* sequence in a 900-bp DNA fragment bounded on the right by the unique *Pvu* II cleavage site and on the left by a *Sma* I cleavage site in *Bam*HI fragment N. The origin is upstream and separable from the promoter-regulator regions of the α ICP 4 gene described elsewhere (13).

DISCUSSION

In this paper we report the construction of a chimeric plasmid carrying a selectable marker gene, *tk*, flanked by inverted copies of the *a* sequence and linked to an origin of HSV DNA replication. In cells converted to TK$^+$ phenotype, the chimeric plasmid retained its original sequence arrangement. Infection of these TK$^+$ cells with HSV-1 or with HSV-2 resulted in the amplification of the resident plasmid sequences and in the inversion of the DNA flanked by inverted copies of the *a* sequences.

The construction of the chimeric plasmids was based on several published observations. Several studies have shown that *trans*-acting HSV gene products can amplify DNA fragments linked to an origin of DNA replication and introduced into cells by cotransfection with helper virus DNA (11, 12, 14). In addition, other studies have shown that *trans*-acting HSV gene products can regulate viral genes stably associated with cellular genomes, provided that the viral genes had retained or were linked to viral promoter-regulator sequences (8, 13). The experiments described in this paper show that *trans*-acting gene products can amplify viral genes that have become stably as-

Table 1. Sizes (kbp) of fragments detected by specific probes in uninfected and infected TK+ L380 cells

Fragment probe	Uninfected L380				Infected L380			
	*Bst*EII	*Eco*RI	*Bam*HI	*Sac* I	*Bst*EII	*Eco*RI	*Bam*HI	*Sac* I
Bgl II–*Sac* I (*tk*)	8.0	8.0	9.3	3.2	**3.1**	**3.1**	9.3	**5.6**
					8.0	8.0		3.2
Hae II (*cab*)	8.0	8.0	9.3	3.2	**3.1**	**3.1**	9.3	**5.6**
	1.3	1.3		6.1	**6.2**	**6.2**		**3.7**
					8.0	8.0		3.2
					1.3	1.3		6.1

The new fragments resulting from inversion of segments bracketed by inverted copies of the *a* sequence as illustrated in Fig. 1 are shown in **boldface**.

sociated with the host cell. Furthermore, provided the resident plasmid sequences contained both an origin of viral DNA replication and inverted copies of the *a* sequence, HSV infection resulted in both amplification and inversion of these sequences. Moreover, we confirmed the presence of origins of DNA replication near the termini of the S component (11).

The precise mechanism of amplification of the resident plasmid sequences in the converted TK+ cells is unknown. We did not use carrier DNA in the transformation of TK− cells to produce L373 or L380, and the resultant structure of the chimeric plasmid sequences in these cells was as head-to-tail concatemers. We have no evidence whether these concatemers were free episomes or were integrated into the host cell genome. If the chimeric plasmid sequences were contained within chromosome-like structures in converted TK+ cells, as has been suggested (15), then amplification would require either the replication of chimeric plasmid DNA *in situ* or the excision, circularization, and replication of one or more unit-length chimeric plasmid DNAs. The observation that in the converted cells the monomers were arranged in head-to-tail concatemers both before and after amplification is particularly puzzling. If input L380 plasmid monomer DNA had associated with other monomers by homologous recombination, during the transformation of TK− cells, it would have been expected that cleavage with enzymes that cut the plasmid only once would have yielded a complex pattern of fragments representing the product of cleavage of various head-to-head as well as head-to-tail concatemers. Concatemers resulting from homologous recombination between identical monomers of pRB380 would have resulted in recombination across the inverted repeat copies of *bac* junction sequence, giving rise to fragments that were not observed in digests of DNA from uninfected L380 cells. A possible

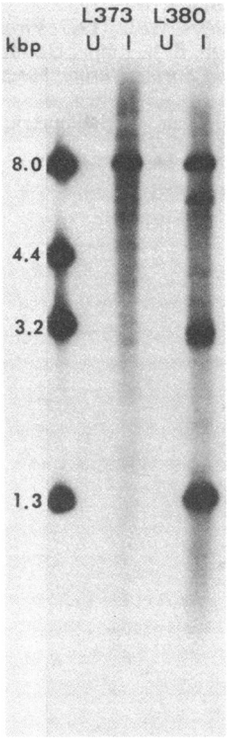

FIG. 3. Autoradiograms of electrophoretically separated restriction fragments homologous to the *Hae* II fragment (*cab*) probe in L373 and L380 cell DNA extracted after infection with HSV-2(G). Electrophoretic separation, transfer, and hybridization were as in Fig. 2. A long autoradiographic exposure is shown to reveal any amplification after viral infection. *Bst*EII fragments from uninfected (U) and HSV-2(G)-infected (I) L373 and L380 cell DNA homologous to the *Hae* II (*cab*) fragment probe, a probe that does not cross-hybridize with the HSV-2(G) L–S junction sequences (unpublished data), are shown.

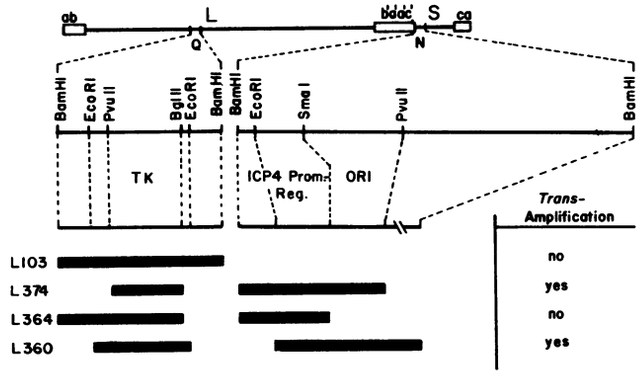

FIG. 4. Mapping of viral origin in the *c* sequences of the S component. The L cell lines converted to TK+ phenotype with chimeric *tk* genes were infected with RΔ305 at a multiplicity of 20 plaque-forming units per cell. The DNA extracted from cells harvested 20 hr after infection was digested with restriction endonucleases, electrophoretically separated, transferred to nitrocellulose strips, and hybridized with the *Bgl* II–*Sac* I *tk* probe as described in Fig. 2. Cell line L103 was derived by conversion of L cells with *Bam*HI fragment Q, cloned as pRB103 (8). The construction of chimeric *tk* genes used to convert L364 and L360 was as described (8, 13). All of the chimeric genes consisted of the coding sequences and a portion of the 5′ noncoding sequences (*Bam*HI–*Bgl* II, *Pvu* II–*Bgl* II, or *Eco*RI–*Bgl* II fragment of *Bam*HI fragment Q) fused to the left *Bam*HI terminus of *Bam*HI fragment N. The portion of the *Bam*HI fragments Q and N contained in each of the chimeric fragments varied as depicted in this figure. To construct L374, cells were converted with a chimeric plasmid (pRB374) without carrier DNA as described in the text. Specifically, a *Bam*HI–*Bgl* II fragment of *Bam*HI fragment Q was fused to *Bam*HI fragment N in the orientation shown in this figure. The fused fragment [pRB316 (8)] was cleaved with *Pvu* II and cloned as pRB374. As illustrated in this figure, on the basis of the amplification of the sequences homologous to the *Bgl* II–*Sac* I *tk* probe, the origin of DNA synthesis maps between the *Sma* I and *Pvu* II cleavage sites shown in the diagram.

explanation for the generation of concatemers in converted TK^+ cells is that, during transformation, the plasmid sequences underwent limited replication.

This report bears on two models advanced to explain the inversions of L and S components in HSV-1 DNA. The first model—that inversions arise at least in part by staggered cleavage of concatemers of unit-length HSV DNA—cannot account for the inversion of the HSV DNA segment in L380 resident chimeric plasmid DNA after infection with RΔ305 virus. The second model (3, 16)—that inversions occur as a consequence of recombination of two molecules through their inverted repeats—is formally feasible. However, to account for the equimolar concentrations of the four isomers in DNA extracted from either virions or infected cells, the model predicts a recombination rate analogous to that achieved by dissociation and random reunion of L and S components (17). This is not the case, as evidenced by the observation that, in a cross between two HSV-1 strains with different phenotypic markers in the L and S components, the distribution of the markers among progeny was not random (17, 18). Another prediction of the model—that the inversions are dependent on the presence of inverted repetitions, *per se*—is not supported by two observations. First, insertions into the genome of inverted repetitions of sequences other than the *a* sequence did not cause additional inversions (9). Second, as shown in this report, the amplification of HSV DNA flanked by inverted repetitions of the *a* sequence during the propagation of the L380 cells was not alone sufficient to cause inversions. The available results support the hypothesis that the inversions specifically involve inverted repeats of the *a* sequence and *trans*-acting gene products specified by the virus. Although this model does not exclude recombination between *a* sequences on different molecules, it favors recombination between *a* sequences within the same DNA molecule.

The *trans*-acting inversion system described in this report will greatly facilitate the identification of the viral gene products and events associated with inversions facilitated by flanking inverted *a* sequences.

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