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Circularization of the Herpes Simplex Virus Type 1 Genome upon Lytic Infection

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For many years, the generally accepted model for the replication of the double-stranded DNA genome of herpes simplex virus type 1 (HSV-1) incorporated initial circularization of linear molecules in the cell nucleus. Ensuing DNA synthesis resulted in the generation of head-to-tail concatemers which were subsequently cleaved into monomeric units and packaged into the nascent viral capsid. Recently, however, it has been proposed that circularization of HSV-1 genomes does not occur at the onset of lytic infection and moreover that this event is specifically inhibited by the HSV-1 transcriptional transactivator, ICP0 (S.A. Jackson and N.A. DeLuca, Proc. Natl. Acad. Sci. USA 100:7871–7876, 2003). To further investigate genome circularization, we have generated HSV-1 derivatives in which the viral *a* sequences, which contain the cleavage-packaging signals, have been replaced by a minimal packaging element located in the thymidine kinase gene. In contrast to wild-type HSV-1, fusion of the genomic termini of these viruses produces a novel fragment in circular or concatemeric DNA which can be detected by Southern blot hybridization. Utilizing these viruses, we demonstrate that fusion of the genomic termini occurred rapidly upon infection and in the presence of inhibitors of viral DNA or protein synthesis. We provide evidence indicating that the end joining represented circularization rather than concatemerization of input molecules and that circularized molecules functioned as templates for replication. Since the termini of these viruses lack direct repeats, our findings indicate that circularization can be mediated by direct end-to-end ligation of linear input genomes.

Herpes simplex virus type 1 (HSV-1), the prototype virus of the family *Herpesviridae*, possesses a linear double-stranded DNA genome of approximately 153 kbp and is widely used as a model for the study of herpesvirus DNA replication and recombination. Like those of other members of the herpesvirus family, the HSV-1 genome is characterized by the presence of unique and repeated sequences (Fig. 1a). Two covalently joined segments, L and S, each comprise a unique region (U_L and U_S) flanked by a set of inverted repeats (TR_L and IR_L, TR_S and IR_S, respectively). A region of approximately 400 bp, the *a* sequence, is present as a direct repeat at the genomic termini and in an inverted orientation at the junction between the L and S segments. The L and S segments of the genome have the capacity to invert relative to each other at high frequency, resulting in the appearance of four equimolar isomeric forms of virion DNA (reviewed in reference 35).

Following infection, the HSV-1 genome is released into the nucleus and may either be retained in a latent state or enter into the lytic cycle (reviewed in reference 32). During the establishment of latency in both in vivo and tissue culture systems, the ends of the viral genome become joined, and there is now strong evidence for persistence as a nonreplicating circular episomal form (5, 17, 18, 27, 32, 34, 42). The lytic cycle, in contrast, is characterized by the generation, following DNA replication, of high-molecular-weight concatemers consisting of tandem head-to-tail repeats of the genome.

Cleavage of the concatemers into monomeric units is tightly coupled to the packaging of the DNA into capsids and is dependent upon *cis*-acting signals that reside within the *a* sequence (for reviews, see references 1 and 2). Amplicons (i.e., bacterial plasmids containing an HSV-1 replication origin and packaging signal [38]) can be replicated and packaged in HSV-1-infected cells, and their use has allowed the minimal packaging signal to be defined (14, 29). This element (Uc-DR1-Ub) comprises a region of about 200 bp spanning the junction of two *a* sequences as would be generated by joining the opposite ends of the genome (Fig. 1b) with Uc and Ub originating from the L and S termini, respectively.

Analysis of the structure of HSV-1 replicative intermediates is complicated due to their fragility and large size, the occurrence of inter- and intramolecular recombination, and the fact that not all infecting genomes are replicated. Although the overall pathway remains incompletely understood, evidence accumulated over many years suggests a replication strategy similar to that employed by bacteriophage lambda (reviewed in references 1 and 21). Two distinct stages are proposed. In the first, the linear genome is circularized and then amplified by a theta mechanism. This is followed by a second stage during which the resulting circular molecules are replicated by a rolling-circle mechanism generating long head-to-tail concatemers. The occurrence of theta replication is supported by the observations that viral DNA accumulates with nonlinear kinetics during the initial stages of replication and that early replication appears to be dependent on cellular topoisomerase II, suggesting the need for the decatenation of circular progeny molecules (13). A switch to a rolling-circle mode at later times is implied by the size and structure of the DNA. The observation that the genome is replicated in an "endless" form (i.e., terminal fragments are absent from replicative intermediates) is entirely consistent with the initial circularization of input

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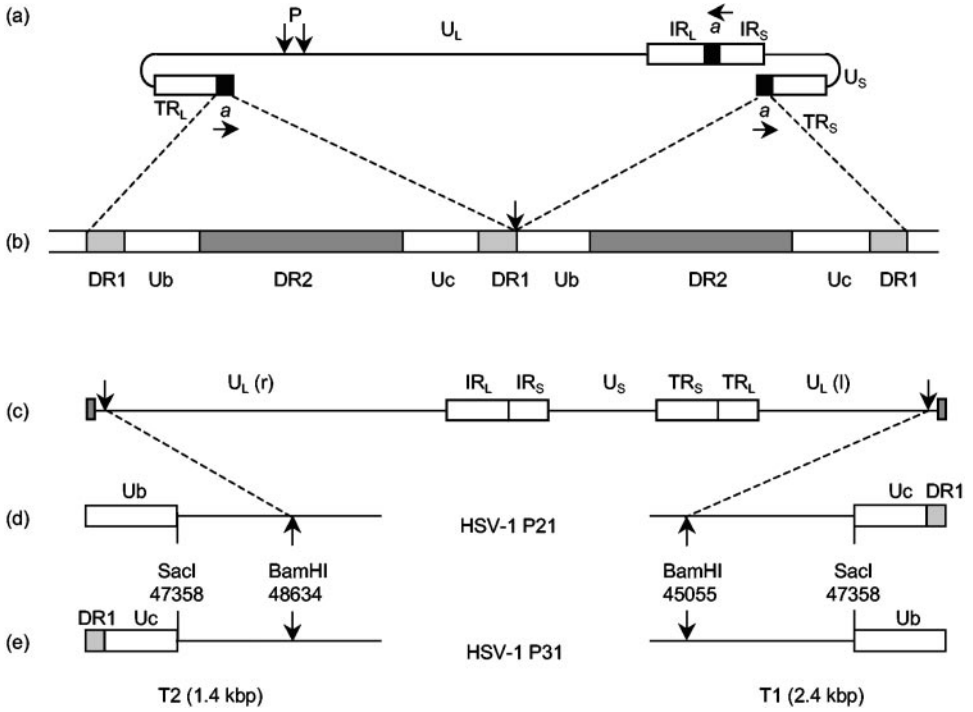

FIG. 1. Structure of HSV-1 P21 and HSV-1 P31 virion DNA. (a) Structure of the HSV-1 genome showing the positions and relative orientations (horizontal arrows) of copies of the *a* sequence (black) and of fragment BamHI P containing the TK gene (marked by arrows pointing downward). For simplicity, only single *a* sequences are shown at the L terminus and L-S junction. (b) Generation of the minimal cleavage/packaging signal Uc-DR1-Ub upon fusion of *a* sequences. The site of cleavage of concatemeric DNA, which almost coincides with one end of the DR1 sequence (3), is indicated by an arrow. (c) General structure for virion DNA of HSV-1 P21 and P31 from which the *a* sequences have been deleted and replaced by a single Uc-DR1-Ub cleavage/packaging signal located within the TK gene. BamHI sites are indicated by arrows, and U_L (r) and U_L (l) indicate the U_L sequences to the right and left, respectively, of BamHI P as shown in panel a. Terminal fragments of these viruses are depicted in (d) and (e). The positions of the sites delimiting BamHI P and of the SacI site into which the Uc-DR1-Ub fragment was inserted are indicated (genome coordinates from reference 23). Both viruses generate large (T1) and small (T2) terminal fragments of approximately 2.4 and 1.4 kbp, respectively. Since the packaging signal was inserted into the TK gene in opposite orientations in the plasmids used to generate these viruses, Uc and almost all of DR1 are in T1 of HSV-1 P21 and in T2 of HSV-1 P31.

genomes. However the anticipated accumulation of unit-length circular molecules has not been reported, and it remains possible that intermolecular ligation or recombination of replicating molecules could be responsible for the generation of concatemers (30).

A central feature of the above model is that HSV-1 genomes should be circularized prior to replication. Restriction enzyme analysis of infecting molecules indicates that terminal genomic fragments remain readily detectable during the early stages of infection, probably reflecting the excess of input genomes that fail to enter the replicative cycle (31). Moreover, circularization of the HSV-1 genome would not generate any novel sequence arrangement, since that corresponding to the fused ends is already represented internally at the junction between the L and S segments. Nevertheless, Poffenberger and Roizman (31) demonstrated the fusion of terminal fragments of input molecules in cells infected with a mutant of HSV-1 unable to perform L-S inversions, although it was not distinguished whether this represented circularization or the joining of two genomes. Interpretation of the data from this study is further complicated by the fact that a number of packaged mutant genomes appeared to contain head-to-tail junctions, and defective genomes were consistently observed with this mutant. End joining was observed early in infection prior to the initiation of DNA synthesis and when viral protein synthesis was blocked with cycloheximide, suggesting that host or virion protein(s) might be responsible (31). Consistent with end joining of input genomes, Su et al. (42) demonstrated an excess of L-S junction over terminal DNA fragments in permissive CV-1 cells infected with wild-type HSV-1 in the presence of the viral DNA synthesis inhibitor phosphonoacetic acid (PAA).

The strongest evidence to date for circularization of input HSV-1 genomes was presented by Garber et al. (10), who observed that shortly after HSV-1 infection, a proportion of viral genomes are converted to a form that is retained in the wells following cell lysis and pulsed-field gel electrophoresis. This DNA lacked detectable terminal fragments and was converted to apparently unit-length molecules by gamma irradiation, suggesting that it represented circular genomes. Moreover, such molecules were also generated when viral protein and DNA synthesis were inhibited by cycloheximide.

Recently, however, conflicting data have been presented by Jackson and DeLuca (17), who employed the gel electrophoresis system of Gardella et al. (11) to investigate the configuration of HSV-1 genomes following lytic or latent infection. In these gels, circular molecules characteristically exhibit lower mobilities than the corresponding linear forms do, and this has

allowed the detection of episomal forms of latent herpesvirus genomes. Unsurprisingly, circular forms of the HSV-1 genome were observed following infection with a mutant virus unable to express any viral genes, which efficiently established a quiescent state. However, corresponding circular genomes were not observed at any stage following productive infection of permissive cells, including when viral DNA synthesis was blocked. Furthermore, the lytic-phase immediate-early protein ICP0 appeared to inhibit the circularization process (17). These results therefore suggest that during productive lytic infection DNA replication does not proceed through a unit-length circular intermediate but rather that linear molecules serve as templates for initial DNA synthesis.

The findings of Jackson and DeLuca (17) demand a reassessment of the early stages of HSV-1 infection. We have approached this problem using HSV-1 mutants from which the *a* sequence has been deleted and replaced by a single copy of a minimal cleavage/packaging signal at an ectopic site in the genome. Such viruses generate a novel fragment upon the fusion of the termini, enabling a convenient end-joining assay similar to that employed by Poffenberger and Roizman (31). Furthermore, coinfection experiments allow circularization to be distinguished from end-to-end ligation of genomes. Our findings indicate that circularization of HSV-1 genomes occurs early in lytic infection and when either DNA or protein synthesis is inhibited. The circular molecules appear to function as templates for replication, and no effect of ICP0 on circularization is apparent.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney 21 clone 13 (BHK) cells were grown in Glasgow minimal essential medium (MEM) supplemented with 10% newborn calf serum, 10% tryptose phosphate broth, 100 U/ml of penicillin, and 100 μg of streptomycin/ml (ETC10). After infection, cells were maintained in Glasgow MEM supplemented with 5% newborn calf serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (EC5). Vero cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and the same antibiotics (EFC5). Virus stocks were prepared from the supernatant medium of BHK cells infected at a low multiplicity of infection and harvested when they exhibited extensive cytopathic effect. The stocks were titrated on Vero cell monolayers.

Generation of HSV-1 P21 and HSV-1 P31. The Uc-DR1-Ub fragment was excised from the amplicon pSA1 (14) by digestion with EcoRI plus HindIII, and the ends filled in, ligated to SacI oligonucleotide linkers (dCGAGCTCG; New England Biolabs), and cleaved with SacI. Plasmid pGX153, which comprises the HSV-1 BamHI P fragment inserted into the vector pAT153, was cleaved at the unique SacI site within the viral thymidine kinase (TK) gene and ligated to the Uc-DR1-Ub fragment. Transformants containing the Uc-DR1-Ub fragment in opposite orientations (pPA1 and pPA2) were identified and used to introduce the packaging signal into the TK gene of an HSV-1 genome from which all copies of the *a* sequence had been deleted. This was achieved using the bacterial artificial chromosome described by Saeki et al. (36), fHSVΔpac, which contains a viral genome that is complete except for the *a* sequences and is noninfectious when introduced alone into mammalian cells, since it lacks a packaging signal. BHK cells were cotransfected with BamHI-cleaved pPA1 or pPA2 and circular fHSVΔpac DNA, the cells were harvested, and progeny viruses were plaque purified. Viruses HSV-1 P21 and HSV-1 P31 resulted from the rescue of fHSVΔpac with DNA from pPA1 and pPA2, respectively. The structures of these viruses were analyzed by restriction enzyme digestion to confirm that the Uc-DR1-Ub fragment had been inserted by homologous recombination into the BamHI P fragment in opposite orientations. Figure 1c shows the general structure for the linear genomes packaged into extracellular infectious virus particles, and Fig. 1d and 1e illustrate the presence of the Ub and Uc elements in the BamHI terminal fragments of the two viruses. It should be noted that the HSV-1 sequences present in fHSVΔpac and the Uc-DR1-Ub fragment all originate from HSV-1 strain 17 syn+ (23).

Infection and isolation of viral DNA. Prior to use, stocks of HSV-1 P21 and HSV-1 P31 were treated with 100 μg/ml DNase I for 15 min at 37°C. It was found that this treatment did not affect the virus titer but was necessary to remove small amounts of contaminating viral DNA which had presumably been released from lysed infected cells (data not shown). Monolayers of BHK cells in 35-mm-diameter petri dishes (1×10^6 cells per plate) were infected with 5 PFU/cell of virus in a volume of 200 μl. After 1 h at 37°C, the inoculum was removed, and the cells were washed with 0.14 M NaCl, exposed to 0.1 M glycine and 0.14 M NaCl (pH 3.0) for 1 min to inactivate residual virus, and then washed with EC5. Incubation was continued for the indicated times at 37°C in 2 ml of EC5 prior to the preparation of DNA. PAA and cycloheximide were used at 200 μg/ml, as indicated below, and were present from 1 h before and at all stages during infection.

The cells were washed with TBS (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM glucose, 25 mM Tris-HCl, pH 7.4) and incubated in 2 ml 1× CLB (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.6% sodium dodecyl sulfate, 0.5 mg of protease [grade XIV; Sigma]/ml) for 1 h at 37°C. The samples were extracted sequentially with phenol and chloroform and precipitated with ethanol, and the nucleic acids were redissolved in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA containing 5 μg of RNase A/ml and 50 U of RNase T1/ml.

Experiments comparing total and DNase-resistant DNA were performed as previously described (14). Identical samples of infected cells were resuspended in RSB (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$) containing 0.5% NP-40. An equal volume of 2× CLB was added either immediately or after incubation in the presence of 200 μg of DNase I/ml, with occasional mixing, for 20 min at 37°C. After the addition of protease, all samples were incubated for 1 h at 37°C, extracted, and precipitated as described above.

Southern hybridization. Gel analysis of the DNAs was performed as previously described (41). Samples of DNA corresponding to the yield from 2×10^5 cells were cleaved with BamHI, and the resulting fragments were separated by agarose gel electrophoresis. After being transferred to a Hybond-N membrane (Amersham), viral DNA was detected by hybridization to a ^{32}P-labeled probe prepared by nick translation of plasmid pGX153 containing the HSV-1 BamHI P fragment. Phosphorimages of Southern blots were acquired with a Personal Molecular Imager system and analyzed with Quantity One software (Bio-Rad). The proportion of genomes with fused ends was determined by expressing the counts in the novel junction fragment as a fraction of the total counts in the two termini plus the junction.

Western blot analysis. Samples representing 5×10^4 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Hybond-ECL membrane (Amersham Biosciences) as described previously (43). The membranes were blocked at room temperature for 90 min using 5% dried milk in TBS and incubated with anti-ICP0 antiserum 11060 (7) or anti-beta-actin antiserum (Sigma) in TBS containing 0.1% Tween-20 and 5% dried milk (TBSTM). After 60 min, the membrane was washed extensively with TBSTM and incubated for 60 min with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Promega) in TBSTM. After further washing, bound antibody was detected by chemiluminescence using ECL reagents from Amersham Biosciences and X-OMAT UV Film (Kodak).

RESULTS

Generation of viruses containing a single minimal cleavage/packaging signal at an ectopic site. To facilitate the study of HSV-1 genome circularization, we first constructed viruses that would generate a novel junction fragment upon the joining of their ends. This was achieved by recombining a minimum packaging signal (the Uc-DR1-Ub fragment) into the nonessential TK gene of a cloned HSV-1 genome, fHSVΔpac (36), from which all copies of the *a* sequence had previously been deleted. Two viruses, HSV-1 P21 and HSV-1 P31, which contained the packaging signal in opposite orientations (Fig. 1 c through e), were isolated. Both viruses produce BamHI terminal fragments of approximately 2.4 and 1.4 kbp, but they differ as to which part of the packaging signal is associated with these fragments. Fusion of the genomic termini, as occurs following circularization or concatemer formation, is predicted to generate a novel 3.8-kbp junction fragment corresponding in size

to the insertion of the 200-bp Uc-DR1-Ub segment into BamHI P of wild-type HSV-1.

The successful isolation of these recombinants confirms previous observations of Martin and Weber (22), who demonstrated that a single ectopic copy of the full-length *a* sequence was sufficient for viability and represents the first demonstration that the minimum packaging signal, Uc-DR1-Ub, is functional in the context of a full-length HSV-1 genome. The yields of HSV-1 P21 and HSV-1 P31 were generally approximately 30-fold lower than those of wild-type HSV-1 and of a control virus derived by the restoration of the deleted *a* sequences of fHSVΔpac (data not shown). This is a significantly smaller impairment in growth than the 4-order-of-magnitude reduction in yield reported by Martin and Weber for their virus (22) and perhaps illustrates the advantage of using an HSV-1 genome cloned as a bacterial artificial chromosome to allow the isolation of the desired recombinant in a single step. As outlined previously (22), several factors may contribute to the reduced growth of viruses with this general structure. Among these, two are likely to be of primary importance: (i) the absence of R_L and R_S sequences flanking the minimal cleavage/packaging signal may reduce the efficiency of this element, and (ii) the inversion of adjacent L components within concatemers will alter the relative orientations of the packaging signals, resulting in up to half of the replicated DNA being unpackageable and possibly interfering with the encapsidation process itself.

Virus stocks for use in experiments were prepared from supernatant medium, and the number of genomes/PFU was estimated by phosphorimager analysis of Southern blots in which DNA from these stocks was analyzed alongside known amounts of a cloned HSV-1 DNA fragment. Values of 24, 28, and 54 genomes/PFU were calculated for three independent stocks.

Demonstration of end joining following infection with HSV-1 P21. To determine whether fusion of the terminal fragments of HSV-1 P21 occurred early in lytic infection, BHK cell monolayers were infected with virus in the absence of inhibitors or in the presence of PAA or cycloheximide to inhibit synthesis of viral DNA or proteins, respectively. DNA was prepared and digested with BamHI, and terminal and junction fragments were detected by hybridization to ^{32}P-labeled BamHI P (Fig. 2). Analysis of the virus inoculum (lane 2) revealed the presence of two major bands corresponding to the terminal fragments, T1 and T2. Minor bands, including fragments comigrating with the junction fragment, J, were often detectable upon longer exposure of input virus samples (data not shown). These may represent a low frequency of defective genomes in the virus stock or possibly a minority of packaged circular molecules, as previously suggested (31). Nevertheless, the amount of junction fragment greatly increased following infection, even when viral DNA or protein synthesis was inhibited (lanes 4 and 5), indicating the joining of the Uc-containing and Ub-containing ends. In the absence of inhibitors, the amount of junction fragment was further increased (lane 3), suggesting that junction fragment-containing DNA functions as a template for DNA replication.

Quantitative analysis of the amounts of fragments T1, T2, and J in samples from cells infected in the presence of PAA or cycloheximide was performed in order to estimate the proportion of genomes with fused ends. Over the course of our investigations, this value was 21% ± 8.7%, whereas the corresponding value for the virus inocula was 1.9% ± 1.2%. It should be noted that the extent of circularization is calculated based on the genomes detectable upon harvesting of the cells rather than on the genomes present in the original inoculum. Although most of the input DNA was usually recovered in the samples from infected cells, on some occasions (for an example, see Fig. 4) DNA was lost, perhaps due to inefficient virus penetration of the cells or intracellular degradation.

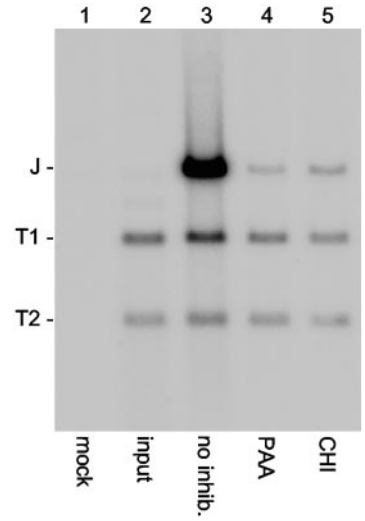

FIG. 2. End joining in cells infected with HSV-1 P21. Monolayers of BHK cells were mock infected (lane 1 [mock]) or infected with 5 PFU/cell HSV-1 P21 (lanes 3, 4, and 5). Following adsorption for 1 h at 37°C, the inoculum was removed and the cells were washed prior to the continuation of incubation for a further 3 h at 37°C. The cells in lanes 1 and 3 (no inhib.) were maintained in the absence of inhibitors, and those in lanes 4 and 5 were maintained in the presence of 200 μg/ml PAA or 200 μg/ml cycloheximide (CHI), respectively, present from 1 h before and throughout infection. Lane 2 (input) corresponds to lysed uninfected cells spiked with the virus inoculum. DNA was prepared and samples were cleaved with BamHI and analyzed by agarose gel electrophoresis and Southern blot hybridization with ^{32}P-labeled pGX153 as described in Materials and Methods. A phosphorimage of the washed membrane was acquired using a Personal Molecular Imager system (Bio-Rad). The positions of the two terminal fragments, T1 and T2 (2.4 and 1.4 kbp, respectively), and the novel junction fragment, J (3.8 kbp), are indicated.

Demonstration of circularization of infecting genomes. The above experiment demonstrates that fusion of the ends of the HSV-1 P21 genome occurs following infection. Although this is consistent with the circularization of input molecules, the alternative possibility of end-to-end ligation of genomes cannot be excluded. Since the HSV-1 cleavage/packaging mechanism generates genomes with complementary single nucleotide 3′ overhangs at their opposite ends (3, 28), precise end-to-end joining may be possible only in a head-to-tail orientation (i.e., coupling of a Uc-containing to a Ub-containing terminus), which would produce a junction fragment identical to that formed by circularization.

To test for the occurrence of end-to-end joining of input genomes, we analyzed mixed infections with HSV-1 P21 and HSV-1 P31. As illustrated in Fig. 1, ligation of the Uc-containing and Ub-containing termini of these two genomes can po-

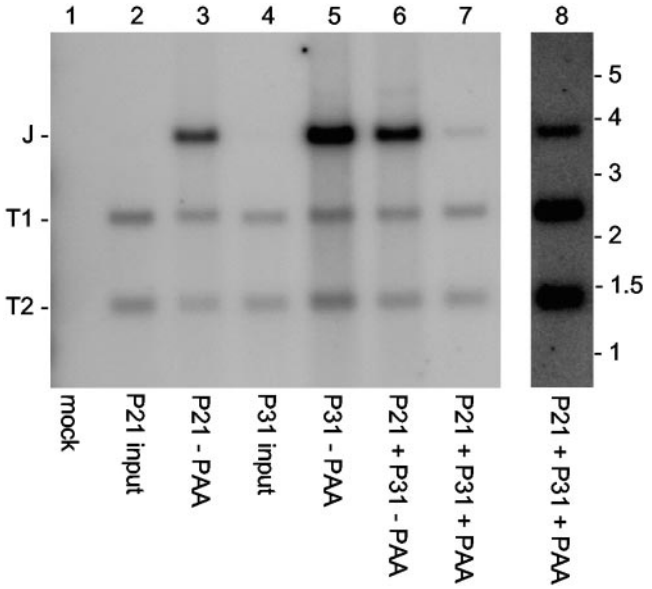

FIG. 3. End joining in cells infected with HSV-1 P21 and HSV-1 P31. Monolayers of BHK cells were mock infected (lane 1) or infected with 5 PFU/cell HSV-1 P21 (lane 3) or HSV-1 P31 (lane 5) or with 2.5 PFU/cell of each virus (lanes 6 and 7). Virus was adsorbed 1 h at 37°C, the cells were washed, and incubation was continued for a further 3 h at 37°C. The cells in lane 7 were maintained in the continued presence of 200 μg/ml PAA. DNA was prepared and analyzed as described in the legend for Fig. 2. The samples in lanes 2 and 4 correspond to lysed uninfected cells spiked with the HSV-1 P21 and HSV-1 P31 inocula, respectively. Terminal (T1 and T2) and junction (J) fragments are indicated on the left, and the positions of DNA size markers (kbp) are shown on the right. Lane 8 is a darker image of lane 7, generated with Quantity One software.

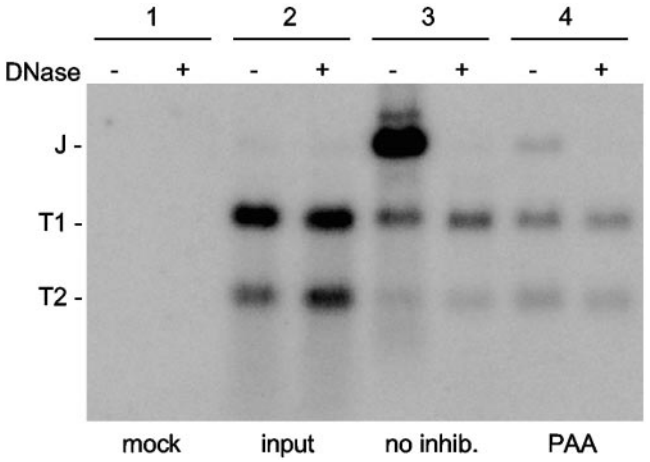

FIG. 4. Susceptibility of HSV-1 P21 DNA to DNase I. Duplicate monolayers were mock infected (lanes 1) or infected with 5 PFU/cell HSV-1 P21 (lanes 3 and 4) in the absence (lanes 3) or continued presence (lanes 4) of 200 μg/ml PAA as described in the legend for Fig. 2. Uninfected cells were spiked with virus inoculum (lanes 2). In each case, the cells from the two plates were pooled in RSB containing 0.5% NP-40 and divided into two identical samples. DNA was prepared either immediately (DNase −) or following incubation for 20 min at 37°C in the presence of 200 μg/ml DNase I (DNase +), and analyzed as described for Fig. 2.

tentially generate two additional junction fragments of 2.8 and 4.8 kbp. Figure 3 demonstrates that in a single infection, HSV-1 P31 generates as expected a novel junction fragment in replicating DNA of the same size as that of HSV-1 P21 (lanes 3 and 5). Following mixed infection in the presence of PAA, the 3.8-kbp junction fragment, but no band of 2.8 or 4.8 kbp, was detected (lanes 7 and 8). Furthermore, when DNA synthesis was allowed to proceed, only the 3.8-kbp band showed significant accumulation (lane 6). These data demonstrate that although both genomes are individually capable of replication, there is no evidence that they are joined end to end in mixed infections. Thus, although not a direct physical demonstration of circle formation, our results strongly suggest that the formation of the junction fragment, J, in the presence of inhibitors of viral protein or DNA synthesis represents circularization of input genomes and not concatemer formation.

The status of linear genomes. The data presented in Fig. 2 and 3 indicate that a proportion of input genomes can circularize following infection and probably serve as templates for DNA replication. In contrast to what was seen with the junction fragment, the amounts of terminal fragments detected in the presence or absence of inhibitors did not differ significantly, suggesting that linear molecules did not function as templates. We therefore performed an experiment to determine whether these genomes represent DNA that has not been released from the virus particle.

Cells were infected with HSV-1 P21 in the presence or absence of PAA or mixed directly with the inoculum. In each instance, two identical samples were lysed in the presence of NP-40, one of which was treated with DNase I prior to the isolation of DNA. It is well established that viral DNA in infected cells is susceptible to DNase digestion unless it has been encapsidated in a virus particle (14). The presence of junction and terminal fragments in the DNA samples were analyzed as before, and the results are shown in Fig. 4. As expected, DNase treatment had no significant effect on the terminal fragments present in the inoculum (Fig. 4, lanes 2), but the junction fragments detected in cells, either in the presence or in the absence of PAA, were fully susceptible, demonstrating that these originated from nonencapsidated molecules (Fig. 4, lanes 3 and 4). DNase had little if any effect on the terminal fragments from these infections. This indicates that the majority of the linear molecules detected at 4 h postinfection (p.i.) were in particles and not contributing to infection. Thus, although we cannot exclude the possibility that a small proportion of linear molecules are functional in DNA synthesis, the results suggest that the majority of DNA replication takes place on circular templates.

Time course of circularization. To determine how rapidly after infection circular genomes were formed, adsorption of HSV-1 P21 to cells was performed for 1 h at 4°C, and DNA analyzed either immediately or at various times after shifting to 37°C. Under these conditions, virus can attach to the cells at 4°C, but penetration occurs only following elevation to 37°C (15).

Infections were performed in either the absence or the presence of PAA, and the results of the gel analysis are shown in Fig. 5. It can be seen that in the presence of PAA the amount of junction fragment, J, had increased by 1 h and remained at a fairly constant level until 5 h after shifting to 37°C. Similar

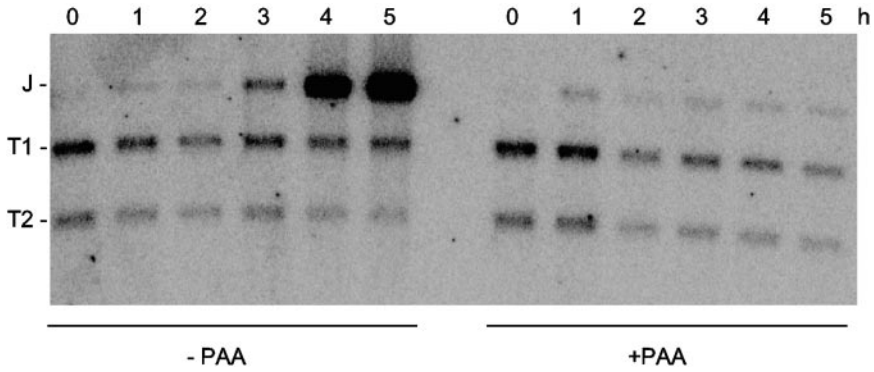

FIG. 5. Time course for DNA circularization. Replicate monolayers of BHK cells were infected with 5 PFU/cell HSV-1 P21 for 1 h at 4°C. The cells were washed and incubation was continued at 37°C for the indicated times (h) in either the absence (−) or the continued presence (+) of 200 μg/ml PAA. The 0-h sample was processed at the end of the 1-h adsorption period at 4°C. DNA was prepared and analyzed as described in the legend for Fig. 2.

amounts of junction fragment were present in the unblocked infections at 1 and 2 h after the upshift, and replication of these molecules had commenced by 3 h. These observations demonstrate that efficient circularization occurs within 1 h of virus penetration of cells and provide further evidence consistent with the circular products functioning as templates for DNA replication.

Expression of ICP0 in cells infected with HSV-1 P21. Since Jackson and DeLuca (17) had previously presented evidence that ICP0 inhibits HSV-1 genomic circularization, we wished to confirm that this protein was expressed during our experiments. Mock-infected cells and cells infected with HSV-1 P21 in the presence of PAA, cycloheximide, or no inhibitor were analyzed for expression of ICP0 by Western blotting (Fig. 6). ICP0 was detected in the unblocked cells and in reduced amounts in the presence of PAA. As expected, expression was completely blocked in the presence of cycloheximide. The inhibition of expression by PAA is consistent with previous observations that ICP0 is efficiently expressed late in infection, presumably from replicated molecules (6), and the observation that significant template amplification had occurred by 4 h p.i. (Fig. 5). Further support for the expression of functional ICP0 by HSV-1 P21 is provided by the observations that in BHK cells, this virus exhibits neither the characteristic multiplicity dependence of infection nor the high number of genomes per infectious particle characteristic of ICP0 mutants (8).

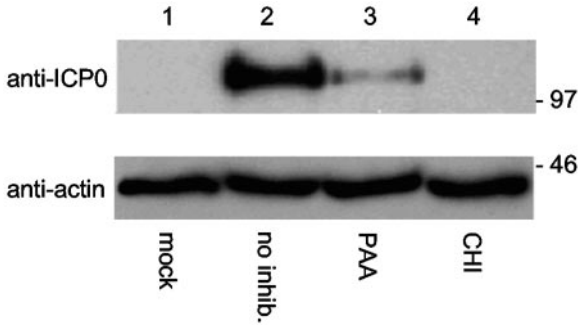

FIG. 6. Expression of ICP0. Monolayers of BHK cells were either mock infected (lane 1) or infected with 5 PFU/cell HSV-1 P21 for 4 h at 37°C in the absence of inhibitor (lane 2) or the continued presence of 200 μg/ml PAA (lane 3) or 200 μg/ml cycloheximide (CHI) (lane 4). Samples representing 5×10^4 cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting utilizing antisera specific for ICP0 and beta-actin as indicated. The positions of protein size markers (kDa) are indicated on the right.

DISCUSSION

In this study, we have utilized HSV-1 variants containing single ectopically located packaging signals to investigate the process of end joining following infection. This study was prompted by the recent work of Jackson and DeLuca (17) who reported that circularization of HSV-1 genomes does not occur at the onset of lytic infection and moreover is specifically inhibited by the HSV-1 transcriptional transactivator ICP0. Our findings are contrary to their conclusions, but rather confirm and extend the earlier reports of Poffenberger and Roizman (31) and Garber et al. (10). It is clear that within 1 h of virus penetration being initiated, a significant proportion of the uncoated DNA has been converted to a circular form and that this does not require viral DNA or protein synthesis. We have also shown that (i) most linear molecules detected following infection have not been uncoated and are probably irrelevant to the infectious process, (ii) the circular molecules are likely to serve as templates for DNA synthesis, and (iii) circularization of input genomes occurs to similar extents in the presence of cycloheximide or PAA as during the early stages of an unblocked infection, even though ICP0 is expressed in the latter two situations. The analysis of mixed infections with HSV-1 P21 and HSV-1 P31 provided independent support for the earlier conclusion of Garber et al. (10) that input genomes are circularized rather than joined end to end. Our findings with HSV-1 are consistent with reports that the genomes of several other herpesviruses, including human and guinea pig cytomegaloviruses and varicella-zoster virus, are circularized prior to initiation of DNA synthesis (20, 24, 25).

The occurrence of HSV-1 genome circularization in the presence of cycloheximide indicates that the process is mediated by a cellular factor(s) or component(s) of the input virion. Joining of the genomic termini has been proposed to occur by either homologous recombination (47) or direct ligation (28).

The incorporation of the Uc-DR1-Ub cleavage/packaging signal into HSV-1 P21 and HSV-1 P31 results in the generation of virion DNA lacking terminal direct repeats, and we thus favor direct ligation as the mechanism by which end joining occurs in these viruses. This process is also likely to be important in other herpesviruses lacking direct terminal repeats (e.g., varicella-zoster virus and guinea pig cytomegalovirus). We cannot, however, exclude the possibility that circularization by homologous recombination can occur when direct terminal repeats are present. The only cellular gene so far implicated as having a possible role in the circularization of input HSV-1 genomes is RCC1 (regulator of chromosome condensation), but it remains unclear whether its involvement is direct or indirect (44).

A central question is why the results of Jackson and DeLuca (17) differ so radically from those presented here and by others (10, 31). Two important aspects relate to the assay for the generation of circular DNA and the possible involvement of ICP0. Jackson and DeLuca's evidence for the lack of circle formation at the early stages of productive HSV-1 infection was based upon the absence of a band migrating at the position expected of a unit-length circle in a Gardella gel (17). This gel system has been successfully employed to detect latent episomal herpesvirus genomes (11, 26, 39, 45), and indeed Jackson and DeLuca (17) detected a band corresponding to circular genomes of viruses lacking a functional ICP0 gene, which are known to have an increased probability of entering into a latent or quiescent state rather than into lytic replication (8, 33, 37, 40). This band accumulated more slowly than the circular molecules detected by ourselves (Fig. 5) and others (10, 31), consistent with a slow conversion of the genomes to a latent, minimally transcribed, episomal form.

Lytic and latent HSV-1 infections represent separate, mutually exclusive pathways that differ in several important respects (12, 32). For example, (i) DNA released from virus particles into the nucleus contains nicks and gaps (9, 16, 46) which may be processed differently in the two pathways; (ii) transcription is repressed during the establishment of latent infection but occurs efficiently in productive infection, even in the presence of PAA or cycloheximide (32, 35); (iii) quiescent HSV-1 genomes become unresponsive to activation of immediate-early gene expression by the viral transcriptional transactivator VP16 (33); and (iv) the association of the genome with histones is likely to differ between the two states, with a more extensive and ordered nucleosomal arrangement occurring during latent infection (4, 19).

Such differences might result in circular genomes generated during productive infection behaving differently in Gardella gels from quiescent episomal forms, perhaps migrating heterogeneously, failing to enter the gel, or being readily converted to a linear form. One explanation as to why Jackson and DeLuca did not recognize circular input genomes in Gardella gels at the early stages of lytic infection is suggested by Fig. 2C of their paper (17). From inspection, it appears that the ratio of junction to terminal fragments in the bands corresponding to linear genomes is greater for DNA isolated from cells infected with HSV-1 mutant d109 (lane d) than from d109 virions (lane b). This suggests that the "linear" band from infected cells contains molecules that may have undergone circularization and either comigrate with linear genomes or have been converted to a linear form by nicking.

It is also interesting that in the studies using pulsed-field or field inversion gel electrophoresis to study the configuration of HSV-1 and human cytomegalovirus genomes early after infection, circularized molecules were retained in the wells (10, 24). A significant difference between these techniques and the Gardella gel assay is that lysis of infected cells occurs in agarose plugs in the former but in the open wells of the latter. If circular molecules formed during productive infection are similarly unable to enter Gardella gels, they might be lost from the wells during electrophoresis or treatment of the gel prior to blotting.

To date, we have not been successful in identifying circular forms of HSV-1 P21 and HSV-1 P31 in Gardella gels, possibly due to inefficient recovery of the very small amounts of viral DNA from the gel, and are unable to distinguish between the possibilities suggested above.

Finally, a major finding of Jackson and DeLuca's study (17) was that ICP0 inhibits circularization of infecting HSV-1 genomes. Our results, however, do not support this conclusion. Specifically, at 4 h p.i., the extents of circularization in the presence of cycloheximide or PAA were indistinguishable (Fig. 2) even though ICP0 was expressed in the latter case (Fig. 6), suggesting that genome circularization occurs independently of the presence of ICP0, consistent with the previous reports of Poffenberger and Roizman (31) and Garber et al. (10). Furthermore, the demonstration of efficient circularization within 1 h of virus uptake (Fig. 5) is difficult to reconcile with a possible role for ICP0 in inhibiting this process, given the time required for capsid transport to the nucleus, uncoating, and transcription and translation of the gene.

In conclusion, we have confirmed that circularization of the HSV-1 genome occurs early in lytic infection and that this process is independent of both viral DNA and protein synthesis. We suggest that Jackson and DeLuca (17) may have reached conflicting conclusions because the earliest replicative intermediates behave unexpectedly in the Gardella gel assay. The results from our study should stimulate further discussion of the mechanism by which the fusion of the genomic termini occurs and encourage a debate as to how Gardella gels are used in the analysis of herpesvirus DNA replication.

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