JOURNAL OF VIROLOGY, Apr. 1996, p. 2075–2085 Vol. 70, No. 4
0022-538X/96/$04.00+0

Herpes Simplex Virus Type 1 Alkaline Nuclease Is Required for Efficient Processing of Viral DNA Replication Intermediates

RIK MARTINEZ,[1] ROBERT T. SARISKY,[2]† PETER C. WEBER,[3] AND SANDRA K. WELLER[1]*

Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut 06030,[1] Department of Microbiology and Immunology, Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, Pennsylvania 17033,[2] and Infectious Diseases Section, Parke-Davis Pharmaceutical Research, a division of Warner-Lambert Company, Ann Arbor, Michigan 48105[3]

Received 28 August 1995/Accepted 19 December 1995

Mutations in the alkaline nuclease gene of herpes simplex virus type 1 (HSV-1) (*nuc* mutations) induce almost wild-type levels of viral DNA; however, mutant viral yields are 0.1 to 1% of wild-type yields (L. Shao, L. Rapp, and S. Weller, Virology 195:146–162, 1993; R. Martinez, L. Shao, J. C. Bronstein, P. C. Weber, and S. Weller, Virology 215:152–164, 1996). *nuc* mutants are defective in one or more stages of genome maturation and appear to package DNA into aberrant or defective capsids which fail to egress from the nucleus of infected cells. In this study, we used pulsed-field gel electrophoresis to test the hypothesis that the defects in *nuc* mutants are due to the failure of the newly replicated viral DNA to be processed properly during DNA replication and/or recombination. Replicative intermediates of HSV-1 DNA from both wild-type- and mutant-infected cells remain in the wells of pulsed-field gels, while free linear monomers are readily resolved. Digestion of this well DNA with restriction enzymes that cleave once in the viral genome releases discrete monomer DNA from wild-type virus-infected cells but not from *nuc* mutant-infected cells. We conclude that both wild-type and mutant DNAs exist in a complex, nonlinear form (possibly branched) during replication. The fact that discrete monomer-length DNA cannot be released from *nuc* DNA by a single-cutting enzyme suggests that this DNA is more branched than DNA which accumulates in cells infected with wild-type virus. The well DNA from cells infected with wild-type and *nuc* mutants contains *Xba*I fragments which result from genomic inversions, indicating that alkaline nuclease is not required for mediating recombination events within HSV DNA. Furthermore, *nuc* mutants are able to carry out DNA replication-mediated homologous recombination events between inverted repeats on plasmids as evaluated by using a quantitative transient recombination assay. Well DNA from both wild-type- and mutant-infected cells contains free U_L termini but not free U_S termini. Various models to explain the structure of replicating DNA are considered.

The molecular mechanisms of DNA replication vary widely from virus to virus. Circular duplex DNAs are generally thought to replicate in a theta structure (12) or through a rolling-circle mechanism (21). The small eukaryotic papovaviruses such as simian virus 40 replicate entirely by theta replication. In contrast, bacteriophage lambda replicates initially as a theta form and switches to rolling-circle replication later in infection (17). The DNA bacteriophage T4 replicates as a linear DNA molecule, and initiation occurs by two markedly different strategies (36). The initial phase of T4 DNA replication occurs at specific origins of replication (33); however, late DNA replication occurs by a recombination-dependent mechanism in which DNA synthesis is primed from the ends of DNA strands that have invaded homologous segments of other DNA molecules (36, 44).

The 152-kb herpes simplex virus type 1 (HSV-1) genome is composed of two components, termed L and S. The L component consists of unique sequences (U_L) flanked by the inverted-repeated sequences *ab* and *b′a′*, whereas, the S component consists of unique sequences (U_S) flanked by inverted-repeated sequences *ac* and *c′a′* (25, 54) (Fig. 1A). During the course of viral DNA replication, the two unique regions invert relative to one another (25). Genome isomerization most likely results from generalized recombination between inverted repeats flanking the L and S components (50, 56, 57). DNA replication results in the formation of large head-to-tail concatemers consisting of tandem repeats of the viral genome (6–8, 26, 29, 30) which can be chased into monomer-length viral genomes (30, 31, 47). Although rolling-circle DNA replication may be involved in the generation of large concatemeric molecules, it is also possible that HSV DNA replication occurs by a mechanism which is inherently recombinogenic in a manner analogous to T4 bacteriophage DNA replication (36, 44). In T4, replication and/or recombination results in the generation of branched forms of viral DNA which must be resolved prior to packaging into preformed phage heads, using the product of the T4 gene 49, endonuclease VII (32, 42). On the basis of the phenotype of HSV-1 mutants defective in the UL12 gene, we previously proposed that the viral alkaline nuclease may carry out an analogous function (53).

HSV alkaline nuclease (UL12) is a relatively abundant 85-kDa phosphoprotein in infected cells (2, 3). UL12 is encoded by a 2.3-kb member of a family of unspliced 3′ coterminal mRNAs (13, 15). We have recently demonstrated that, as originally proposed by Costa et al. (13), another member of this family of mRNAs (1.9 kb) encodes an N-terminally truncated protein (UL12.5) which shares its carboxy terminus with the alkaline nuclease protein (38). A viral frameshift mutant (ANF1) that was constructed is predicted to abolish the gene product of the 2.3-kb mRNA (full-length alkaline nuclease) but leave intact the UL12.5 product of the 1.9-kb mRNA. Since no

* Corresponding author. Mailing address: Department of Microbiology, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030. Phone: (860) 679-2310. Fax: (860) 679-1239. Electronic mail address: Weller@panda.uchc.edu.

† Present address: Department of Pharmacology and Molecular Science, Johns Hopkins School of Medicine, Baltimore, MD 21205.

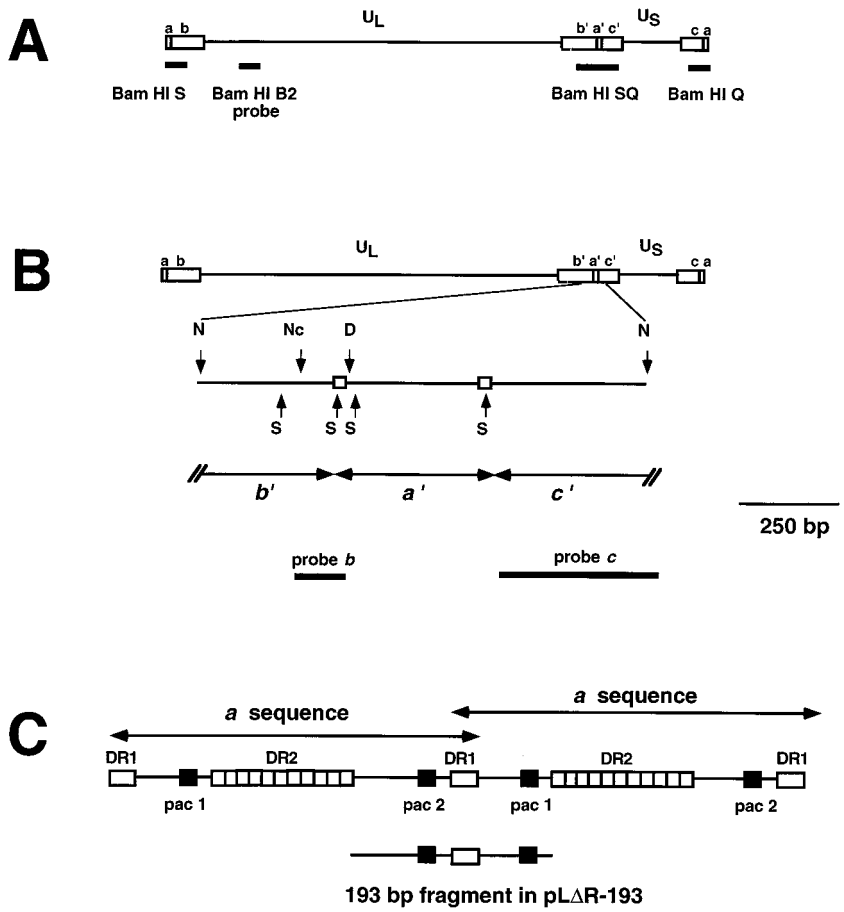

FIG. 1. Structure of the HSV-1 genome showing the locations of various probes and fragments used in this study. (A) Diagram of the HSV-1 genome showing the U_L and U_S regions of the genome as well as the flanking repeated sequences *ab*, *b'a'c'*, and *ca*. The location of the *Bam*HI B2 probe used in this study is shown (described in Materials and Methods). The locations of the *Bam*HI SQ, S, and Q fragments are also shown. (B) Diagram of the HSV-1 genome showing an expanded view of the *Nar*I fragment in pNar. Restriction sites are shown for *Nar*I (N), *Sma*I (S), *Nco*I (Nc), and *Dra*I (D). Boxes on the second line denote DR1 sequences which flank the *a* sequence. The third line shows the locations of the *a*, *b*, and *c* sequences within pNar. On the fourth line, the positions of probes *b* and *c* are shown. (C) A tandem HSV-1 *a*-sequence junction with locations of sequences DR1, DR2, and the pac1 and pac2 elements. The portion of the *a* sequence present in pLΔR-193 is shown on the bottom line. This sequence represents the minimal signal sufficient for mediating cleavage and packaging of viral DNA (46).

exonuclease activity was observed in cells infected with AN-F1, it appears that UL12.5 may have little or no enzymatic activity (38). We previously reported the construction of a null mutation (AN-1) in the HSV-1 alkaline nuclease gene (53, 62). Both *nuc* mutants (AN-1 and AN-F1) form very small plaques on Vero cells, and mutant virus yields on nonpermissive cells are approximately 0.1 to 1% of that seen for wild-type virus; however, they synthesize wild-type levels of viral DNA and late proteins.

Although cleavage of viral DNA into monomeric units and packaging of viral DNA into capsids in the nucleus occurred efficiently in cells infected with *nuc* mutants, three lines of evidence suggest that the maturation of DNA-containing capsids into infectious virions is defective in *nuc* mutants (38, 53, 65). (i) In contrast to wild-type-infected cells, very small amounts of staphylococcal nuclease (SN)-protected DNA were detected in cytoplasmic extracts from *nuc* mutant-infected cells. (ii) Very few if any mature, DNA-containing capsids were observed in the cytoplasm upon analysis by electron microscopy or sucrose gradient sedimentation. (iii) Analysis of mutant-infected cells by sucrose gradient sedimentation indicated an elevated ratio of A to B capsids. Type B capsids are believed to be precursors to the DNA-containing C capsids found in the cytoplasm (27, 28); A capsids are believed to result from abortive, unsuccessful attempts to package viral DNA into B capsids (1, 34, 55). These data indicate that *nuc* mutants may be defective for the production of capsids competent to mature into the cytoplasm. We have proposed that the role of alkaline nuclease in the viral life cycle is to resolve or debranch recombination intermediates which are formed during the DNA replication process (53, 65). The model posits that if replication/recombination intermediates are not resolved properly, viral DNA can be cleaved and taken up into capsids, but these capsids are unstable and not competent to mature into the cytoplasm.

We set out to analyze the DNA which accumulates in cells infected with wild-type and *nuc* mutant viruses. Previous studies have shown that pulsed-field gel electrophoresis (PFGE) and field inversion gel electrophoresis are powerful tools in the analysis of large viral DNAs (4, 20, 41, 51, 70). Several intriguing observations have been made regarding the structure of replicating viral DNA. First, replication intermediates in HSV-1-infected cells are present in complex nonlinear structures which cannot enter a pulsed-field gel. After digestion with a restriction enzyme which has a single recognition site within the HSV genome, some but not all of the viral DNA is released

into a unit-length molecule (51). Second, analysis of newly replicated DNA by PFGE indicates that inversion has occurred at the earliest times that replicated DNA can be detected (4, 40, 51, 70). In this report, PFGE was used to analyze the replication intermediates which accumulate in cells infected with wild-type or *nuc* mutant HSV-1. We find that complex nonlinear structures arise during replication of both wild-type and *nuc* mutant viruses and that inversion events have occurred early in the process in both viruses. After digestion with a single-cutting enzyme, no discrete fragments are released from replicating DNA from cells infected with *nuc* mutants, whereas discrete fragments are released in DNA from cells infected with wild-type virus. This finding indicates that the DNA which accumulates in these cells is even more complex in structure than that in cells infected with wild-type virus.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (Vero; American Type Culture Collection) were grown and maintained as described previously (64). Cell line 6-5, which is permissive for *nuc* mutants, was generated and maintained as described previously (53). The KOS strain of HSV-1 was used as the wild-type virus. AN-1 is a null mutant in the alkaline nuclease gene in which 917 bp from the N-terminal half of the gene (corresponding to residues 70 to 375) were deleted and replaced by an insertional mutagen consisting of the *lacZ* gene of *Escherichia coli* under the control of the regulatory elements of the large subunit of ribonucleotide reductase (ICP6) (65). AN-F1 and AN-F3 are independent isolates containing a 2-bp insertion which results in the premature termination of translation of the 2.3-kb alkaline nuclease mRNA (38). The growth phenotypes of the null and frameshift mutants are identical (38). AN-1-2a is a recombinant virus in which the deletion-insertion of AN-1 has been replaced by the wild-type UL12 gene by marker rescue; the growth properties of this virus resemble those of wild-type KOS (65).

Plasmids and bacteria. Plasmids pLR and pLΔR-193, carrying HSV-1 ori_S and different sets of inverted-repeat sequences, were previously described (50). pLR is a construct containing two copies of the IS*50* element of Tn*5*. pLΔR-193 contains a 193-bp (Fig. 1C) fragment from the *a* sequence inserted into a deleted version of the IS*50* element (all but 236 bp of the $IS50_R$ element deleted). The 193-bp fragment contains the pac2-DR1-pac1 region from two tandemly repeated *a* sequences and lacks the DR2 sequences. This fragment is similar to one which has been shown to function as a minimal cleavage and packaging signal (46). Plasmid pSG124 containing the *Eco*RI A fragment of HSV-1 strain KOS (map coordinates 0.495 to 0.635) was previously described (22). Plasmids pSG10, containing the *Eco*RI D fragment of HSV-1 strain KOS (map coordinates 0.086 to 0.194), and pSG10-B2, containing an internal *Bam*HI fragment (map coordinates 0.145 to 0.165) from pSG10, were described previously (63). Plasmid pNN9, containing two copies of the 5.9-kb *Bam*HI K fragment from strain KOS cloned into pUC19, was generously provided by Mark Challberg (National Institutes of Health, Bethesda, Md.). pNar contains a 1,209-bp *Nar*I fragment (sequence coordinates 125585 to 126793 [39]) from pNN9 cloned into the *Nar*I site of pSL301 (Invitrogen); this *Nar*I fragment contains the entire *a* sequence (400 bp) and parts of the flanking *b* (389 bp) and *c* (420 bp) sequences from HSV-1 strain KOS (Fig. 1B). The published sequence for HSV-1 strain 17 (39) lists an additional *Nar*I site in this region at position 125789 which is not present in strain KOS (38a). Recombinant plasmids were propagated in *E. coli* UT481 or DH5α by standard procedures (37).

DNA isolation and Southern blotting. Virion DNA was isolated from purified virions following equilibrium centrifugation in CsCl as described previously (66). Cellular DNA was isolated as previously described (66). For analysis of DNA by Southern blotting, virion or infected-cell DNA was digested with a restriction endonuclease(s) as directed by the manufacturer, subjected to agarose gel electrophoresis, and transferred to a GeneScreen Plus nylon membrane (New England Nuclear Corp., Boston, Mass.) as recommended by the supplier. Blots to be rehybridized with separate probes were stripped by boiling in TE (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]) with 1% sodium dodecyl sulfate for 30 min.

DNA fragments to be used as probes for hybridization were labeled as described previously (18), using the random hexamer-primed method (Boehringer Mannheim, Indianapolis, Ind.). Virion DNA was digested with *Bam*HI and *Eco*RI prior to labeling. In several experiments, subfragments of recombinant plasmids were isolated following restriction enzyme digestion, agarose gel electrophoresis, and electroelution of the appropriate fragment (37). For fragments smaller than 150 bp in length, β-agarase (New England Biolabs [NEB], Beverly, Mass.) was used to dissolve the agarose as directed by the manufacturer. For the transient recombination assay, a radiolabeled 3.4-kb *Hin*dIII fragment of Tn*5* was used as a probe as described previously (50). Other HSV genomic fragments used as probes were a probe from U_L, the *Bam*HI fragment B2 from pSG10-B2 (coordinates 0.145 to 0.165; Fig. 1A), the 8-kb *Bam*HI H fragment (coordinates 0.522 to 0.572) isolated from pSG124, and the 5.0-kb *Hin*dIII N fragment from the middle of U_S (see Fig. 7). For the detection of viral termini, two probes were used: a 132-bp *Nco*I-to-*Dra* fragment from pNar, a fragment from within the repeated *b* sequences which detects the *Bam* S terminal fragment flanking U_L (Fig. 1B, probe *b*); and a 429-bp *Sma*I-*Nar*I fragment from pNar, a fragment within the repeated *c* sequences which detects the *Bam* Q terminal fragment flanking U_S (Fig. 1B, probe *c*).

PFGE. PFGE was performed on Bio-Rad (Melville, N.Y.) DR-II and DR-III apparatuses. Vero or 6-5 cells were infected at a multiplicity of infection (MOI) of 5 PFU per cell in 60-mm-diameter tissue culture plates and harvested at various times after infection. Cells were scraped off the plates, centrifuged in medium at 2,000 rpm for 5 min in a Beckman model TJ-6 tabletop centrifuge, rinsed in phosphate-buffered saline (PBS), resuspended in approximately 600 μl of 55°C 1% low-melting-point agarose (SeaKem LMP) in PBS, and cast into two blocks of approximately 270 μl each in a casting mold provided by the manufacturer (Bio-Rad). Liquid stocks of HSV virions were cast into blocks by using 2% SeaKem LMP agarose and contained approximately 10^8 PFU per block. Blocks were stored at 4°C in 50 mM EDTA (pH 8.0). Lysis of the cells or virions in blocks was performed by incubation in 1% laurylsarcosine–0.4 M EDTA (pH 9.0) with proteinase K at 1 mg/ml for 24 h at 37°C. Blocks were then rinsed in 1× TE at 50°C, five times for 15 min each, to remove the detergent and the proteinase K and stored at 4°C in TE.

Enzyme digestions were carried out by incubating one-quarter of a lysed and rinsed block in enzyme digestion buffer and enzyme (30 to 60 U) overnight at 4°C and then for 4 to 6 h at 37°C. Restriction enzymes *Bam*HI and *Xba*I were obtained from NEB, and restriction enzyme *Spe*I was obtained from U.S. Biochemical (Cleveland, Ohio). When double digestions were performed, the rinses and equilibration steps were repeated for the second enzyme and buffer condition. The digested blocks were sealed into the well of a 1.3 or 1.5% SeaKem GTG agarose gel made in 0.5× TBE (1× TBE is 0.089 M Tris [pH 8.0], 0.089 M boric acid, and 0.002 M EDTA [pH 8.0]). Run conditions were 6 V/cm (approximately 200 V) for 18 to 22 h at 14°C, with a linear pulse increase from 2 to 70 s over the course of the run. Lysed and rinsed HSV virions, lambda ladder (NEB), and lambda DNA digested with *Hin*dIII were used as molecular weight markers. In most cases, gels were stained in ethidium bromide at 0.5 μg/ml for 1 h at room temperature, photographed, and then prepared for Southern blotting (37) by incubation in 0.25 M HCl for 45 min, 0.6 M NaCl–0.4 M NaOH for 30 min, and 1.5 M NaCl–0.5 M Tris (pH 7.5) for 30 min. DNA was transferred to GeneScreen Plus membranes in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate [pH 7.0]).

Well DNA was obtained by subjecting lysed, rinsed blocks to PFGE, using parameters described above. This procedure results in the separation of viral DNA into two bands, one which does not leave the well and one which migrates as a unit-length linear genome. Viral DNA from the well was isolated by excising the block from the well of an unstained gel. For some experiments, this process was repeated to rule out possible contamination of unit-length DNA. Well DNA was then subjected to restriction enzyme digestion and electrophoresis as described above.

SN digestion. Vero cells were infected with KOS, *nuc* mutant AN-1 or AN-F1, or the recombinant AN-1-2a at an MOI of 5 PFU per cell for 18 h at 37°C. Wild-type- or *nuc* mutant-infected cells were harvested and resuspended in cold lysis buffer (150 mM NaCl, 10 mM Tris [pH 7.5], 1.5 mM $MgCl_2$) with 0.2% Nonidet P-40 and incubated on ice for 3 min. The nuclei were spun (2,000 rpm for 10 min) and embedded in agarose, and each agarose block was divided in half. One half of each block was incubated overnight at 4°C and then for 2 h at 37°C in SN at 6 U/10^6 cells in SN digestion buffer (53). Treated and untreated samples still in blocks were lysed for 24 h at 37°C in 0.4 M EDTA (pH 9.5)–1% lauryl sarcosine–1 mg of proteinase K per ml. Blocks were rinsed in TE five times for 15 min each, to remove the detergent and the proteinase K, and then stored at 4°C in TE. These samples were then subjected to PFGE as described above.

Analysis of inversion events in a transfection-superinfection assay. Vero cells were transfected in triplicate with 6 μg of each plasmid by calcium phosphate precipitation as described previously (50). The medium was changed after 18 h, and the cells were superinfected with HSV-1 (KOS) or AN-1 at an MOI of 10 to 20. After 24 h, the infected-cell DNA was isolated as described previously (61). The DNA was resuspended and digested with the appropriate restriction endonucleases, reprecipitated, and subjected to agarose gel electrophoresis and blotting onto GeneScreen Plus as described previously (50). Filters were hybridized to Tn*5*-specific probes as described above. Autoradiograms were quantified with a Betagen imager.

RESULTS

Characterization of replicating DNA from wild-type- and *nuc* mutant virus-infected cells. To analyze the structure of viral DNA which accumulates in wild-type- and nuclease mutant-infected cells, PFGE was used. PFGE has been used previously to separate large viral DNA genomes and to investigate the structures of nonlinear DNAs such as circles, concatenates, or branched molecules which are unable to enter these gels (9,

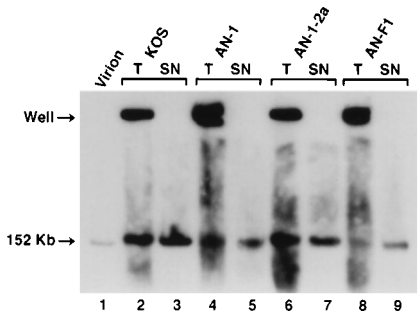

FIG. 2. Southern blot analysis of a pulsed-field gel of total and encapsidated DNA from KOS-, AN-1-, AN-1-2a-, and AN-F1-infected Vero cells. Vero cells were infected with the indicated virus at an MOI of 5 PFU per cell for 18 h. Samples for PFGE were prepared by suspending infected cells in a block containing melted low-melting-point agarose as described previously (51). Blocks were either used as is (total [T]) or digested with SN as described in Materials and Methods. Virion DNA was prepared by lysis of purified KOS virions in agarose blocks. PFGE was performed with a CHEF DR-II apparatus (Bio-Rad). Linear fragments up to 864 kb could be resolved under these conditions. The Southern blot was probed with a ^{32}P-labeled *Bam*HI B2 probe. The positions of well and 152-kb monomer DNAs are indicated. Lambda ladder PFGE markers consisting of concatemers of lambda DNA (ranging in size from 48.5 to 1,018.5 kb) (NEB) were used as molecular weight markers.

10, 49). To avoid shearing of large viral DNA molecules, infected cells were embedded in blocks of agarose and manipulated in situ. DNA from cells infected with KOS, AN-1, AN-1-2a, or AN-F1 were harvested at 18 h postinfection and subjected to PFGE as described in Materials and Methods. The gel was blotted and hybridized to a ^{32}P-labeled *Bam*HI B2 probe (Fig. 1A). Figure 2 shows that total HSV-1 DNA from wild-type KOS and a wild-type recombinant AN-1-2a consists of two major bands (lanes 2 and 6, respectively). The upper band has been termed well DNA and does not enter the gel, even if excised and rerun on a new gel (data not shown). The lower band, which comigrates with the 152-kb band of the virion DNA (lane 1), represents the cleaved linear monomer form that is packaged into capsids. In lanes 3 and 7, total DNA from KOS- or AN-1-2a-infected cells was treated with SN as described in Materials and Methods. Only the 152-kb monomer band is protected, indicating that it has most likely become encapsidated, whereas the well DNA is sensitive to SN digestion. Other groups have also reported the accumulation in infected cells of HSV-1 DNA which fails to enter a pulsed-field or field inversion gel (4, 40, 51, 70). Severini et al. showed that well DNA represents replicating HSV-1 DNA, as determined by pulse-labeling with [^{3}H]thymidine (51). At 3 h postinfection, at an MOI of 5 PFU per cell, the viral DNA signal is barely detectable as either monomer or well DNA; between 5 and 8 h, faint signals consisting primarily of well DNA are observed (data not shown). These results are consistent with the previous report of Zhang et al. showing that even at an MOI of 20 PFU per cell, input DNA is barely detectable at early time points (3 to 5 h postinfection) (70). Therefore, the well and monomeric DNA seen at 18 h postinfection (Fig. 2) is believed to represent newly replicated and not input DNA.

The profile of DNA which accumulates in *nuc* mutant-infected cells is identical to that seen in wild-type virus-infected cells (Fig. 2). Specifically, the SN-treated DNAs from AN-1- and AN-F1-infected cells (Fig. 2, lanes 5 and 9, respectively) were similar to DNA from wild-type virus-infected cells, indicating that the encapsidated DNA from *nuc* mutant-infected cells did not differ in size or structure from that in wild-type virus-infected cells. Furthermore, the relative amounts of protected DNA were similar in all four types of infected cells when the signals were quantified and normalized to amount of viral DNA synthesized (data not shown). These results confirm our previous observations that efficient encapsidation occurs in the nuclei of infected cells (53).

Analysis of well DNA from wild-type- and *nuc* mutant-infected cells. The structure of the replicative well DNA was also examined by PFGE following digestion with restriction enzymes. DNA samples from cells infected with KOS, AN-1, AN-1-2a, or AN-F1 at various times postinfection were subjected to PFGE, and the band at the well was excised and digested to completion with *Spe*I, which cuts the HSV-1 genome once. Digested well DNA was subjected to PFGE. This treatment would be expected to produce linear monomers from either circular or concatemeric viral DNA. If genomic inversion has occurred within the concatemeric DNA, three bands, 152, 118, and 186 kb, would be expected (Fig. 3). In the experiment shown in Fig. 4, only two of the three fragments, 152 and 186 kb, would be expected to react with the HSV-1 *Bam*HI B2 probe. At 8 h postinfection, the 186- and 152-kb bands are visible in KOS- and AN-1-2a-infected cells (Fig. 4, lanes 1 and 3, respectively). An additional band of 118 kb is visible on ethidium bromide-stained gels (not shown). The fact that both the 186- and 152-kb bands are seen indicates that genomic inversions have occurred by 8 h postinfection. These results confirm the recent report of Zhang et al. that inverted concatemers were present in wild-type virus-infected cells at early times after infection (70). Furthermore, the presence of the 186- and 118-kb bands indicates that these linear molecules arise from replicating concatemeric DNA and could not possibly be the product of circularized input DNA.

At later time points (13, 24, and 48 h postinfection), the 186- and 152-kb bands are very prominent in well DNA from KOS- and AN-1-2a-infected cells (Fig. 4, lanes 5, 7, 9, 11, 13, and 15),

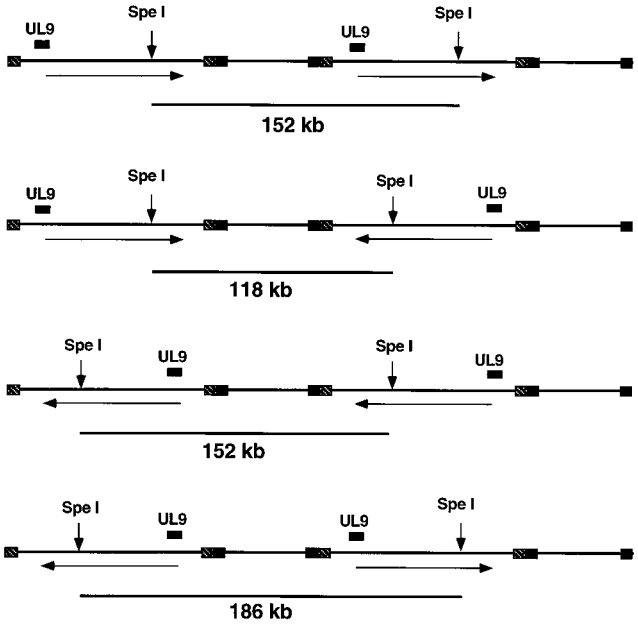

FIG. 3. Schematic diagram of the HSV-1 genome showing expected fragments of *Spe*I digestion. The positions of *Spe*I internal fragments expected from replicating DNA are shown, as are four different concatemers representing various inversion events. Concatemers in which the orientations of both long arms remain constant generate a 152-kb band; concatemers in which the long arms have inverted with respect to each other generate a 118- or 186-kb band. The *Bam*HI B2 probe (corresponding to the position marked UL9) will detect the 152- and 186-kb bands but not the 118-kb band.

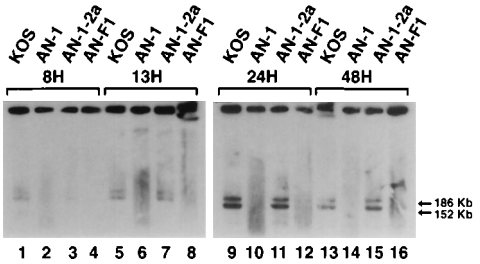

FIG. 4. Southern blot analysis of well DNA from wild-type- and *nuc* mutant-infected cells digested with *Spe*I. Cells were infected with KOS, AN-1, AN-1-2a, and AN-F1 at an MOI of 5 PFU per cell for various times after infection, and well DNA was isolated as described in Materials and Methods. Well DNA was digested in an agarose block with *Spe*I, which cleaves HSV-1 DNA once per monomer unit, and subjected once again to PFGE as described in the legend to Fig. 2. If linear monomers are released from well DNA, three fragments, 186, 152, and 118 kb, are expected as a result of genomic inversions. The blots were probed with the *Bam*HI B2 probe, which will detect only the 186- and 152-kb genome isomers (see Fig. 3). Lambda ladder PFGE markers consisting of concatemers of lambda DNA (ranging in size from 48.5 to 1,018.5 kb) (NEB) were used as molecular weight markers.

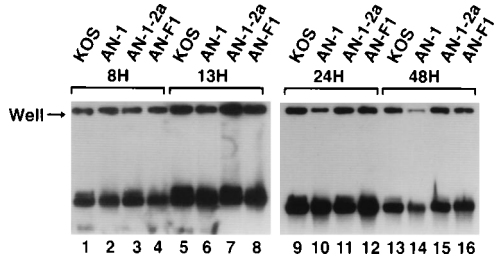

FIG. 5. Southern blot analysis of well DNA from KOS-, AN-1-, AN-1-2a-, and AN-F1-infected cells digested with *Xba*I. Cells were infected and well DNA was isolated as described in the legend to Fig. 2. Agarose blocks were digested with *Xba*I, which cleaves HSV-1 DNA four times per monomer unit. ^{32}P-labeled genomic HSV DNA digested with *Bam*HI and *Eco*RI was used as a probe. Lambda ladder PFGE markers and lambda DNA digested with *Hin*dIII were used as molecular weight markers.

although a considerable amount of viral DNA remains in the well. The fact that most of the DNA remained in the well even after digestion with a single-cutting enzyme strongly suggests that the replicating DNA is composed of complex nonlinear DNA. The DNAs remaining in the wells following *Spe*I digestion are not the products of incomplete digestion; this was determined by treating *Spe*I-digested DNA with *Bam*HI and analyzing the products by conventional agarose gel electrophoresis and Southern blotting. The blot was probed with a DNA fragment (*Bam*HI-H) which spans the *Spe*I site. In each of the DNA samples shown in Fig. 4, digestion with *Bam*HI yielded the predicted digestion products, indicating that *Spe*I digestion was nearly complete; in this experiment, very little DNA corresponding to partial *Bam*HI digestion products were seen, indicating that *Bam*HI digestion was also complete (data not shown).

When the well DNA from Vero cells infected with AN-1 or AN-F1 was digested with *Spe*I, no discrete fragments were released from the well even at late times after infection (Fig. 4, lanes 2, 4, 6, 8, 10, 12, 14, and 16). *Spe*I digestion was shown to be complete as described above (data not shown). Some additional smearing of DNA was seen at positions corresponding to between 50 and 375 kb. These results indicate that in cells infected with *nuc* mutants, the viral DNA is structurally different from that in cells infected with wild-type virus. Taken together, these results indicate that complex nonlinear DNA accumulates in cells infected with both wild-type HSV-1 and *nuc* mutants of HSV-1. Severini et al. have proposed that replicating DNA is composed of large branched structures (51), and our results are consistent with this hypothesis. Furthermore, our results suggest that the DNA which accumulates in cells infected with *nuc* mutants is even more complex or branched than that found in cells infected with wild-type virus. We suggest that the processing or debranching of viral DNA is aberrant in cells infected with *nuc* mutants.

To further characterize the well DNA, we have also digested it with enzymes which cleave viral DNA more frequently. For instance, *Xba*I cleaves HSV DNA four times, generating fragments of between 24 and 64 kb. In Fig. 5, well DNA from cells infected with KOS, AN-1, AN-1-2a, or AN-F1 was isolated as described above. After digestion with *Xba*I, DNA was subjected to PFGE, blotted, and hybridized to a probe consisting of whole HSV genomic DNA as described in Materials and Methods. Figure 5 shows that linear fragments can be released from well DNA from both mutant- and wild-type-infected cells following *Xba*I digestion. The predominant band of released linear DNA represents several different fragments ranging from 24 to approximately 62 kb which are not resolved on this gel. A significant amount of replicating DNA remains in the well, indicating that some DNA is still in a complex configuration, possibly containing branches which prevent the DNA from migrating into the gel. In Fig. 6, well DNA was digested with *Bam*HI, which cleaves the HSV-1 genome at least 41 times and generates fragments smaller than 12 kb. In this case, almost all viral DNA can be released from well DNA from mutant- and wild-type-infected cells (Fig. 6). The fact that most of the DNA can be released by *Bam*HI digestions indicates either that small fragments with branches migrate normally on gels and are not retained in the well or that in small fragments, branch migration may occur readily (48). Since *Bam*HI is a frequent cutter, putative branch points would likely be found close to an end and be able to migrate, generating linear fragments which can enter a gel.

Taken together, these results indicate that replicating DNA assumes a complex, possibly branched structure. If the DNA is in fact in a branched configuration, we estimate that the frequency of branches must be at least once per genome in *nuc* mutant-infected cells. Although the frequency is less in cells infected with wild-type virus, it is impossible to accurately estimate the number of branches per genome. This is due in part to the fact that although some DNA was released, a

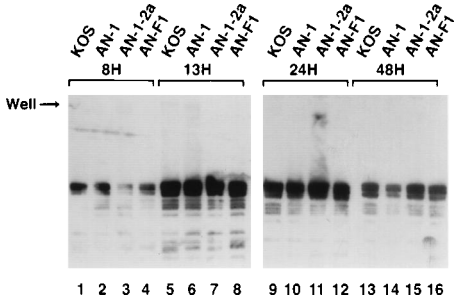

FIG. 6. Southern blot analysis of well DNA from wild-type- and *nuc* mutant-infected cells digested with *Bam*HI. Cells were infected and well DNA was isolated as described in the legend to Fig. 2. Agarose blocks were digested with *Bam*HI, which cleaves HSV-1 DNA 41 times per monomer unit. ^{32}P-labeled genomic HSV DNA digested with *Bam*HI and *Eco*RI was used as a probe. Lambda ladder PFGE markers and lambda DNA digested with *Hin*dIII were used as molecular weight markers.

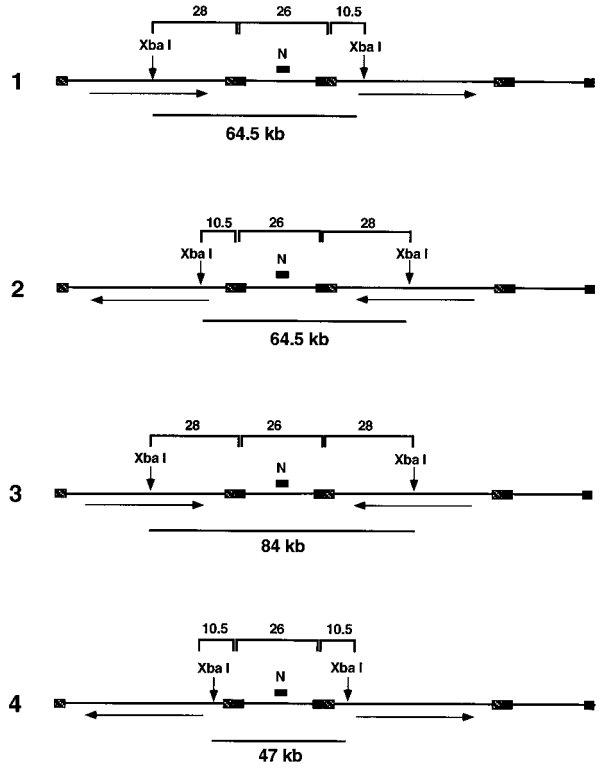

FIG. 7. Schematic diagram of the HSV-1 genome showing several possible concatemers after digestion with *Xba*I. Concatemers in which the orientations of both long arms remain constant generate a 64.5-kb band (lines 1 and 2); concatemers in which the long arms have inverted with respect to each other generate an 84- or 47-kb band (lines 3 and 4). Horizontal arrows represent the orientations of the long-arm segments. The lengths of the segments contributing to the observed *Xba*I fragments are shown above each line. The position of the hybridization probe, the *Hin*dIII N fragment from the middle of U_S, is shown.

significant amount of DNA from cells infected with wild-type virus remained in the well. The possibility of branch migration when the branch point is near an end liberated by digestion with a restriction enzyme also makes it difficult to precisely estimate the frequency of these structures. Furthermore, it is not clear how a molecule with a single branch would migrate in PFGE.

Inversion in replicating DNA from wild-type- and *nuc* mutant-infected cells. In the experiment described above (Fig. 4), well DNA from KOS-infected cells was digested with *Spe*I. The appearance of both the 186- and 152-kb bands indicates that genomic inversions have occurred by 8 h postinfection. Since *Spe*I failed to release discrete bands from AN-1- and AN-F1-infected cells, it was impossible to determine whether inversions had occurred in these cells. Because *Xba*I digestion was more successful in liberating well DNA from *nuc* mutant-infected cells, we used *Xba*I-digested well DNA from KOS-, AN-1-2a-, AN-F1-, or AN-F3-infected cells to determine whether *nuc* mutants were able to carry out genomic inversions. In the experiment shown in Fig. 8, the 5-kb *Hin*dIII N fragment from the middle of U_S was used as a hybridization probe. This probe will detect a 64.5-kb band if excised from concatemeric DNA in which the two U_L segments are in the same orientation with respect to one another (either parental or inverted orientation) (Fig. 7, lines 1 and 2); on the other hand, if two adjacent U_L segments are inverted with respect to one another, an 84- or a 47-kb band will be detected (Fig. 7, lines 3 and 4). In Fig. 8, lane 2, well DNA harvested at 8 h postinfection with KOS was digested with *Xba*I; the three expected bands at 84, 64.5, and 47 kb (bands 1, 2, and 3, respectively) are seen, although the 47-kb band (band 3) is somewhat underrepresented. An identical pattern was seen in cells infected with the wild-type recombinant virus, AN-1-2a (Fig. 8, lane 3). In cells infected with AN-F1 or AN-F3, the same three bands can be seen; however, in this case the 47-kb band (band 3) is even more prominent than it was in well DNA from wild-type virus-infected cells (Fig. 8, lanes 4 and 5). Both *nuc* mutant DNAs exhibit smearing in the region of the gel surrounding the observed bands. The smearing is reminiscent of the smearing seen after *Spe*I digestion (Fig. 4) and may represent increased fragility in the DNA as a result of unusual structures in the *nuc* mutant DNA. The appearance of *Xba*I fragments 1, 2, and 3 indicates that inversions occur in *nuc* mutant DNA at a frequency similar to that seen in the wild-type DNA.

To confirm that *nuc* mutants do not exhibit a defect in inversion events, we used a quantitative recombination assay originally developed to assess the role of the *a* sequences in HSV-1 genome isomerization (50). Two of the constructs used in that study have been used here to analyze the ability of *nuc* mutants to carry out DNA inversion-type events. pLR, a construct containing an HSV origin of replication and Tn*5* sequences, was shown to undergo inversion as a result of recombination between its reiterated IS*50* sequences. pLΔR-193 contains inverted copies of a small subfragment from within the *a* sequence; these reiterations are much smaller than the IS*50* elements of pLR and are able to promote high-frequency recombination only because they can be recognized and cleaved by the cleavage and packaging machinery (50). Both plasmids had previously been shown to undergo high-frequency recombination in cells infected with wild-type virus; however, high-frequency inversion did not occur within pLΔR-193 when the superinfecting virus was an HSV-1 mutant which is defective for the cleavage and packaging process. We previously showed that AN-1 is capable of carrying out genome cleavage and packaging (53). In this study, we were interested in whether AN-1 was capable of directing high-frequency recombination and also in whether the inversion frequency of pLR and pLΔR-193 would differ in HSV-1 (KOS) and AN-1.

Vero cells were transfected with pLR and pLΔR-193 and

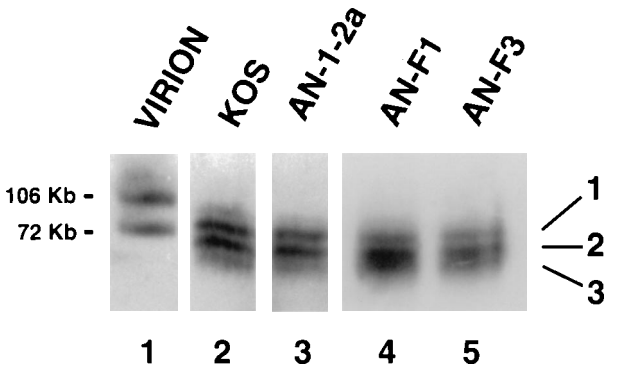

FIG. 8. Southern blot analysis of well DNA from wild-type- and *nuc* mutant-infected cells digested with *Xba*I to analyze genomic inversions. Cells were infected with KOS, AN-1-2a, AN-F1, and AN-F3 at an MOI of 5 PFU per cell for various times after infection. Well DNA was purified twice by PFGE to rule out contamination with free monomeric DNA and subjected to *Xba*I digestion, PFGE, and Southern blotting. Lane 1, virion DNA digested with *Spe*I to serve as size markers; lane 2, well DNA from KOS-infected cells; lane 3, well DNA from AN-1-2a-infected cells; lane 4, well DNA from AN-F1-infected cells; lane 6, well DNA from AN-F3-infected cells. Bands 1, 2, and 3 correspond to 84-, 64.5-, and 47-kb fragments, respectively.

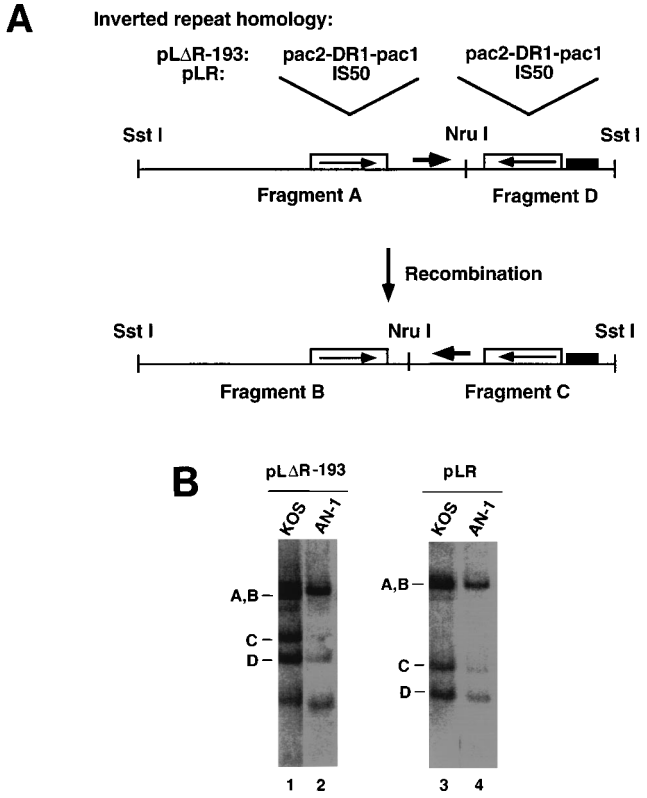

FIG. 9. Ability of AN-1 and KOS to induce inversion events in plasmids. (A) Schematic diagram of plasmids pLΔR-193 and pLR, which were used to assess inversion events. pLΔR-193 contains a 193-bp region from the *a* sequences including pac2, DR1, and pac1 (illustrated in Fig. 1C). pLR contains the IS*50* repeat elements from Tn*5*. The repeat homology in each case was cloned in an inverted orientation into pUC19, which also contains ori_S, an HSV-1 origin of replication. The third line shows the predicted fragments (A and D) expected if the parental plasmid is digested with *Nru*I and *Sst*I. The bottom line shows the predicted fragments (B and C) if the plasmid had undergone an inversion event. (B) Cells were transfected with either pLΔR-193 (lanes 1 and 2) or pLR (lanes 3 and 4). Transfected cells were superinfected with KOS (lanes 1 and 3) or AN-1 (lanes 2 and 4). Total DNA was digested with *Nru*I and *Sst*I, and the DNA was subjected to electrophoresis and Southern blot transfer as described in Materials and Methods.

superinfected the following day with either wild-type HSV-1 strain KOS or AN-1. DNA was isolated and examined in Southern blots to determine if plasmid DNA had undergone sequence inversion as a result of DNA replication-directed recombination. The plasmids typically yield two *Sst*I-*Nru*I fragments (A and D in Fig. 9A) but will generate two novel fragments (B and C) if a sequence inversion event has occurred. Representative blots are shown in Fig. 9B. The signal in each of the bands was quantified as described in Materials and Methods, and the results from duplicate experiments are shown in Table 1. Inversion frequency represents the percentage of the C-fragment signal relative to the total signal of the C and D fragments for a given lane. The frequency of inversion of AN-1 relative to the KOS inversion frequency for a given plasmid is shown. In addition, the total signal of all bands within each lane was determined, and the efficiency of DNA replication of plasmid in AN-1-infected cells relative to that for KOS-infected cells for a given plasmid is shown. These results indicate that both pLR and pLΔR-193 are capable of undergoing high levels of recombination in both wild-type- and AN-1-infected cells. Both plasmids undergo inversion at maximal levels in KOS-infected cells, and this recombination is reduced by only 13 to 19% in AN-1-infected cells. The latter effect is probably due to the fact that both plasmids are significantly underreplicated in AN-1-infected cells. We previously reported that DNA synthesis of viral genomes in AN-1-infected cells varies somewhat from experiment to experiment between 50 and 90% of wild-type levels. The present experiment indicates that the efficiency of replication of HSV-1 origin-containing plasmids may be lower than the efficiency of genome replication. We do not have an explanation for this observation. Nevertheless, the high levels of recombination seen in AN-1-infected cells clearly indicate that alkaline nuclease is not required for HSV-1 genome isomerization. This finding is in agreement with the previous finding that the UL12 gene is not a member of the minimum complement of genes required for mediated HSV-1 recombination events in a transient expression assay (61). Thus, the results of the transfection assays confirm the results obtained by directly examining replicating DNA, which indicate that inversion is not compromised in AN-1-infected cells.

Presence of U_L termini in replicating DNA from wild-type and *nuc* mutant virus-infected cells. Previous reports showed that after electrophoretic removal of linear viral genomes, terminal fragments in well DNA were barely detectable (51, 70). Upon further study, however, Zhang et al. were able to detect genomic termini corresponding to the U_L segment of the HSV-1 genome (70). We have confirmed this observation by using *Spe*I-digested well DNA from cells infected with wild-type virus (data not shown). We wanted to extend this analysis to well DNA from cells infected with *nuc* mutants; however, because *Spe*I digestion does not release fragments efficiently from replicating DNA from these cells, we decided to use a more frequent cutter, *Bam*HI. Wild-type and *nuc* mutant DNAs were analyzed following *Bam*HI digestion and Southern blot hybridization using defined probes which are expected to hybridize specifically to U_L or U_S. In the experiment shown in Fig. 10, virion DNA, total DNA, or twice-purified well DNA from KOS-, AN-1-, and AN-F1-infected cells taken at 24 h postinfection was digested with *Bam*HI and hybridized sequentially with probes *b* and *c*. Probe *b* was from the reiterated *b* fragment and would be expected to hybridize to the SQ junction and to the *Bam*HI S fragment from U_L but not to the *Bam*HI Q fragment from U_S (Fig. 1B). Probe *c* was from the reiterated *c* sequence and would be expected to hybridize to the SQ junction and to the *Bam*HI Q fragment from U_S but not to the *Bam*HI S fragment from U_L (Fig. 1B). In this region of the genome, considerable heterogeneity has been reported. One source of heterogeneity results from multiple reiterations

TABLE 1. Inversion frequencies of plasmids pLΔR-193 and pLR in KOS- and AN-1-infected cells

Transfected plasmid	Strain	Inversion frequency[a] (SD)	% of wt recombination[b] (SD)	% of wt replication[c] (SD)
pLΔR-193	KOS	51.3 (0.8)	100	100
	AN-1	41.8 (4.4)	81.5 (8.6)	18.3 (4.0)
pLR	KOS	50.3 (3.4)	100	100
	AN-1	43.9 (2.9)	87.3 (5.8)	29.8 (16.1)

[a] Percentage of the C-fragment signal relative to the total signal of the C and D fragments for a given lane.

[b] Percentage of the AN-1 recombination frequency relative to the frequency for KOS (wild type [wt]). The KOS values were set at 100% for comparison purposes.

[c] Efficiency of DNA replication for each plasmid in AN-1-infected cells compared with KOS-infected cells. The KOS values were set at 100% for comparison purposes.

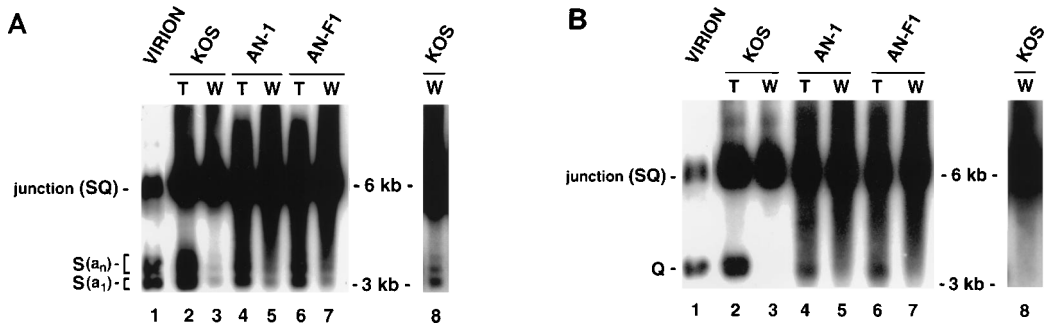

FIG. 10. Southern blot analysis of virion, total, and well DNAs from wild-type- and *nuc* mutant-infected cells. Cells were infected with KOS, AN-1, or AN-F1 at an MOI of 5 PFU per cell for 24 h. Virion, total, and twice-purified well DNAs were subjected to *Bam*HI digestion, conventional gel electrophoresis, and Southern blotting. Two different probes were used in this experiment. In panel A, a 132-bp *Nco*I-to-*Dra*I fragment from pNar, within the repeated *b* sequences, detects the *Bam* S terminal fragment flanking U_L (Fig. 1B, probe *b*). Bands marked S(a_1) and S(a_n) represent S fragments with single and multiple *a* sequences (see text). In panel B, a 429-bp *Sma*I-*Nar*I fragment from pNar, within the repeated *c* sequences, detects the *Bam* Q terminal fragment flanking U_S (Fig. 1B, probe *c*). Molecular weight markers were 1-kb markers (ranging in size from 12 to 0.075 kb) (GIBCO-BRL, Grand Island, N.Y.).

of the *a* sequence itself. The L-S junction (*Bam*HI SQ) and the U_L terminus (*Bam*HI S) contain multiple copies of the *a* sequence, while the U_S terminus (*Bam*HI Q) possesses only one copy (35, 60). Virion and total DNAs from all infected cell samples contain several bands detected with probe *b* (Fig. 10A, lanes 1, 2, 4, and 6): the band marked S(a_1) likely corresponds to an S band with a single copy of the *a* sequence, and the group of bands marked S(a_n) each differ from the bottom band and from each other by approximately 250 bp. These bands likely correspond to reiterations of the *a* sequence (a_n). On the other hand, when probe *c* was used (Fig. 10B, lanes 1, 2, 4, and 6), a single band corresponding to the Q fragment was observed. The only difference between wild-type and *nuc* mutant total DNAs is an increased amount of heterogeneously sized DNA in the *nuc* mutants, migrating as a smear.

Well DNA from KOS-infected cells shows a strong band corresponding to the SQ internal junction fragment and contains very little DNA corresponding to terminal fragments (Fig. 10A and B, lanes 3 and darker exposures of the same lanes shown in lanes 8). By comparing the well DNAs which hybridize to probes *b* and *c* (Fig. 10A and B, lanes 3 and 8), it is clear that small amounts of DNA from the U_L terminus but no DNA from the U_S terminus are present in replicating DNA. The multiple bands seen in Fig. 10A, marked S(a_1) and S(a_n), likely correspond to S fragments with single or multiple copies of the *a* sequence. Well DNA from AN-1- or AN-F1-infected cells (Fig. 10A and B, lanes 5 and 7) is also similar to well DNA from cells infected with KOS. Thus, bands corresponding to the U_L terminus, marked S(a_n) and S(a_1), are seen (Fig. 10A, lanes 5 and 7). Although considerable smearing is observed in *nuc* mutant well DNA with both probes, the absence of a discrete Q band in Fig. 10B, lanes 5 and 7, confirms that no U_S terminus is present. Thus, it appears that well DNA from KOS- and *nuc* mutant-infected cells contains a U_L terminus but not a U_S terminus. The major difference between wild-type and mutant well DNAs is in the considerable size heterogeneity observed in DNA from the *nuc* mutants. As mentioned above, the heterogeneity may reflect fragile sites in the mutant DNA as a result of extensive branching or other alterations in structure. Thus, we have demonstrated that U_L and not U_S segments are found at the termini of replicating well DNA in *nuc* mutants as well as wild-type well DNA. Although other explanations are possible, we consider it most likely that the observed ends are generated by an asymmetry in the cleavage and packaging process, resulting in U_L termini being left behind. The presence of U_L termini in *nuc* mutant-infected cells provides another piece of evidence that the cleavage machinery is functional in these mutants.

DISCUSSION

The precise role of the viral alkaline nuclease in the life cycle is unknown; however, the fact that well-conserved homologs of the alkaline nuclease are found in all herpesviruses suggests that it may carry out an important function in the virus life cycle. The major conclusions drawn from this study are as follows: (i) both wild-type and mutant HSV-1 DNAs exist in a complex nonlinear form (possibly branched) during replication; (ii) digestion with a single-cutting enzyme cannot release discrete monomer-length DNA from replicating DNA isolated from *nuc* mutant-infected cells whereas it can do so from wild-type virus-infected cells; (iii) replication intermediates from cells infected with the wild type and *nuc* mutants contain genomic inversions indicating that alkaline nuclease is not essential for genomic inversion; (iv) plasmids containing inverted repeats undergo high-frequency recombination in cells infected with the wild type and *nuc* mutants, indicating that alkaline nuclease is not required for replication driven recombination in plasmids; (v) DNA encapsidated by *nuc* mutants is similar in size and structure to DNA encapsidated by wild-type virus; and (vi) replicating DNA in cells infected with the wild type and *nuc* mutants contains U_L termini and is devoid of U_S termini. Our previous data indicated that although DNA synthesis, cleavage of viral DNA into monomeric units, and encapsidation of monomeric units in the nucleus occur in *nuc* mutants, the capsids are not competent to migrate into the cytoplasm (53, 65). These results and those of others suggest that DNA with a complex structure accumulates during HSV DNA replication (51). In *nuc* mutants, the structure is even more complex than that seen in wild-type virus-infected cells; this leads to the production of aberrant capsids which are not competent for egress.

Models for the generation of replication intermediates. Several models for the formation of concatemers during DNA replication can be considered. (i) It has been suggested that HSV DNA replicates through a rolling-circle mechanism (5, 29); however, a simple rolling-circle model does not account for recent reports describing the structure of viral DNA which arises during DNA replication in HSV-infected cells (described below). In addition, the rolling-circle model does not account for the rapid amplification of viral DNA seen in the initial hours after infection (23, 24). (ii) It is possible that the

initial rounds of HSV DNA replication occur by theta replication structures initiated at one or more of the three origins of replication. At a later stage, a rolling-circle structure could be formed which would generate the head-to-tail concatemers seen late in infection. Another version of this model (70) posits that theta-type amplification of a circular template followed by a recombination event similar to those which occur between two copies of a repeated sequence in the yeast 2μm circle may occur (19, 45, 58). The inversion occurs between a replicated copy of an inverted repeat unit and an unreplicated copy, resulting in a situation in which two forks chase each other, generating concatemeric DNA. (iii) Concatemers could be formed by a recombination mechanism similar to that used during late T4 bacteriophage DNA replication (36, 44). Strand invasion provides 3′ hydroxyl primers for initiation of further DNA replication, and these recombination intermediates can be converted into replication forks (44). Branch migration and subsequent resolution of these recombination intermediates are required for efficient late viral DNA synthesis and packaging in T4. In models ii and iii, on the basis of DNA replication strategies in either the 2μm circle and bacteriophage T4, respectively, recombination is an obligatory event in the generation of the head-to-tail concatemers of DNA. The notion that replication and recombination in HSV are intimately intertwined and possibly dependent on one another is attractive for several reasons. First, replication and recombination seem to be temporally linked (16, 70). Second, replication and recombination appear to be functionally linked in that Sarisky and Weber showed that inversion events are mediated by the viral DNA replication machinery (50). Third, a precedent exists for a linkage between replication and recombination in other DNA viruses such as T4 (36) and adenovirus (69).

The demonstration that genomic inversions can be detected at 3 to 4 h postinfection (70) has several implications for the models of DNA replication. First, this result is not consistent with simple rolling-circle replication (model i, above), since this model makes no provision for inversion. Second, the replication and/or recombination machinery must include a nicking activity responsible for initiating the inversion event in a circular genome. Although it is possible that the cleavage and packaging system of HSV can generate a free DNA end capable of strand invasion, Zhang et al. have shown that inversion occurs before the viral enzymes responsible for cleavage and packaging are functional (70). Furthermore, the demonstration that the seven HSV replication functions are sufficient for recombination (61) further argues that the inversion events do not require the cleavage and packaging machinery. Another possible candidate for an activity which could initiate recombination through the formation of double-stranded breaks is a cellular endonuclease described by Wohlrab et al. which cleaves DNA in the DR2 array of the *a* sequence of HSV-1 (67, 68). However, inversions occur even in the absence of the DR2 array (50, 56). Furthermore, Martin and Weber (37a) have recently constructed a virus whose L-S junctions lack *a* sequences. This virus still undergoes normal isomerization, indicating that neither cleavage and packaging at the *a* sequence nor nicking of its DR2 repeats is required for genomic inversion events. Thus, while generation of a single- or double-stranded end of DNA by either the cleavage and packaging enzymes or by a DR2-specific endonuclease has the potential to promote recombination, these cleavage events cannot explain the early inversion events which occur independently of these two mechanisms. A third possibility is that the process of HSV DNA replication per se is recombinogenic. According to this model, breaks which occur normally at an HSV replication fork, either at the 3′ end of the leading strand or at the 3′ end of an Okazaki fragment on the lagging strand, may provide the required break to initiate a strand invasion leading to a recombination event. We propose that DNA replication which initiates at an HSV origin is more likely to be involved in such an event than in other systems such as simian virus 40 DNA replication.

Resolution of replication intermediates. We propose that as a result of one or more of the replication and/or recombination mechanisms outlined above, branched structures arise. These structures may initially resemble three-way or Y junctions. Direct evidence for the presence of Y junctions in replicating HSV DNA has recently been provided by Severini et al., using agarose two-dimensional gel electrophoresis and electron microscopy (52). Three-way junctions can be converted to four-way junctions (X or Holliday junctions) by branch migration (43). Severini et al. also have evidence for X junctions in replicating HSV DNA by two-dimensional gel electrophoresis, although Y junctions were more prevalent (52). Regardless of how they are formed, and whether Y or X junctions predominate, these branched structures must be resolved prior to packaging genomic DNA into viral capsids. Bacteriophage T4 encodes a phage protein endonuclease VII (product of gene 49) which can process or resolve Holliday junctions to produce unconnected linear DNA molecules (33, 42). A similar enzyme, called endonuclease I, is encoded by T7 gene 3 (14). Severini et al. have recently demonstrated that branched structures which accumulate in HSV-infected cells can be resolved by T7 phage endonuclease I, which is known to specifically linearize Y and X junctions (52). Therefore, although it is not clear exactly how they are generated, considerable evidence indicates that replicating HSV DNA contains branches which must be resolved before infectious virus can be produced.

We have previously suggested that the alkaline nuclease functions to resolve recombination intermediates which arise during DNA replication (53, 65). However, it is possible that structures which result from recombination in infected cells require host enzymes as well. The high frequency of recombination observed between infecting viruses (11) and in plasmid recombination systems suggests that numerous Holliday junctions likely exist within the replicating DNA. Brown et al. have estimated that the average frequency of recombination between infecting genomes is 0.43/kbp, or almost 70 per genome length (11). In the quantitative plasmid recombination assay, inversion frequencies are very high (50). Branched DNA accumulates in cells infected with wild-type virus (4, 40, 51, 70), and we have now shown that even more complex structures accumulate in cells infected with *nuc* mutants. Although it has not been possible to accurately estimate the number of branches per genome, the frequencies of branches even in cells infected with *nuc* mutants do not seem to be as high as would be suggested by the overall recombination frequencies. It is possible that host cell resolvases function to eliminate at least some of the Holliday junctions in viral DNA. This explanation is consistent with the observation that plasmid recombination occurs with very high efficiency in cells infected with AN-1. The possible involvement of a cellular resolvase is also consistent with the observation that transfected DNA replication genes are capable of mediating inversion in the absence of alkaline nuclease.

The observation that the DNA accumulating in cells infected with *nuc* mutants has an aberrant structure indicates that the cellular enzymes are incapable of resolving all unusual structures which occur in replicating DNA. This may indicate that replicating HSV DNA exists in a complex structure unique to replicating herpesvirus DNA which is different from standard Holliday junctions and not recognized by host resolvases. A

comprehensive biochemical analysis of the activities of the alkaline nuclease and continued investigation of the structure of replicating viral DNA will be required to fully understand the role that the alkaline nuclease plays in the maturation of herpesvirus DNA.

DNA encapsidated by *nuc* mutants is similar in size and structure to DNA encapsidated by wild-type virus. Our previous results indicated that *nuc* mutants have a severe defect in the egress of DNA-containing capsids from the nucleus and are consistent with the observation that no C capsids were found in the cytoplasm of AN-1-infected cells on sucrose gradients (53). These findings are reminiscent of a report in 1982 by Vlazny et al. describing the formation of defective genomes (59); these investigators demonstrated that viral DNA molecules substantially smaller than unit length can be packaged in the nucleus but do not appear in the cytoplasm. Apparently, a selection mechanism exists such that only capsids containing a full complement of viral DNA actually mature into the cytoplasm; this mechanism may involve a conformational change or "locking step" involving the capsid. We previously demonstrated that a significant amount of AN-1 DNA in the nucleus of infected cells does become protected in a nuclease sensitivity assay (53). In this study, we used PFGE to determine the size and structure of viral DNA protected in the nuclei of AN-1-infected cells. One hypothesis was that this encapsidated DNA was abnormal in some way, for instance, perhaps shorter than unit length as was seen by Vlazny et al. (59). Our PFGE analysis indicates that this DNA is indistinguishable from wild-type monomeric DNA. Thus, the DNA encapsidated by the mutant is certainly not smaller than unit length; however, we cannot rule out alterations such as small branches which may not be detected by PFGE. It is possible that the full-length monomeric DNA which is taken up into the capsids in *nuc* mutant-infected cells contains branches that are capable of branch migration and that the branches simply migrated off each linear molecule during extraction. Spontaneous branch migration is known to occur readily in molecules containing a branch point flanked by DNA sequence homology (48). Further experiments designed to freeze putative branch points will be required in order to more fully understand the nature of the viral DNA which becomes nuclease resistant in *nuc* mutant-infected cells.

The two major defects in *nuc* mutants are (i) the inability to correctly process viral DNA genomes (discussed above) and (ii) the failure of DNA-containing capsids to migrate into the cytoplasm. It is possible that the alkaline nuclease plays a role in the conformational or maturational changes required for egress of DNA-containing capsids which is completely separate from its role in resolution of recombination intermediates. However, we favor the notion that the defect in processing of viral DNA is responsible for the failure of DNA-containing capsids to mature into the cytoplasm.

ACKNOWLEDGMENTS

We are grateful to members of the laboratory for critical discussions and reading of the manuscript. We thank Alberto Severini for making results available prior to publication.

This work was supported by ACS grant VM9 and by grant AI37549. S.K.W. was an American Heart Association/Genentech Established Investigator during the initial stages of this work.

REFERENCES

1. **Addison, C., F. J. Rixon, and V. G. Preston.** 1990. Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. J. Gen. Virol. **71:**2377–2384.
2. **Banks, L., D. J. Purifoy, P. F. Hurst, R. A. Killington, and K. L. Powell.** 1983. Herpes simplex virus non-structural proteins. IV. Purification of the virus-induced deoxyribonuclease and characterization of the enzyme using monoclonal antibodies. J. Gen. Virol. **64:**2249–2260.
3. **Banks, L. M., I. W. Halliburton, D. J. M. Purifoy, R. A. Killington, and K. L. Powell.** 1985. Studies on the herpes simplex virus alkaline nuclease: detection of type-common and type-specific epitopes on the enzyme. J. Gen. Virol. **66:** 1–14.
4. **Bataille, D., and A. Epstein.** 1994. Herpes simplex virus replicative concatemers contain L components in inverted orientation. Virology **203:**384–388.
5. **Becker, Y., Y. Asher, E. Weinberg-Zahlering, S. Rabkin, A. Friedmann, and E. Kessler.** 1978. Defective herpes simplex virus DNA: circular and circular-linear molecules resembling rolling circles. J. Gen. Virol. **40:**319–335.
6. **Ben-Porat, T., and F. J. Rixon.** 1979. Replication of herpesvirus DNA. IV: analysis of concatemers. Virology **94:**61–70.
7. **Ben-Porat, T., F. J. Rixon, and M. L. Blankenship.** 1979. Analysis of the structure of the genome of pseudorabies virus. Virology **95:**285–294.
8. **Ben-Porat, T., and S. A. Tokazewski.** 1977. Replication of herpesvirus DNA. II. Sedimentation characteristics of newly synthesized DNA. Virology **79:** 292–301.
9. **Beverley, S. M.** 1988. Characterization of the 'unusual' mobility of large circular DNAs in pulsed field-gradient electrophoresis. Nucleic Acids Res. **16:**925–939.
10. **Beverley, S. M.** 1989. Estimation of circular DNA size using gamma-irradiation and pulsed-field gel electrophoresis. Anal. Biochem. **177:**110–114.
11. **Brown, S. M., J. H. Subak-Sharpe, J. Harland, and A. R. MacLean.** 1992. Analysis of intrastrain recombination in herpes simplex virus type 1 strain 17 and herpes simplex virus type 2 strain HG52 using restriction endonuclease sites as unselected markers and temperature-sensitive lesions as selected markers. J. Gen. Virol. **73:**293–301.
12. **Cairns, J.** 1963. The chromosome of Escherichia coli. Cold Spring Harbor Symp. Quant. Biol. **28:**43–46.
13. **Costa, R. H., K. G. Draper, L. Banks, K. L. Powell, G. Cohen, R. Eisenberg, and E. K. Wagner.** 1983. High-resolution characterization of herpes simplex virus type 1 transcripts encoding alkaline exonuclease and a 50,000-dalton protein tentatively identified as a capsid protein. J. Virol. **48:**591–603.
14. **deMassy, B., F. W. Studier, L. Dorgai, E. Appelbaum, and R. A. Weisberg.** 1984. Enzymes and sites of genetic recombination: studies with gene-3 endonuclease of phage T7 and with site-affinity mutants of phage lambda. Cold Spring Harbor Symp. Quant. Biol. **49:**715–726.
15. **Draper, K. G., G. Devi-Rao, R. H. Costa, E. D. Blair, R. L. Thompson, and E. K. Wagner.** 1986. Characterization of the genes encoding herpes simplex virus type 1 and type 2 alkaline exonucleases and overlapping proteins. J. Virol. **57:**1023–1036.
16. **Dutch, R. E., R. C. Bruckner, E. S. Mocarski, and I. R. Lehman.** 1992. Herpes simplex virus type 1 recombination: role of DNA replication and viral a sequences. J. Virol. **66:**277–285.
17. **Enquist, L. W., and A. M. Skalka.** 1978. Replication of bacteriophage lambda DNA. Trends Biochem. Sci. **3:**279–283.
18. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. **132:**6–13.
19. **Futcher, A. B.** 1986. Copy number amplification of the 2 micron circle plasmid of Saccharomyces cerevisiae. J. Theor. Biol. **119:**197–204.
20. **Garber, D. A., S. M. Beverley, and D. M. Coen.** 1993. Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. Virology **197:**459–462.
21. **Gilbert, W., and D. Dressler.** 1968. DNA replication: the rolling circle model. Cold Spring Harbor Symp. Quant. Biol. **33:**473–484.
22. **Goldin, A. L., R. M. Sandri-Goldin, M. Levine, and J. C. Glorioso.** 1981. Cloning of herpes simplex virus type 1 sequences representing the whole genome. J. Virol. **38:**50–58.
23. **Hammerschmidt, W., and J. Mankertz.** 1991. Herpes viral DNA replication: between the known and unknown. Semin. Virol. **2:**257–269.
24. **Hammerschmidt, W., and B. Sugden.** 1990. DNA replication of herpesviruses during the lytic phase of their life-cycles. Mol. Biol. Med. **7:**45–57.
25. **Hayward, G. S., R. J. Jacob, S. C. Wadsworth, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. Proc. Natl. Acad. Sci. USA **72:**4243–4247.
26. **Hirsch, I., G. Cabral, M. Patterson, and N. Biswal.** 1977. Studies on intracellular replicating DNA of herpes simplex virus type 1. Virology **81:**48–61.
27. **Irmiere, A., and W. Gibson.** 1983. Isolation and characterization of a non-infectious virion-like particle released from cells infected with human strains of cytomegalovirus. Virology **130:**118–133.
28. **Irmiere, A., and W. Gibson.** 1985. Isolation of human cytomegalovirus intranuclear capsids, characterization of their protein constituents, and demonstration that the B-capsid assembly protein is also abundant in noninfectious enveloped particles. J. Virol. **56:**277–283.
29. **Jacob, R. J., L. S. Morse, and B. Roizman.** 1979. Anatomy of herpes simplex virus DNA. XII. Accumulation of head to tail concatemers in the nuclei of infected cells and their role in the generation of four isomeric arrangements of viral DNA. J. Virol. **29:**448–457.
30. **Jacob, R. J., and B. Roizman.** 1977. Anatomy of herpes simplex virus DNA.

VIII. Properties of the replicating DNA. J. Virol. **23:**394–411.
31. **Jongeneel, C. V., and S. L. Bachenheimer.** 1981. Structure of replicating herpes simplex virus DNA. J. Virol. **39:**656–660.
32. **Kemper, B., F. Jensch, M. von Depka-Prondzynski, H. J. Fritz, U. Borgmeyer, and K. Mizuuchi.** 1984. Resolution of Holliday structures by endonuclease VII as observed in interactions with cruciform DNA. Cold Spring Harbor Symp. Quant. Biol. **49:**815–825.
33. **King, G. J., and W. M. Huang.** 1982. Identification of the origins of T4 DNA replication. Proc. Natl. Acad. Sci. USA **79:**7248–7252.
34. **Lee, J. Y., A. Irmiere, and W. Gibson.** 1988. Primate cytomegalovirus assembly: evidence that DNA packaging occurs subsequent to B capsid assembly. Virology **167:**87–96.
35. **Locker, H., and N. Frenkel.** 1979. The *Bam*I, *Kpn*I, and *Sal*I restriction enzyme maps of the DNAs of herpes simplex virus strains Justin and F: occurrence of heterogeneities in defined regions of the viral DNA. J. Virol. **32:**429–441.
36. **Luder, A., and G. Mosig.** 1982. Two alternative mechanisms for initiation of DNA replication forks in bacteriophage T4: priming by RNA polymerase and by recombination. Proc. Natl. Acad. Sci. USA **79:**1101–1105.
37. **Maniatis, T., E. F. Fritsch, and J. Sambrook (ed.).** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37a. **Martin, D. W., and P. C. Weber.** Unpublished data.
38. **Martinez, R., L. Shao, J. C. Bronstein, P. C. Weber, and S. K. Weller.** 1996. The product of a 1.9-kb mRNA which overlaps the HSV-1 alkaline nuclease gene (UL12) cannot relieve the growth defects of a null mutant. Virology **215:**152–164.
38a. **Martinez, R., and S. K. Weller.** Unpublished data.
39. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**1531–1574.
40. **McVoy, M. A., and S. P. Adler.** 1994. Human cytomegalovirus DNA replicates after early circularization by concatemer formation, and inversion occurs within the concatemer. J. Virol. **68:**1040–1051.
41. **McVoy, M. M., and S. P. Adler.** 1991. Analysis of human cytomegalovirus (HCMV) DNA replicative intermediates: DNA forms not predicted by the rolling circle model, p. 53–56. *In* M. P. Landini (ed.), Progress in cytomegalovirus research. Elsevier, Amsterdam.
42. **Mizuuchi, K., B. Kemper, H. Hays, and R. A. Wiesberg.** 1988. T4 endonuclease VII cleaves Holliday structures. Cell **29:**357–365.
43. **Morgan, A. R., and A. Severini.** 1990. Interconversion of replication and recombination structures: implications for terminal repeats and concatemers. J. Theor. Biol. **144:**195–202.
44. **Mosig, G.** 1987. The essential role of recombination in phage T4 growth. Annu. Rev. Genet. **21:**347–371.
45. **Murray, J. A. H., M. Scarpa, N. Rossi, and G. Cesareni.** 1987. Antagonistic controls regulate copy number of the yeast 2m plasmid. EMBO J. **6:**4205–4212.
46. **Nasseri, M., and E. S. Mocarski.** 1988. The cleavage recognition signal is contained within sequences surrounding an a-a junction in herpes simplex virus DNA. Virology **167:**25–30.
47. **O'Callaghan, D. J., M. C. Kemp, and C. C. Randall.** 1977. Properties of nucleocapsid species isolated from an in vivo herpesvirus infection. J. Gen. Virol. **37:**585–594.
48. **Panyutin, I. G., and P. Hsieh.** 1994. The kinetics of spontaneous DNA branch migration. Proc. Natl. Acad. Sci. USA **91:**2021–2025.
49. **Petrillo-Peixoto, M. L., and S. M. Beverley.** 1988. Amplified DNAs in laboratory stocks of *Leishmania tarentolae*: extrachromosomal circles structurally and functionally similar to the inverted-H-region amplification of methotrexate-resistant *Leishmania major.* Mol. Cell. Biol. **8:**5188–5199.
50. **Sarisky, R. T., and P. C. Weber.** 1994. Requirement for double-strand breaks but not for specific DNA sequences in herpes simplex virus type 1 genome isomerization events. J. Virol. **68:**34–47.
51. **Severini, A., A. R. Morgan, D. R. Tovell, and L. J. Tyrrel.** 1994. Study of the structure of replicative intermediates of HSV-1 DNA by pulsed-field gel electrophoresis. Virology **200:**428–435.
52. **Severini, A., D. G. Scraba, and D. L. J. Tyrrel.** Branched structures in the replicative intermediates of herpes simplex virus type 1 DNA. Submitted for publication.
53. **Shao, L., L. M. Rapp, and S. K. Weller.** 1993. Herpes simplex virus 1 alkaline nuclease is required for efficient egress of capsids from the nucleus. Virology **196:**146–162.
54. **Sheldrick, P., and N. Berthelot.** 1975. Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symp. Quant. Biol. **2:** 667–678.
55. **Sherman, G., and S. L. Bachenheimer.** 1988. Characterization of intranuclear capsids made by ts morphogenic mutants of HSV-1. Virology **163:** 471–480.
56. **Smiley, J. R., J. Duncan, and M. Howes.** 1990. Sequence requirements for DNA rearrangements induced by the terminal repeat of herpes simplex virus type 1 KOS DNA. J. Virol. **64:**5036–5050.
57. **Smiley, J. R., C. Lavery, and M. Howes.** 1992. The herpes simplex virus type 1 (HSV-1) a sequence serves as a cleavage/packaging signal but does not drive recombinational genome isomerization when it is inserted into the HSV-2 genome. J. Virol. **66:**7505–7510.
58. **Som, T., K. A. Armstrong, F. C. Volkert, and J. R. Broach.** 1988. Autoregulation of 2 micron circle gene expression provides a model for maintenance of stable plasmid copy levels. Cell **52:**27–37.
59. **Vlazny, D. A., A. Kwong, and N. Frenkel.** 1982. Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA. Proc. Natl. Acad. Sci. USA **79:** 1423–1427.
60. **Wagner, M. J., and W. C. Summers.** 1978. Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. J. Virol. **27:**374–384.
61. **Weber, P. C., M. D. Challberg, N. J. Nelson, M. Levine, and J. C. Glorioso.** 1988. Inversion events in the HSV-1 genome are directly mediated by the viral DNA replication machinery and lack sequence specificity. Cell **54:** 369–381.
62. **Weller, S. K.** 1995. Herpes simplex virus DNA replication and genome maturation, p. 189–213. *In* B. S. G. M. Cooper and R. Temin (ed.), Implications of the DNA provirus: Howard Temin's scientific legacy. ASM Press, Washington, D.C.
63. **Weller, S. K., E. P. Carmichael, D. P. Aschman, D. J. Goldstein, and P. A. Schaffer.** 1987. Genetic and phenotypic characterization of mutants in four essential genes that map to the left half of HSV-1 UL DNA. Virology **161:** 198–210.
64. **Weller, S. K., K. J. Lee, D. J. Sabourin, and P. A. Schaffer.** 1983. Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. J. Virol. **45:**354–366.
65. **Weller, S. K., R. M. Seghatoleslami, L. Shao, D. Rowse, and E. P. Carmichael.** 1990. The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a lacZ insertion mutant. J. Gen. Virol. **71:**2941–2952.
66. **Weller, S. K., A. Spadaro, J. E. Schaffer, A. W. Murray, A. M. Maxam, and P. A. Schaffer.** 1985. Cloning, sequencing, and functional analysis of oriL, a herpes simplex virus type 1 origin of DNA synthesis. Mol. Cell. Biol. **5:** 930–942.
67. **Wohlrab, F., S. Chatterjee, and R. D. Wells.** 1991. The herpes simplex virus 1 segment inversion site is specifically cleaved by a virus-induced nuclear endonuclease. Proc. Natl. Acad. Sci. USA **88:**6432–6436.
68. **Wohlrab, F., M. J. McLean, and R. D. Wells.** 1987. The segment inversion site of herpes simplex virus type 1 adopts a novel DNA structure. J. Biol. Chem. **262:**6407–6416.
69. **Young, C. S. H., G. Cachianes, P. Munz, and S. Silverstein.** 1984. Replication and recombination in adenovirus-infected cells are temporally and functionally related. J. Virol. **51:**571–577.
70. **Zhang, X., S. Efstathiou, and A. Simmons.** 1994. Identification of novel herpes simplex virus replicative intermediates by field inversion gel electrophoresis: implications for viral DNA amplification strategies. Virology **202:** 530–539.