VIROLOGY 197, 459–462 (1993)

Demonstration of Circularization of Herpes Simplex Virus DNA Following Infection Using Pulsed Field Gel Electrophoresis

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Received May 6, 1993; accepted July 12, 1993

Studies of the configuration of intracellular herpes simplex virus DNA have been limited by the inability of restriction enzyme analyses to distinguish circular DNA from other configurations. To address this issue, we used pulsed-field gel electrophoresis of virus-infected, cycloheximide-treated cells and detected accumulation of viral DNA that failed to migrate out of the sample wells of the gels. This DNA lacked terminal restriction fragments and could be converted to unit-length linear species by γ-irradiation, demonstrating the circularization of viral DNA following infection. © 1993 Academic Press, Inc.

The genome of herpes simplex virus (HSV) is a linear double-stranded DNA molecule of about 150 kilobase pairs (kb). It consists of two covalently linked components designated L and S, each of which comprises unique sequences bracketed by inverted repeats. The L and S components can invert relative to each other so that four different isomers of viral DNA are present in most preparations of HSV DNA (reviewed in *1*). This has complicated analyses of the configuration of intracellular HSV DNA during viral replication and viral latency, especially since restriction enzyme analysis alone can only distinguish DNA containing terminal fragments (linear DNA) from DNA lacking terminal fragments ("endless DNA"). Endless DNA is consistent with circularization, with concatamerization, and with integration into host cell DNA, either as a linear molecule at random sites or following circularization or concatamerization.

Early work addressing the mode of HSV replication suggested that HSV replicates by a rolling circle mechanism (reviewed in *1, 2*). Inherent to this mechanism for HSV is circularization of the input linear viral genome. However, evidence for circularization of HSV DNA prior to replication has thus far been limited, in part because only a small fraction of input parental DNA is replicated (*3*). Although electron microscopic studies detected a few unit-sized circles of appropriate buoyant density in HSV-infected cells, they also detected a panoply of other molecules including unit-length linear molecules with branches and loops (*3–5*). Evidence consistent with circularization of HSV DNA has also been presented in studies of a mutant unable to undergo L–S inversions. This mutant permitted the detection of an increased fraction of unique restriction fragments corresponding to head-to-tail junctions shortly following infection, even in the absence of protein synthesis (*6*). However, this study did not exclude the formation of noncircular structures such as concatamers from input genomes. The interpretation is also complicated by the mutant genome being smaller than wild-type, by the finding that a percentage of mutant virions contained head-to-tail junctions, and by the presence of defective genomes in the mutant population.

To address the issue of circularization of HSV DNA following infection, we have adapted an assay entailing pulsed-field gel electrophoresis (PFGE) and γ-irradiation that was used to demonstrate the existence of large unit-length circles in drug resistant strains of the protozoan parasite, *Leishmania* and in mammalian cells (*7–10*). Although there have been other reports applying PFGE to herpesvirus DNAs (*11–16*), none of these examined the circularization of DNA following infection. For our studies, Vero cells were infected with HSV wild-type strain KOS as previously described (*17*). At given times postinfection, samples were prepared for PFGE by scraping infected cells from dishes, pelleting by centrifugation, and resuspending in Dulbecco's modified Eagle's medium to which an equal volume of 1% low-melt agarose (SeaPlaque) in 75 m*M* $NaPO_4$, pH 8, 65 m*M* NaCl, 1% glucose was added. Sixty-microliter sample plugs containing approximately 10^6 cells per plug were cast, deproteinized, washed, and stored as described (*9, 18*). PFGE analyses were carried out as described (*7–9*) using lamda DNA oligomer size markers $(48.5\ kb)_n$ (*19*). Gels were soaked for 15 min. in 0.25N HCl to depurinate DNA, followed by denaturation and capillary transfer of DNA to Gene-

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0042-6822/93 $5.00
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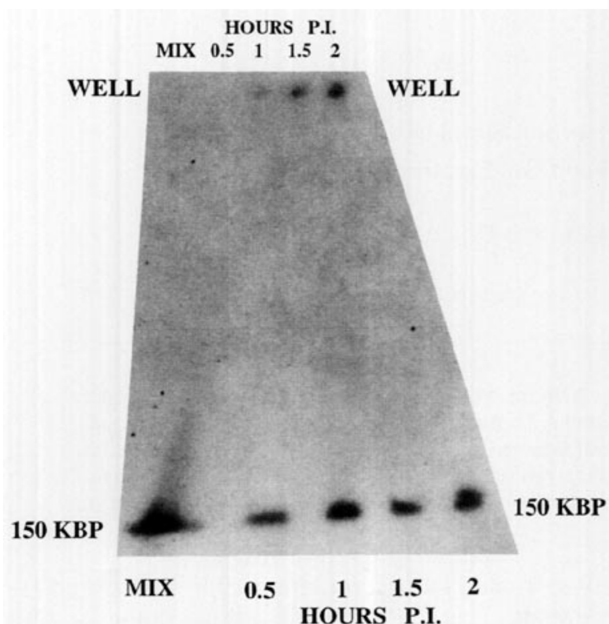

FIG. 1. Accumulation of HSV DNA in PFGE wells at early times postinfection. Vero cells infected with HSV at 15 PFU/cell were prepared for PFGE at the indicated times postinfection with 10^6 cells per 60-μl sample plug. An additional sample (MIX) was prepared by mixing concentrated virus and cell suspensions at a similar multiplicity just before casting sample plugs and deproteinizing. Following PFGE for 20 hr with a switch time of 50 sec on an apparatus similar to that of Schwartz and Cantor (*25*), the sample plugs were sealed in the gel and the DNA was transferred to a nylon membrane and probed with radiolabeled HSV DNA fragments. "150 KBP" indicates the position of a lambda DNA trimer. With the apparatus used, DNAs loaded in adjacent lanes did not migrate in parallel, but rather diverged. The 150-kb signal in the MIX lane is more intense than the others as unadsorbed virus was not removed.

Screen Plus nylon membranes according to the manufacturer's protocols. Membranes were hybridized with the indicated probes labeled with [α-^{32}P]dCTP using the random primer method (*20*).

Previous work studying large circular DNAs in drug-resistant *Leishmania* showed that such DNAs often failed to migrate from sample wells during PFGE (*7–9*). *To determine if any HSV DNA species exhibited this* property early in infection, we performed a time-course experiment in which Vero cells were infected at 37° with wild-type HSV strain KOS for 30, 60, 90, and 120 min. Infected cells were analyzed by PFGE, sample plugs were sealed in place with agarose, the gels were Southern blotted, and filters were probed with a radiolabeled HSV *Eco*RI fragment pool designed to minimize cross-hybridization to cellular sequences (*21*). Two major hybridizing species were detected in this experiment (Fig. 1): (*1*) Hybridization at the position of linear HSV virion DNA (~150 kb), which could derive from unadsorbed virions that were not removed, adsorbed virions that did not penetrate, and internalized DNA that did not circularize and (2) hybridization at the position of the well. The latter was barely detectable at 0.5 hr postinfection and increased upon further incubation. To control for the possibility that the "well hybridization" represented nonspecific trapping of virion DNA, we electrophoresed a mixture of crude virions with uninfected cells cast together into an agarose plug for PFGE (Fig. 1, lane MIX). Although substantial hybridization at the position of unit-length linear DNA *was detected, no hybridization at the position of the* well was observed. We also detected no hybridization at the position of the well when virus was permitted to adsorb to, but not enter cells, but did when virus was permitted to enter cells in the presence of cycloheximide (see below and unpublished results). Therefore, this accumulation did not require viral protein synthesis. We interpret this as conversion of linear input viral DNA to a different configuration.

We hypothesized that the "well hybridization" represented *circularized HSV DNA. This is consistent with* reports that HSV virion DNA contains nicks (reviewed in *1*) and that large (>30 kb) nicked circular DNAs remain in sample wells during PFGE (*9*). We therefore asked whether the HSV DNA that remained in the well had assumed an "endless" state, lacking terminal fragments. Vero cells were incubated with HSV for 1 hr on ice to permit adsorption, but not penetration (*22, 23*). These were then either processed immediately into sample plugs for PFGE (zero time point) or incubated at 37° to permit HSV penetration for 2 or 9 hr in the presence of cycloheximide and then processed. Certain sample plugs were subjected to PFGE to re*move ("strip") the unit-length linear species from the* plugs. Stripped and "unstripped" sample plugs were incubated with restriction endonuclease *Bam*HI overnight and then subjected to ordinary agarose gel electrophoresis and Southern blot hybridization analysis with a probe specific for the HSV terminal and junction *Bam*HI fragments (Fig. 2). Stripped plugs from the zero time point samples yielded no detectable hybridization (Fig. 2, lane 2), which demonstrated that the stripping procedure removes linear HSV DNA, while an un*stripped plug from the zero time point sample yielded*, as expected, both the 6-kb junction and the smaller terminal *Bam*HI fragments typical of linear virion DNA (lane 1). In contrast, stripped sample plugs from either the two (lane 3) or nine (lane 4) hr time points hybridized only to the junction fragment without detectable hybridization to the terminal fragments. These results demonstrate that HSV DNA accumulating in sample wells in the presence of cycloheximide assumes an endless configuration. This is consistent with cycloheximide-insensitive formation of novel junction fragments observed in a noninverting HSV mutant (*6*).

To determine whether the endless viral DNA accu*mulating in sample wells was circular, we used limited* γ-irradiation to introduce an average of one double-stranded break per DNA molecule (Fig. 3). Vero cells

were infected with KOS in the presence of cycloheximide for 9 hr and prepared for PFGE. Sample plugs were stripped, recovered, γ-irradiated for varying lengths of time corresponding to doses of 0–200 krad as described (*7, 8*), and subjected to a second round of PFGE. Southern blot hybridization analysis showed that with increasing γ-ray dose, hybridization to material in the well decreased. Concomitantly, hybridization to a discrete species that migrated at the position of linear 150 kb DNA appeared, particularly at 25 and 50 krad. At higher doses, hybridization to a more rapidly migrating smear occurred, presumably due to the introduction of >1 double-strand break per DNA molecule. We conclude that the endless HSV DNA which hybridizes at the position of the well contains unit-length circles.

These circular HSV DNA molecules may function as replication templates. Consistent with this, later during infection the amount of HSV DNA in the PFGE sample well increases dramatically, prior to a increase of similar magnitude in the amount of linear DNA (unpublished results). Comparable results have been found during human cytomegalovirus infection (*16*). This dramatic increase in "well hybridization" was sensitive to phosphonoacetic acid (unpublished results) and its molecular configuration is under investigation. Nevertheless, we have not ruled out the possibility that DNA

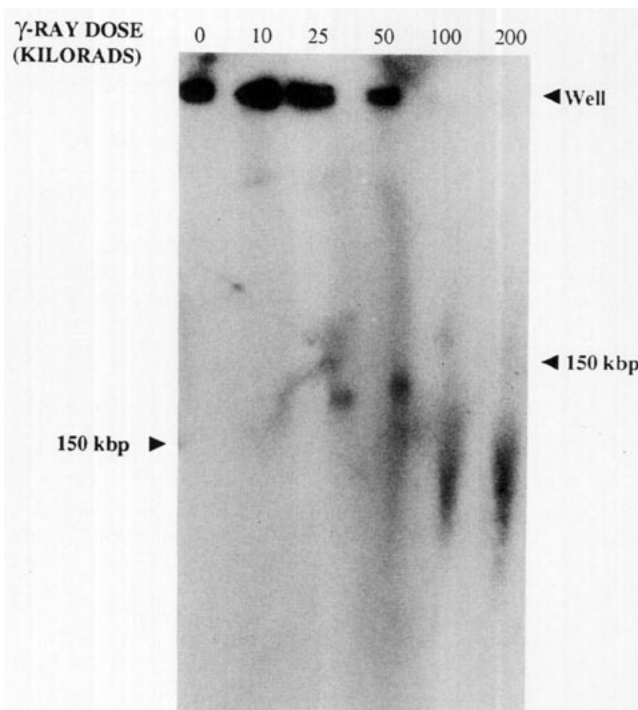

FIG. 3. Conversion of HSV DNA in PFGE wells to unit-length linear molecules. Stripped sample plugs were prepared from virus-infected cycloheximide-treated cells 9 hr postinfection as described in the legend to Fig. 2 and treated with the indicated doses of γ-irradiation. The irradiated plugs were then subjected to PFGE on a contour-clamped homogeneous electric field apparatus for 20 hr with a 60-sec switch time alongside unstripped plugs and lambda-DNA oligomers as size markers, the DNA was transfered to a nylon membrane, and probed with radiolabeled HSV DNA fragments. The positions of size markers on each side of the experimental lanes are indicated; note that the same size DNA migrated more slowly on the right side of the gel than on the left.

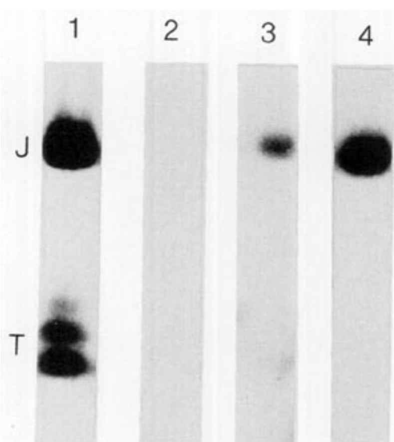

FIG. 2. HSV DNA in wells is endless. Vero cells pretreated 2 hr with 50 μg/ml cycloheximide were incubated with 15 PFU/cell HSV on ice for 1 hr and then prepared for PFGE immediately (lanes 1 and 2) or incubated at 37° for 2 hr (lane 3) or 9 hr (lane 4) in the presence of 50 μg/ml cycloheximide. PFGE sample plugs were either subjected to PFGE on a continuous-clamped homogeneous electric field apparatus for 20 hr with a 60-sec switch time to remove linear DNAs (stripped) (lanes 2–4) or not (unstripped) (lane 1) and then recovered and incubated with restriction endonuclease *Bam*HI overnight. The plugs were then subjected to normal agarose gel electrophoresis, the DNA was transferred to a nylon membrane and probed with a radiolabeled cloned HSV DNA *Bam*HI fragment containing the L–S junction sequences deleted of internal *Sac*I fragments that cross-hybridize to cellular DNA (*24*). The positions of HSV junction (J) and terminal (T) *Bam*HI fragments are indicated based on molecular weight markers electrophoresed in parallel.

molecules other than circles might serve as initial templates for viral replication. The PFGE assay described here, especially because it permits the separation of nonlinear from linear molecules, should be valuable both for investigating the configuration of viral DNA during productive replication and for investigating its configuration during the establishment, maintenance, and reactivation of latent HSV infections.

ACKNOWLEDGMENTS

We thank K. Kerns for the plasmid probe and C. Coburn for help with the experiment shown in Fig. 2, Drs. Adler and McVoy for providing us with *16*, J. Brown, P. Digard, and J. Scales for help with the figures, and S. K. Weller for helpful comments. This work was supported in part by NIH research Grants PO1 AI24010 and SO7 RR05381 to D.M.C., RO1 AI21903 to S.M.B. and Training Grant T32 GM07196 for D.A.G.

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