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Rolling Circle DNA Replication by Extracts of Herpes Simplex Virus Type 1-Infected Human Cells

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Whole-cell extracts of herpes simplex virus type 1-infected human cells (293 cells) can promote the rolling circle replication of circular duplex DNA molecules. The products of the reaction are longer than monomer unit length and are the result of semiconservative DNA replication by the following criteria: (i) resistance to *Dpn*I and susceptibility to *Mbo*I restriction enzymes, (ii) shift in density on a CsCl gradient of the products synthesized in the presence of bromo-dUTP to a position on the gradient consistent with those of molecules composed mainly of one parental DNA strand and one newly synthesized DNA strand, and (iii) the appearance in the electron microscope of molecules consisting of duplex circles with multiunit linear appendages, a characteristic of a rolling circle mode of DNA replication. The reaction requires ATP and is dependent on herpes simplex virus type 1-encoded DNA polymerase.

Studies in vivo have demonstrated that the linear genome of herpes simplex virus type 1 (HSV-1) circularizes upon the infection of susceptible cells and replicates predominantly by a rolling circle mechanism (1, 4, 7). We have recently reported that a multiprotein complex consisting of HSV-1-encoded DNA polymerase, UL42 protein, helicase-primase, and the single-stranded DNA-binding protein ICP8, isolated from extracts of Sf21 insect cells coinfected with baculoviruses recombinant for the genes encoding these proteins, can promote the rolling circle replication of circular plasmids that is independent of an HSV-1 origin and the HSV-1-encoded origin-binding protein (8). Although this system has provided an insight into the enzymes that may participate in the rolling circle replication of the HSV-1 genome in vivo, it can be viewed as artificial in that Sf21 cells do not normally serve as a host for HSV-1. We have therefore sought extracts of HSV-1-infected primate cells that can initiate and sustain rolling circle DNA replication in a manner analogous to that of Sf21 cell extracts. We have, in fact, found that whole-cell extracts of HSV-1-infected 293 cells can promote the rolling circle replication of plasmid DNA molecules lacking an HSV-1 origin. Rabkin and Hanlon have shown that partially purified extracts from HSV-1-infected mammalian cells can mediate DNA synthesis with a circular template containing a preformed replication fork (6).

MATERIALS AND METHODS

Materials. Cell culture media and reagents were obtained from GIBCO-BRL. Restriction endonucleases *Dpn*I and *Hin*dIII and pUC18 plasmid DNA were from Boehringer Mannheim. The *Mbo*I restriction enzyme was from New England Biolabs. Deoxynucleotides, ribonucleotides, DEAE-Sephacel, and heparin-Hitrap were from Pharmacia. [α-^{32}P]dCTP (3,000 Ci/mmol) was from NEN.

Buffers. Buffer A (lysis buffer) contained 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-NaOH (pH 7.6), 1 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid], 10 mM sodium bisulfite, 2 μg each of leupeptin and pepstatin per ml, and 1 mM dithiothreitol. Buffer B (dialysis buffer) contained 40 mM HEPES-NaOH (pH 7.6), 10% glycerol, 2 μg each of leupeptin and pepstatin per ml, and 1 mM dithiothreitol.

Cell cultures. Monolayers of 293 cells (obtained from Teresa Wang, Stanford University) were grown at 37°C in Dulbecco's modified Eagle medium in 185-cm^2 flasks containing 10% fetal calf serum supplemented with 2 mM glutamine. At 80% confluence, cells were infected with HSV-1 strain RΔ305 at a multiplicity of infection of 5 for 2 to 15 h. At the end of infection, cells were dislodged, washed once with phosphate-buffered saline (GIBCO), and frozen in liquid nitrogen.

Preparation of extracts. Frozen cells (5 g) that had been infected with HSV-1 were thawed at 4°C. Lysis buffer (10 ml) was added, and cells were incubated for 20 min. Cells were homogenized 20 times with a B-type pestle, and KCl was added to a final concentration of 0.5 M. The homogenate was swirled gently at 4°C for 40 min and then centrifuged at 100,000 × *g* for 35 min. The supernatant was dialyzed for 6 h against buffer B, frozen in liquid nitrogen, and stored at −80°C. The protein concentration was 10 to 15 mg/ml, as determined with the Bradford reagent (2).

The complex of enzymes that can mediate rolling circle DNA replication was fractionated by a two-step procedure. Crude extract (20 mg of protein) was loaded onto a 10-ml DEAE-Sephacel column equilibrated with buffer B containing 0.1 M NaCl at a flow rate of 1 column volume/h. The column was washed with 2 column volumes of buffer B containing 0.1 M NaCl and then eluted with 0.25 M NaCl. The eluate (4 ml) was diluted fivefold with buffer B to reduce the salt concentration to <0.05 M NaCl and loaded onto a 5-ml heparin-Hitrap column at a flow rate of 0.5 ml/min. The column was washed with 2 column volumes of buffer B containing 0.05 M NaCl, and bound proteins were eluted with 0.8 M NaCl. The eluate was dialyzed against buffer B containing 0.1 M NaCl and frozen.

Detection of HSV-1-encoded proteins by Western blot (immunoblot) analysis. Extract (100 μg) prepared as described above was subjected to 8% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Proteins were then blotted onto a nitrocellulose membrane (pore size, 0.45 μm; Schleicher & Schuell) with a semidry blotter (Milliblot Graphite Electroblotter; Millipore; at 0.4 A/cm^2) in a buffer containing 10% methanol, 96 mM glycine, and 12.5 mM Tris. HSV-1-encoded proteins were detected with specific antisera (8) by enhanced chemiluminescence (ECL; Amersham).

Analysis of DNA replication. Reaction mixtures (50 μl) contained 30 mM HEPES-NaOH (pH 7.5); 0.5 mM dithiothreitol; 8 mM magnesium acetate; 4 mM ATP; 50 μM (each) dATP, dGTP, and dTTP; 10 μM dCTP; 5 μCi of [α-^{32}P]dCTP; 250 μM (each) CTP, GTP, and UTP; 40 mM creatine phosphate; 5 μg of creatine kinase; 160 fmol of pUC18 plasmid DNA (molecules); and 100 μg of enzyme fraction. Incubation was at 37°C for 2 h. DNA synthesis was measured by the incorporation of [^{32}P]dCMP into acid-insoluble material. To measure rolling circle DNA replication, reaction mixtures were treated with 100 μg of proteinase K per ml–0.5% SDS for 15 min. DNA was extracted first with phenol-chloroform and then with chloroform. DNA was precipitated by the addition of ammonium acetate to 2.5 M, followed by 2.5 volumes of ethanol, and allowed to stand overnight at −20°C. DNA was pelleted by centrifugation in an Eppendorf centrifuge for 30 min at 4°C and washed with an ice-cold solution of 80% ethanol. DNA was dried in a Speed-Vac, resuspended in 50 μl of TE (10 mM Tris HCl [pH 7.5], 0.1 mM EDTA), and digested for 8 h with *Dpn*I (10 U per reaction mixture) or *Mbo*I (5 U per reaction mixture) and *Hin*dIII (5 to 10

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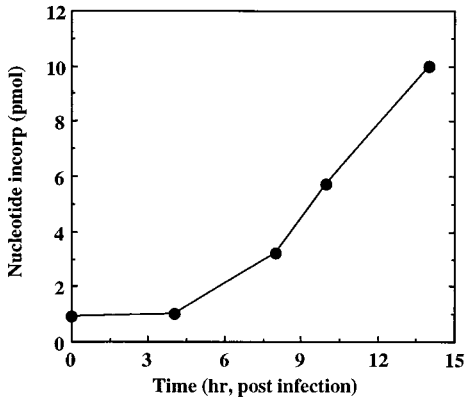

FIG. 1. DNA synthesis by extracts of HSV-1-infected 293 cells. DNA synthesis was determined by measuring the incorporation (incorp) of [^{32}P]dCMP into acid-insoluble material, as described in Materials and Methods.

U per reaction mixture) restriction enzymes. The digest was analyzed by agarose gel electrophoresis. Agarose gel electrophoresis was performed on 1% agarose with TBE (89 mM Tris-borate, 1 mM EDTA) containing 0.5 μg of ethidium bromide per ml as the electrode buffer at 2.5 V/cm for 12 h. After electrophoresis, gels were dried and autoradiographed with an intensifying screen.

For the analysis of products by CsCl density gradient sedimentation, the dTTP in the reaction mixture was replaced by bromo-dUTP (BrdUTP) (Sigma). Deproteinized replication products were loaded onto a solution of 3 ml of CsCl (refractive index = 1.405; density = 1.76 g/ml at 25°C in TE). The density gradient was formed by centrifugation for 70 h at 25°C with a Beckman SW50.1 rotor at 30,000 rpm. After centrifugation, 100-μl fractions were collected. One hundred microliters of TE containing glycogen (0.2 μg/ml) and 400 μl of ethanol were added to each fraction, and mixtures were allowed to stand at −20°C for 12 h. Then samples were centrifuged in an Eppendorf centrifuge, washed with cold 80% ethanol, dried in a Speed-Vac, and resuspended in 60 μl of TE. The amount of labeled DNA in each fraction was measured by the incorporation of [^{32}P]dCMP into acid-insoluble material. The density marker was pUC18 propagated in *Escherichia coli* in medium containing BrdU at 50 μM (light DNA, 1.60 g/ml; heavy light DNA, 1.72 g/ml).

EM. Samples to be examined by electron microscopy (EM) were fixed with 0.6% glutaraldehyde for 10 min at 20°C and then passed over a Biogel A-5m column equilibrated with 40 mM HEPES-NaOH [pH 7.5]–8 mM magnesium acetate–4 mM ATP to remove fixatives and unbound proteins. Samples were mixed with spermidine hydrochloride to yield a final concentration of 2 mM and applied to thin carbon films, which were washed, dehydrated, and rotary shadowcast with tungsten (3). Micrographs were taken with a Philips CM12 electron microscope. Contour lengths were measured on micrographs by using a Summagraphics digitizing tablet coupled with a Macintosh computer programmed with software developed at the University of North Carolina by J.D.G.

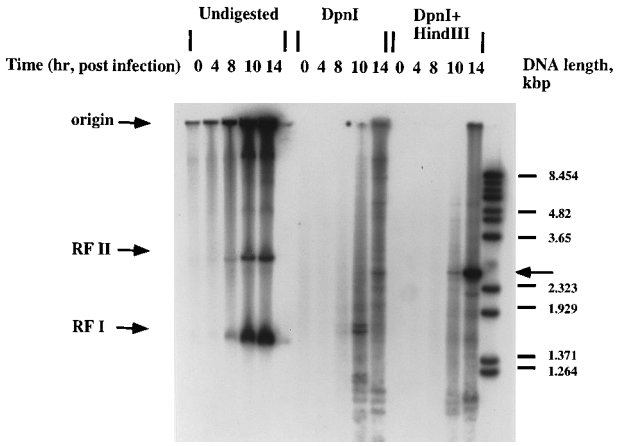

FIG. 2. Analysis of products of rolling circle DNA replication by agarose gel electrophoresis. DNA replication, the treatment of products with the indicated restriction enzymes, and agarose gel electrophoresis were performed as described in Materials and Methods. The arrow on the right indicates the position of a monomer linear DNA.

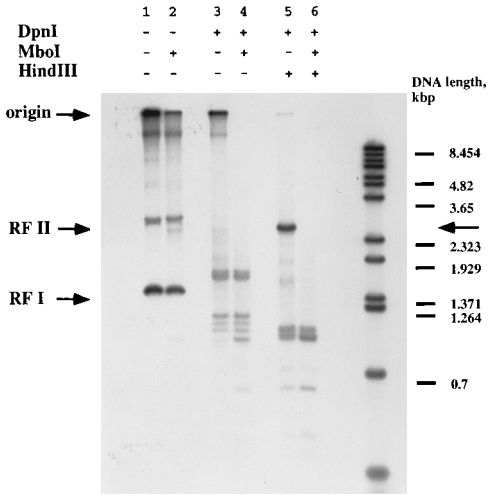

FIG. 3. Analysis of the state of methylation of DNA products. DNA replication, the treatment of products with the indicated restriction enzymes, and agarose gel electrophoresis were performed as described in Materials and Methods. Lanes: 1, untreated products; 2, products digested with *Mbo*I; 3, products digested with *Dpn*I; 4, products digested with *Dpn*I and *Mbo*I; 5, products digested with *Dpn*I and *Hin*dIII; 6, products digested with *Dpn*I, *Mbo*I, and *Hin*dIII. The arrow on the right indicates the position of a monomer linear DNA.

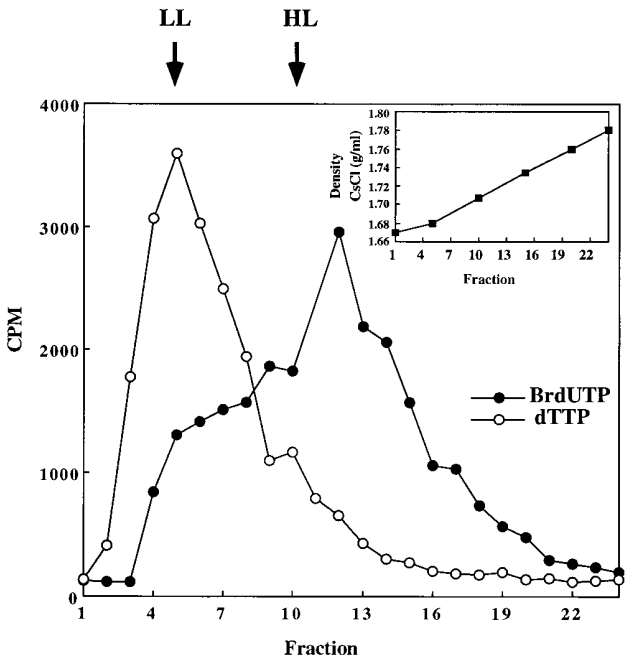

FIG. 4. Analysis of products of rolling circle DNA replication by CsCl density gradient sedimentation. DNA replication was performed as described in Materials and Methods. As indicated, dTTP was replaced by BrdUTP. The products were deproteinized and loaded (60 μl) onto 3 ml of CsCl (refractive index = 1.405; density of 1.76 g/ml in TE at 25°C). Gradients were formed by centrifugation with a Beckman SW50.1 rotor for 30 h at 30,000 rpm at 25°C. DNA products were precipitated with ethanol, and the incorporation of [^{32}P]dCMP into acid-insoluble material was measured. The densities of aliquots were measured directly with a Milton Roy Co. refractometer. The positions of the migrations of light DNA (LL; 1.69 g/ml) and heavy light DNA (HL; 1.72 g/ml) were estimated by using pUC18 propagated in *E. coli* in the presence of BrdU.

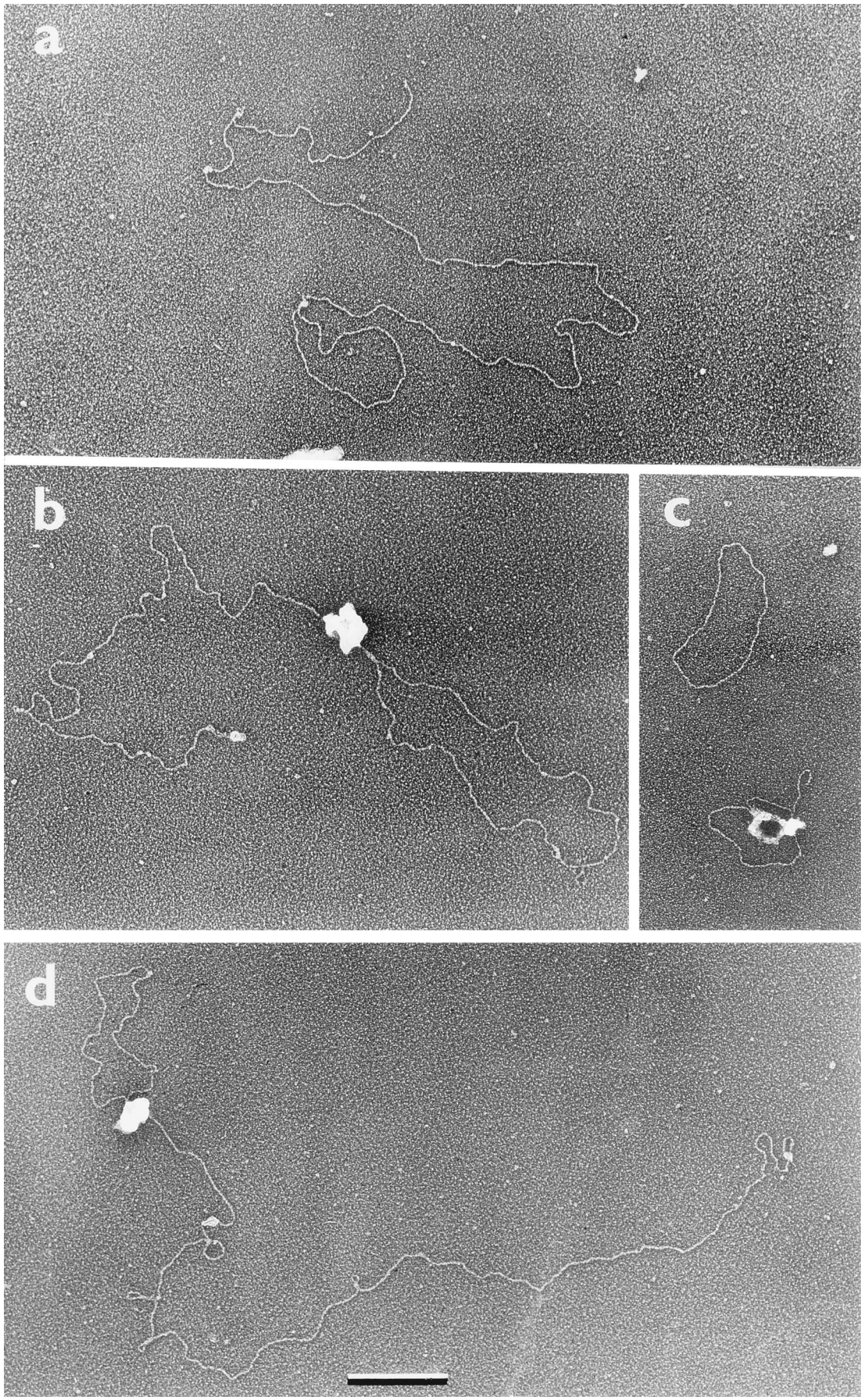
a
b
c
d

FIG. 5. Products of rolling circle DNA replication observed by EM. The processing of complexes for EM was performed as described in Materials and Methods. (a) Circular DNA molecule with a double-stranded DNA appendage of 3.6 monomer units in length. (b and c) Replication complexes obtained after glutaraldehyde cross-linking of products obtained with whole-cell extracts. The lengths of the double-stranded DNA appendages are 1.9 (b) and 0.2 (c) monomer units. (d) Replication complexes obtained after glutaraldehyde cross-linking of products obtained with extracts partially purified as described in Materials and Methods. The length of the double-stranded DNA appendage is 3.7 monomer units. Bar, 0.2 μm.

RESULTS

Rolling circle DNA replication by extracts of HSV-1-infected 293 cells. 293 cells were infected with HSV-1, and at various times after infection, cell extracts were prepared and assayed for the ability to promote replication of the pUC18 plasmid. As shown in Fig. 1, the capacity of these extracts to promote DNA replication, as measured by the conversion of [^{32}P]dCTP to an acid-insoluble product, increased over time postinfection, reaching a maximum at approximately 14 h postinfection. The increase in replication activity closely follows the increase in the appearance of HSV-1-encoded replication enzymes, as judged by Western blot analysis of crude extracts obtained at different times after infection (data not shown) (5). Extracts obtained from cells at 14 h postinfection were used for all of the experiments described below.

Analyzed by agarose gel electrophoresis, the labeled products of the reaction consisted of the replicative form I (RF I) and RF II of the plasmid and a heterogeneous mixture of DNA molecules ranging in size up to 9 kb. Upon treatment with the *Dpn*I restriction enzyme, RF I and RF II disappeared; however, the heterogeneous mixture of DNA chains largely persisted. Treatment with *Dpn*I and subsequently with the *Hin*dIII restriction enzyme, which cleaves pUC18 once, reduced these chains to predominantly monomer unit-length DNA molecules (Fig. 2). This result, which is indicative of semiconservative rolling circle replication of the circular plasmid, is the same as that previously observed with extracts of insect cells multiply infected with baculoviruses recombinant for the HSV-1-encoded DNA polymerase, UL42 protein, helicase-primase, and ICP8 (8). The treatment of the reaction products with the *Mbo*I restriction enzyme, whose specificity is the opposite of that of *Dpn*I, i.e., it cleaves unmethylated DNA but not fully methylated DNA, showed that *Dpn*I-resistant products of rolling circle replication were fully sensitive to *Mbo*I (Fig. 3).

CsCl density gradient analysis of products of rolling circle DNA replication. To confirm that the heterogeneous mixture of DNA molecules did result from semiconservative DNA replication, the dTTP in the reaction mixture was replaced by BrdUTP and the products were analyzed by CsCl density gradient sedimentation. As shown in Fig. 4, the products of the reaction carried out in the presence of BrdUTP sedimented predominantly in the HL position, i.e., at a position expected for a DNA molecule containing one light thymine-containing strand and one heavy BrU-containing strand. Some material, presumably the products of repair replication (Fig. 2), did sediment at the LL position. As expected, all of the products synthesized in the presence of dTTP sedimented in the LL position.

Analysis of products of rolling circle DNA replication by EM. Since the products of rolling circle DNA replication should have a characteristic appearance, i.e., a circular duplex with the dimensions of plasmid pUC18 and a linear duplex tail extending from the circle, we examined the products by EM. As shown in Fig. 5, structures consistent with the rolling circle mode of DNA replication were observed. As expected from agarose gel analysis, there was variability in tail length, with a maximum of 3.7 monomer units (Fig. 5d). Molecules in which the linear tail was substantially truncated (less than 1 monomer unit) were often observed, presumably as a consequence of nucleases present in HSV-1-infected cell extracts (Fig. 5c shows an appendage of 0.2 monomer unit in length). Glutaraldehyde cross-linking of the products generated by either a whole-cell extract or a partially purified enzyme preparation revealed the presence of a protein mass, possibly the enzyme complex, situated at the junction of the circular template and the linear extension (Fig. 5b, c, and d).

Requirements of the reaction. Rolling circle DNA replication by extracts of HSV-1-infected 293 cells required ATP (data not shown) and was dependent on HSV-1 DNA polymerase. As shown in Fig. 6, the formation of *Dpn*I-resistant, high-molecular-weight products of rolling circle replication was sensitive to phosphonoacetic acid, a specific inhibitor of HSV-1 DNA polymerase. At 10 μM phosphonoacetic acid, the synthesis of high-molecular-weight products was nearly eliminated. In contrast, the appearance of labeled RF I and RF II, presumably the products of repair replication, was largely unaffected by this concentration of inhibitor.

DISCUSSION

We have shown that whole-cell extracts of HSV-1-infected 293 cells promote the rolling circle replication of circular plasmids. This reaction closely resembles the rolling circle replication mediated by the partially purified multienzyme complex obtained from Sf21 insect cells infected with baculoviruses recombinant for the HSV-1 genes encoding the viral DNA polymerase, UL42 protein, helicase-primase, and ICP8. Although we do not know whether an analogous complex exists in the extracts of 293 cells, EM analysis of the products of rolling circle DNA replication cross-linked to protein has revealed the presence in some products of a protein mass situ-

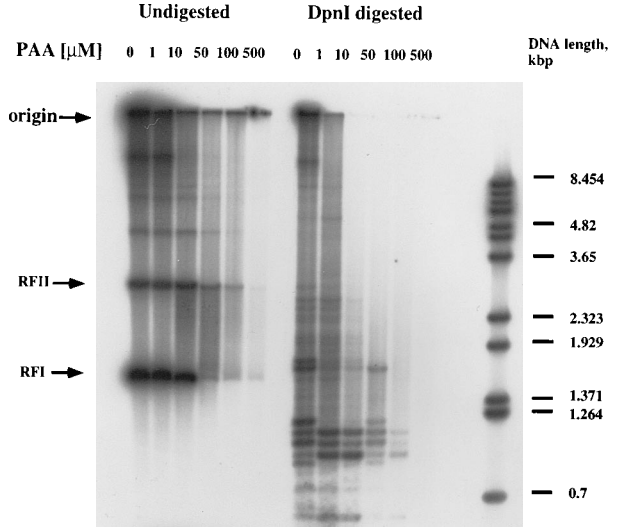

FIG. 6. Inhibition of rolling circle DNA replication by phosphonoacetic acid (PAA). DNA replication, the treatment of products with restriction enzymes, and agarose gel electrophoresis were performed as described in Materials and Methods. PAA was added at the indicated concentrations.

ated at the juncture of the circular template and the linear extension. These structures are similar in appearance to the structures we observed with the partially purified complex isolated from multiply infected Sf21 cells (10).

An attractive feature of the insect cell system is the ease with which the requirement for each HSV-1-encoded enzyme can be determined (8). In the case of extracts of HSV-1-infected 293 cells, experiments with the appropriate conditional lethal HSV-1 mutants will be required to establish the viral gene products required for rolling circle DNA replication. However, with the use of phosphonoacetic acid at the appropriate concentration as a specific inhibitor of HSV-1 DNA polymerase, we have been able to demonstrate that this virus-encoded enzyme is essential for rolling circle DNA replication.

As in the case of the insect cell system, we do not know the form of plasmid DNA that serves as the template for rolling circle replication. A simple nick does not appear to serve as the site of initiation of rolling circle replication since graded pretreatment of the supercoiled pUC18 plasmid with DNase I did not increase the low-level efficiency of the reaction (1 to 2% of the template molecules replicated) (9).

Our EM analysis of the reaction products has confirmed the rolling circle mode of DNA replication. The linear portion of the rolling circle product, which ranges up to 4 monomer units in length, is fully double stranded with few, if any, single-stranded gaps. Leading- and lagging-strand synthesis during the process of rolling circle replication in this system must therefore be tightly coordinated.

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