Novel Rearrangements of Herpes Simplex Virus DNA Sequences Resulting from Duplication of a Sequence Within the Unique Region of the L Component

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We constructed insertion mutants of herpes simplex virus type 1 that contained a duplication of DNA sequences from the *Bam*HI-L fragment (map units 0.706 to 0.744), which is located in the unique region of the L component (U_L) of the herpes simplex virus type 1 genome. The second copy of the *Bam*HI-L sequence was inserted in inverted orientation into the viral thymidine kinase gene (map units 0.30 to 0.32), also located within U_L . A significant fraction of the progeny produced by these insertion mutants had genomes with rearranged DNA sequences, presumably resulting from intramolecular or intermolecular recombination between the *Bam*HI-L sequences at the two different genomic locations. The rearranged genomes either had an inversion of the DNA sequence flanked by the duplication or were recombinant molecules in which different regions of the genome had been duplicated and deleted. Genomic rearrangements similar to those described here have been reported previously but only for herpes simplex virus insertion mutants containing an extra copy of the repetitive *a* sequence. Such rearrangements have not been reported for insertion mutants that contain duplications of herpes simplex virus DNA sequences from largely unique regions of the genome. The implications of these results are discussed.

The genome of herpes simplex virus (HSV) is 96×10^6 daltons (Da) and is composed of two segments, long (U_L) and short (U_S), of unique DNA. These two segments are each flanked by inverted repeats, the ends of which are common to both segments and are repeated in direct orientation at the ends of the genome (4, 18, 20, 26, 28) (see Fig. 1). Recombination apparently occurs between these inverted repeats, resulting in the inversion of the unique segments flanked by the repeats. Because U_L and U_S can invert independently of each other, populations of HSV genomes usually contain equimolar quantities of the four possible isomers (5, 10, 29, 37).

Previous studies showed that, when specific HSV type 1 (HSV-1) DNA sequences are duplicated by insertion of a second copy into the thymidine kinase (*tk*) gene of the virus, inversions of the DNA sequence flanked by the duplicated sequence can occur. These inversions could be detected in the genomes of progeny virus, provided that the inserted sequence and the sequence at the normal locus were in inverted orientation and that the inserted sequence contained the repetitive sequence designated *a* (17, 18, 20, 30). Inversions could not be detected when inserted sequences were from unique regions of the genome (7, 14, 17). We report here, however, that duplication of a sequence from the U_L component can also result, at apparently high frequency, either in inversion of the DNA sequences flanked by inverted copies of the duplication or in other DNA rearrangements resulting from intermolecular recombination between an inserted *Bam*HI-L sequence and the same sequence at the normal locus.

The studies described here were initiated for the purpose of characterizing a putative HSV-1 gene product that governs the process of HSV-induced cell fusion. Mutant HSV-

1(MP) causes the fusion of infected cells under conditions in which a wild-type sibling strain, HSV-1(mP), does not (12). The mutation was mapped first to map units 0.70 to 0.83 (27) and more recently to map units 0.735 to 0.740 (2, 22), a region contained within the *Bam*HI-L fragment of the HSV-1 genome. Insertion mutants were constructed which contained the *Bam*HI-L fragment of HSV-1(mP) DNA inserted into the *tk* gene of HSV-1(MP) DNA and vice versa; this was done to characterize a product expressed from the insert that could either cause HSV-1(mP) to induce cell fusion or complement the mutation of HSV-1(MP). The insertion mutants isolated did not serve the original purpose, because marker rescue and marker transfer from the inserted locus to the normal locus (and vice versa) appeared to occur at high frequency, possibly by mechanisms proposed and discussed previously (4, 13, 32). This precluded the correlation of phenotype with expression of products from the inserted DNA. This study therefore focuses on the novel DNA arrangements occurring in these mutants.

MATERIALS AND METHODS

Cells and viruses. Vero and HEP-2 cells were obtained from the American Type Culture Collection, Rockville, Md. HEP-2 cells were grown in Dulbecco modified Eagle medium with 10% fetal calf serum, and Vero cells were grown in medium 199 with 5% fetal calf serum. The viral strains used were HSV-1(MP) and HSV-1(mP) (6, 11, 12).

Plasmids. All vector sequences present in the plasmids used in this study were from pBR322 (1). pRB142 and pRB103, containing the *Bam*HI-D' and *Bam*HI-Q fragments, respectively, from HSV-1(F) DNA (23), were kindly supplied by Bernard Roizman, University of Chicago. pKPG103R was subcloned from pRB103 by digestion with *Sal*I and *Bgl*II followed by religation at 37°C for 1 h. To obtain pKPG103L, pRB103 was digested with *Hind*III and *Bgl*II, the larger fragment was isolated from an agarose gel, and the ends were filled in with DNA polymerase I Klenow fragment and religated at 16°C for 8 h (15). pKPG103R and

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FIG. 1. Maps of the HSV-1(MP) and HSV-1(mP) genomes and of the plasmids used in these studies. The organization of the genomes with respect to U_L and U_S and the repetitive sequences ($\square$) is shown for both HSV-1(MP) and HSV-1(mP). The *tk* gene ($\blacksquare$), the *Bam*HI-L sequence of HSV-1(MP) (hatched), and the *Bam*HI-L sequence of HSV-1(mP) (dotted) are shown in the appropriate viral genomes and plasmids. The arrows indicate the directions of the two *Bam*HI-L sequences with respect to each other in the insertion mutants. The restriction endonucleases used for cloning these HSV-1 sequences were *Bgl*II (Bg) and *Bam*HI (Ba). To construct pTK133, the cloned *Bam*HI-L sequence from HSV-1(MP) present in plasmid pTYL133 was recloned into the single *Bgl*II site of the *tk* gene in pRB103. The same procedure was used to construct pTK106, except that the *Bam*HI-L fragment was from pTYL106, which contains the *Bam*HI-L fragment from HSV-1(mP). The *Bgl*II-*Bam*HI (Bg/Ba) ligation joints are resistant to cleavage by both enzymes. The sizes (10^6 Da) of the ligated DNA fragments are indicated.

pKPG103L contain the *Bam*HI-Q sequences that are to the right and left, respectively, of the single *Bgl*II site in this fragment (Fig. 1). pTK133 and pTK106 contain the *Bam*HI-L fragment (map units 0.706 to 0.744) from HSV-1(MP) and HSV-1(mP) DNAs, respectively, inserted into the *Bgl*II site of pRB103. Construction of these plasmids is described in the legend to Fig. 1. pKPG101 contains a *Sal*I fragment (map units 0.702 to 0.752) from HSV-1(MP) DNA. pKPG101 was digested with *Bam*HI and religated to form pKPG102, which contains HSV-1 DNA sequences from the *Bam*HI site to the *Sal*I site at 0.744 and 0.752 map units, respectively.

Construction of recombinant viruses. Rabbit skin cells were cotransfected either with mixtures of HSV-1(MP) DNA and *Bam*HI-digested pTK106 DNA or with mixtures of HSV-1(mP) DNA and *Bam*HI-digested pTK133 DNA (8, 14, 36). When the total cytopathic effect was observed, progeny virus were plated under agarose containing thymidine arabinoside (araT) at 100 μ g/ml (17). A second plating under agarose with araT was done to purify the virus.

DNA preparation and analysis. Plasmid DNA was purified in cesium chloride gradients by equilibrium centrifugation (25) of cleared lysates (9). HSV-1(MP) and HSV-1(mP) DNAs used for transfections or cloning were purified in NaI density gradients as described by Walboomers and ter Scheggt (35) and modified by Zezulak and Spear (38). Recombinant viral DNAs used for Southern blotting experiments were obtained from Vero cells infected with recombinant virus that had been plaque purified twice. These viral DNAs were prepared by phenol extraction of the cytoplasmic fraction of infected cells lysed with Nonidet P-40 (38). The DNAs were digested with restriction enzymes as specified by the manufacturer (New England BioLabs, Inc.), electrophoresed on agarose gels, and blotted to nitrocellulose (31). Hybridizations were done at 65°C in 6 $\times$ SSC (0.9 M NaCl and 0.09 M sodium citrate)–30% formamide–salmon sperm DNA (50 μ g/ml)–1 $\times$ Denhardt solution (15)–sodium dodecyl sulfate 0.5% for 8 to 15 h. Nitrocellulose filters were

washed three times in 2 $\times$ SSC, one time in 1 $\times$ SSC, and finally in 0.4 $\times$ SSC. Nick-translation kits (New England Nuclear Corp.) were used to make 32 P-labeled DNA probes from undigested plasmids.

RESULTS

Selection and characterization of viruses with a duplication of the *Bam*HI-L sequence. pTK133 and pTK106 were constructed by inserting the *Bam*HI-L fragment of either HSV-1(MP) or HSV-1(mP) DNA into the single *Bgl*II site of the cloned *Bam*HI-Q fragment present in pRB103 (Fig. 1). Insertions of DNA at this *Bgl*II site result in a nonfunctional *tk* gene (14, 16, 24, 34). After cotransfection of cells with HSV-1(mP) or HSV-1(MP) DNA and one of the plasmids shown in Fig. 1, recombinant viruses containing the appropriate insertion in their *tk* genes were selected by their resistance to araT (17). AraT-resistant isolates were plaque purified, and the DNA was extracted from cytoplasmic fractions of the infected cells. Most of the viral DNA in the cytoplasmic fractions was packaged in progeny virions.

DNAs from most of the araT-resistant plaque isolates we analyzed were shown by Southern blot analysis to contain the *Bam*HI-L sequence inserted into the *tk* gene. The genomes of eight of the viruses analyzed (Fig. 2) were all missing the *Bam*HI-Q fragment (2.2×10^6 Da); they contained instead a new fragment (5.7×10^6 Da) that hybridized with both the *Bam*HI-L probe and the *Bam*HI-Q or *tk* probe and was the size predicted for the new *Bam*HI fragment generated by the insertion. All viruses (including the parent MP strain) had a band at 3.5×10^6 Da, which is the *Bam*HI-L fragment at its normal location on the genome.

Two novel fragments were detected in *Bam*HI digests of DNA from the insertion mutants. The amounts of these fragments relative to those predicted differed from mutant to mutant. The novel fragments, 5.2×10^6 and 4.0×10^6 Da, hybridized to both the *Bam*HI-Q and *Bam*HI-L probes.

FIG. 2. Analysis of genome structures of araT-resistant progeny obtained from cells cotransfected with HSV-1(MP) DNA and pTK106 or with HSV-1(mP) DNA and pTK133. *Bam*HI restriction fragments of virion DNA from HSV-1(MP) and from nine araT-resistant isolates were electrophoresed on a 0.7% agarose gel and transferred to nitrocellulose. The fragments containing sequences homologous to the *Bam*HI-L sequence (L probe) and the *Bam*HI-Q sequence containing the *tk* gene (Q probe) were identified by hybridization. The sizes (10^6 Da) of the fragments detected are indicated.

Their sizes were as predicted for fragments from genomes resulting from the recombinational events depicted in Fig. 3. Genomes with an inversion of the DNA sequence between the duplicated *Bam*HI-L sequences (product 5) could be generated by intramolecular recombination or by a double-crossover intermolecular recombination event as shown. Many of the defective products of intermolecular recombination (products 1 through 4) could theoretically be packaged into virions. These products were approximately genome length (since the two copies of the *Bam*HI-L sequence in the insertion mutants were approximately equidistant from the ends of the genome), and they contained cleavage and packaging signals that have been shown to be present in the repetitive *a* sequence located at both ends of the genome and at the L-S junction (33; N. Frenkel, L. P. Deiss, and R. R. Spaete, Abstr. Herpesvirus 1984, no. 1357, p. 198).

To test the hypothesis that the anomalous fragments containing sequences that hybridized to both the *Bam*HI-L

and *Bam*HI-Q fragments were from recombinant genomes (Fig. 3), we digested DNA from the progeny of two insertion mutants with *Sal*I. This enzyme does not cleave within the *Bam*HI-L or *Bam*HI-Q sequence and generates fragments in which sequences contiguous to the anomalous *Bam*HI fragments can be identified. Figure 4 shows the two separate regions of the genome in which *Bam*HI-L sequences were present in opposite orientations in the insertion mutants. Also shown is the change in contiguous DNA sequences that would result from recombination between the two *Bam*HI-L sequences. The sizes of the *Sal*I fragments characteristic of each *Bam*HI-L locus in parental or recombinant form and the probes to which they would hybridize are also indicated. All four of the *Sal*I fragments depicted in Fig. 4 were detected (Fig. 5), providing evidence for the predicted recombinational events.

DNA from the insertion mutants were also digested with *Eco*RI or *Hpa*I. Both of these enzymes cut within the

FIG. 3. Results of intermolecular and intramolecular recombination between the two *Bam*HI-L sequences present at different loci in the insertion mutants. Left, Genome structures of the mutants resulting from insertion of the *Bam*HI-L sequence into the *tk* gene in opposite orientation to the resident *Bam*HI-L sequences. Symbols: $\square$, repetitive sequences (superscript letters refer to the same strand of DNA within a molecule: *a*, *b*, and *c* are sequences homologous to *a'*, *b'*, and *c'*, respectively); ||||| , *Bam*HI-L sequence (orientation is shown by the direction of the cross-hatching); X, recombination sites generating the products shown on the right. The molecules are drawn to scale with respect to the particular features identified, except that a portion of each genome has been omitted from most of these representations where indicated by breaks between parallel diagonal lines.

FIG. 4. Restriction fragments generated by *SalI* cleavages of rearranged or nonrearranged genomes from the insertion mutants. Top, Parental *SalI* fragments that will hybridize to a *BamHI*-L probe if no recombination has taken place between the *BamHI*-L sequences at the two different loci. Bottom, Novel *SalI* fragments that will hybridize to a *BamHI*-L probe if recombination has taken place between the two *BamHI*-L sequences at the different loci. The vertical arrows show the positions of the *SalI* recognition sequence; numbers between the vertical arrows indicate the sizes of *SalI* fragments (10^6 Da). Horizontal arrows indicate that the *BamHI*-L sequences ($\square$) are in inverted orientation. The letters above the lines are the names of *BamHI* fragments from HSV-1 DNA (or portions thereof) used as probes in the Southern blot analysis (Fig. 5). Symbols: ▨, the HSV-1 DNA sequence contained in probe Q(l) or plasmid pKPG103L; ▩, the HSV-1 DNA sequence in probe Q(r) or plasmid pKPG103R; ▤, the HSV-1 DNA sequence of the *BamHI*-D' fragment present in pRB142 (map units 0.700 to 0.706); ▦, the HSV-1 DNA sequence, designated B(l), contained in pKPG102.

BamHI-L sequence so that no restriction fragments specific to the recombinant genomes would be generated. As predicted, these restriction digests showed no novel bands (data not shown).

DISCUSSION

Evidence has been presented that the normal inversion of the U_L and U_S components of wild-type HSV genomes is due to site-specific recombination between inverted copies of the reiterated α sequences and is mediated by viral gene products (3, 4, 17–21, 30). The evidence for this site specificity was obtained by inserting fragments from either the unique or the reiterated regions of the genome into the *tk* gene of the HSV genome (17). Insertions of DNA from the reiterated regions of the genome resulted in novel inversions between inverted copies of the reiterated sequences. In contrast, insertions of three different *SacI* fragments from U_L (map units 0.64 to 0.65) or U_S (map units 0.910 to 0.925 or 0.925 to 0.935) did not result in any novel DNA rearrangements that could be detected in cytoplasmic packaged genomes.

Two other HSV DNA fragments from unique regions of the genome, in addition to the *BamHI*-L fragment, have been inserted into the *tk* gene of HSV. No evidence for DNA rearrangements was obtained when a *SalI*-to-*BamHI* fragment containing the gene for gC-1 (map units 0.62 to 0.64) was inserted in either orientation into the HSV-1(MP) genome (14). In contrast, when the homologous HSV-2 sequence encoding gC-2 (map units 0.62 to 0.64) was inserted into the HSV-1(MP) genome in inverted orientation to the gC-1 gene, one of the two novel restriction fragments which would be predicted by inversion was detected in a *BamHI* digest of packaged viral DNA (K. M. Zzulak, Ph.D. thesis, University of Chicago, Chicago, Ill., 1983). Perhaps the second novel fragment was not detected because its size was very similar to that of a *BamHI* fragment from the parental viral DNA. Thus, it is possible that inverted duplication of

the sequence encoding gC-2 as well as the *BamHI*-L sequence results in DNA rearrangements.

Several factors other than the actual frequency of recombination between the duplicated sequences could influence the ability to detect rearranged genomes, and these factors could help to explain the different consequences of inserting the *BamHI*-L sequence and other unique sequences. In all the experiments described here and elsewhere, the DNA that was analyzed was DNA packaged in virions. Thus, detection of recombination products can be limited by the requirements for packaging. These requirements include the presence of the α sequence on at least one end of the genome (33; N. Frenkel et al., *Herpesvirus* 1984) and an appropriate genome size. Although the range of permissible sizes is not known with certainty, mutants containing deletions or insertions that are greater than about 8% of the genome length have not yet been reported. The detection of recombination products may also depend in part on the ability of virions containing rearranged genomes to replicate. Defective rearranged genomes would not be amplified during successive rounds of replication except under conditions of high multiplicity of infection.

It seems unlikely, however, that packaging constraints alone can explain the different consequences of duplicating various sequences from unique regions of the genome. Intramolecular recombination between duplicated sequences in opposite orientations should always yield packageable genomes. Recombinant products that might have resulted from intermolecular recombination in this study (i.e., products 1 through 4, Fig. 3) are an appropriate

FIG. 5. *SalI* restriction endonuclease digestion and Southern blot analysis of DNA from the *BamHI*-L insertion mutants. This Southern blot includes *SalI* digests of DNA from HSV-1(MP) (sample 1) and from the insertion mutants MP-TK106-7 (sample 2) and mP-TK133-2 (sample 3). Samples of these *SalI*-digested DNAs were loaded into four or five wells, and the appropriate lanes were hybridized with the labeled probes indicated at the top of each panel (as described in the legend to Fig. 4). The first panel shows *HindIII*-digested λ DNA hybridized with labeled λ DNA. The bands detected here were used as size markers to determine the sizes (10^6 Da) of the bands in the other panels as shown on the far right.

size for packaging only because the *tk* gene and the normal genomic location of the *Bam*HI-L sequence happen to be equidistant from the ends of the genome. This is probably also true for at least two of the other insertion mutants for which no evidence of genomic rearrangement was obtained. These insertion mutants include those described by Lee et al. (14) and Mocarski et al. (17), which had sequences from map units 0.62 and 0.64 and 0.64 to 0.65, respectively, duplicated in the *tk* gene.

The possible defectiveness of rearranged genomes also does not fully explain the different consequences of duplicating *Bam*HI-L sequences and other unique sequences. Smiley et al. (30) detected packaged molecules from which half of the genome had been deleted due to recombination between two copies of the *a* sequence in direct orientation in infections at relatively low multiplicity (1 PFU per cell). The deleted genomes were an appropriate size to be packaged as dimers.

It seems likely, therefore, that an unusually high rate of recombination between the duplicated *Bam*HI-L sequences is at least partly responsible for the results reported here. It is possible that a site-specific mechanism is responsible for these rearrangements, because similar rearrangements have not been detected in HSV genomes with duplications of other unique sequences. This argument was used previously to propose the existence of a site-specific mechanism for recombination between *a* sequences (17). If both of these genome regions contain signals for site-specific recombination, then presumably the signals are for different systems; or, if the same system is used, it does not promote recombination between the *a* and the *Bam*HI-L sequences. Alternatively, the recombinational events we observed may require homology but not a specific nucleotide sequence, with the frequency of recombination between homologous sequences depending on factors not yet defined.

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