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Rolling circle DNA replication *in vitro* by a complex of herpes simplex virus type 1-encoded enzymes

(theta replication/helicase/primase/DNA polymerase)

RAMI SKALITER AND I. R. LEHMAN

Department of Biochemistry, Beckman Center, Stanford University School of Medicine, Stanford, CA 94305-5307

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ABSTRACT **Extracts of insect cells infected with baculoviruses recombinant for the herpes simplex virus 1 (HSV-1)-encoded enzymes that are required for its replication can promote the rolling circle replication of circular plasmid templates. Replication is independent of a HSV-1 origin of replication (ori_s) or the HSV-1 origin binding protein and is inhibited by the origin binding protein when the plasmid contains ori_s. Replication is dependent on a complex composed of the HSV-1-encoded DNA polymerase and its processivity enhancing factor (the UL42 protein), ICP8 (the HSV-1-encoded single-strand DNA binding protein), and the HSV-1-encoded helicase-primase. The complex can be purified by size-exclusion and anion-exchange chromatography.**

The herpes simplex virus type 1 (HSV-1) genome consists of a linear 152-kb double-stranded DNA molecule (1). The viral DNA is circularized upon infection and replicates in the nucleus of infected cells. The newly replicated viral DNA is found in large head-to-tail concatamers (2, 3), suggesting that replication occurs via a rolling circle mechanism. The mechanism by which HSV-1 DNA replication is initiated is unknown; however, the circularized HSV-1 DNA molecule with its three origins of replication may be a template for theta replication (4).

HSV-1 encodes seven gene products that are necessary and sufficient for the replication of origin-containing plasmids in transient replication assays performed in both mammalian and insect cells (4, 5). These proteins include a highly processive heterodimeric DNA polymerase (the product of the *UL30* and *UL42* genes) (6, 7), a heterotrimeric helicase-primase (the product of the *UL52*, *UL5*, and *UL8* genes) (8), a single-strand DNA binding protein (ICP8, the product of the *UL29* gene) (15), and an origin binding protein (the product of the *UL9* gene) (4, 9, 10). These proteins have been overexpressed in the baculovirus expression system (11) and purified to near homogeneity. The purified enzymes by themselves are incapable of initiating origin-dependent HSV-1 DNA replication, indicating that other, host-derived, factors may be required.

In this report, we show that extracts of insect cells infected with baculoviruses that are recombinant for the seven essential gene products can promote the rolling circle replication of circular plasmid templates. Replication requires the HSV-1-encoded DNA polymerase-UL42 protein, ICP8, and helicase-primase. However, it is independent of the UL9 origin binding protein and is, in fact, inhibited by the UL9 protein when the plasmid template contains a HSV-1 origin of replication (ori_s).

MATERIALS AND METHODS

Materials. Cell culture media and reagents were obtained from GIBCO/BRL. Restriction endonucleases and the pUC18 plasmid DNA were from Boehringer Mannheim. Plasmid pUO7 was prepared by cloning an 822-bp fragment containing HSV-1 origin of replication ori_s (12) into the *Bam*HI site in pUC18. Deoxynucleotides, ribonucleotides, DEAE-Sepharose, and S-500 resin were from Pharmacia. [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) was from NEN. The recombinant baculovirus AcUL29 was a gift from N. Stow (Medical Research Council Virology Unit, Glasgow). The recombinant AcUL42 was a gift from M. Challberg (National Institutes of Health, Bethesda, MD). The recombinant baculoviruses AcUL30, AcUL52, AcUL5, AcUL8, and AcUL9 were constructed by B. D. Song and M. Dodson (13) of this department. The UL30- and UL42-specific rabbit antisera were prepared by T. Hernandez of this department. The UL52- and UL5-specific rabbit antisera were gifts from M. Dodson (University of Arizona). The ICP8-, UL9-, and UL8-specific rabbit antisera were raised against the native proteins.

Buffers. Buffer A (lysis buffer) contained 20 mM Hepes·NaOH (pH 7.6), 1 mM EDTA, 1 mM EGTA, 5 mM potassium acetate, and 1 mM dithiothreitol (DTT). Buffer B contained 20 mM Hepes·NaOH (pH 7.6), 20 mM potassium acetate, 1 mM EDTA, and 1 mM DTT. Buffer C (size-exclusion chromatography buffer) contained 30 mM Hepes·NaOH (pH 7.6), 100 mM KCl, 1 mM EDTA, and 1 mM DTT. Buffer D contained 40 mM Tris·HCl (pH 7.5), 100 mM KCl, 10% (vol/vol) glycerol, 1 mM EDTA, and 1 mM DTT. TE contained 10 mM Tris·HCl (pH 7.5) and 1 mM EDTA.

Cell Cultures. *Spodoptera frugiperda* (Sf21) cells were maintained and propagated in suspension in SFM 900 medium (GIBCO/BRL) at 27°C with constant rotation at 125 rpm. *Autographa californica* nuclear polyhedrosis virus recombinant for the seven essential HSV-1 replication enzymes (UL30, UL42, UL9, UL29, UL52, UL5, and UL8) and the parental baculovirus were prepared by infecting Sf21 cells in 500 ml of medium at a concentration of 1–2 × 10^6 in a 2-liter glass Erlenmeyer flask with 10 ml of stock virus (1–10 × 10^7 cells per ml) for 3 days. Cells were pelleted at 1500 × *g* for 10 min and the virus in the supernatant (final titer, 1–10 × 10^7 cells per ml) was used directly.

Sf21 cells (2 × 10^6 cells per ml) were infected with different combinations of the seven recombinant viruses each at a multiplicity of infection of 1–5 by adding 70 ml of stock virus to 500 ml of SFM 900 medium containing 5% fetal bovine serum in a 2-liter Erlenmeyer flask. Cells were grown at 27°C with constant rotation at 125 rpm for 40 hr. The cells were then centrifuged at 1500 × *g* for 10 min, washed with phosphate-buffered saline, and frozen in buffer A. To obtain productive expression of the HSV-1-encoded replication proteins, the cells should be infected during exponential growth at a cell density no greater than 2 × 10^6 cells per ml.

Preparation of Extracts. Frozen cells that had been infected with the recombinant viruses were thawed at 4°C. The cells

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Abbreviations: HSV-1, herpes simplex virus type 1; ori_s, HSV-1 origin of replication; DTT, dithiothreitol; RF, replicative form.

were homogenized 20 times with a B-type pestle and KCl was added to a final concentration of 0.25 M. The homogenate was swirled gently at 4°C for 30 min and then centrifuged at 100,00 × *g* for 35 min. The supernatant was dialyzed for 6 hr against buffer B, frozen in liquid nitrogen, and stored at −80°C.

Verification of Expression of HSV-1 Proteins by Western Analysis. The extract (50 μg) prepared as described above was subjected to 8% polyacrylamide gel electrophoresis in the presence of SDS. The proteins were then blotted onto a nitrocellulose membrane (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. The HSV-1-encoded proteins were detected with specific antisera using enhanced chemiluminescence as the detection method (ECL, Amersham).

Fractionation of Enzyme Complex. The extract (20 mg of protein) was loaded onto an S-500 column (50 × 1.5 cm) (2 × 10^7–4 × 10^4 Da for dextrans) in buffer C and 1.8-ml fractions were collected (retention time, 5 min per fraction). Elution was followed by measurement of absorbance at 280 nm and by SDS/polyacrylamide gel electrophoresis and Western analysis of the fractions. Fractions with DNA replication activity were pooled and loaded onto a 2.5-ml DEAE-Sepharose column that had been equilibrated with buffer D. The column was washed with 2 column volumes of buffer D and eluted with a 10 column volume linear gradient of 0.1–0.5 M KCl in buffer D. Alternatively, the column was eluted first with 0.25 M and then with 0.5 M KCl in buffer D (2 column volumes each).

Analysis of DNA Replication. The reaction mixture (50 μl) contained 30 mM Hepes·NaOH (pH 7.5), 0.5 mM DTT, 8 mM magnesium acetate; 4 mM ATP; 50 μM dATP, dGTP, and dTTP; 10 μM dCTP; 5 μCi of [α-^{32}P]dCTP; 250 μM CTP, GTP, and UTP; 40 mM creatine phosphate; 5 μg of creatine kinase; 80 fmol of plasmid DNA (molecules); and 5 μg of enzyme fraction. Incubation was at 37°C for 2 hr. DNA synthesis was measured by incorporation of [^{32}P]dCMP into acid-insoluble material. After incubation, the volume of the reaction mixture was increased to 200 μl with TE, and the DNA was extracted first with phenol/chloroform and then with chloroform. The DNA was precipitated by the addition of ammonium acetate to 2.5 M, followed by 2.5 vol of ethanol, and allowed to stand overnight at −20°C. The 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. The DNA was dried in a Speed-Vac and resuspended in 50 μl of TE. It was digested for 8–12 hr with the *Dpn* I (10 units per reaction) and/or *Hin*dIII restriction enzymes (10–15 units per reaction). Partial digestion of the product was performed by treatment with *Dpn* I (10 units) for 6–8 hr and then with 5 units of *Hin*dIII for 0–60 min. The digest was analyzed by agarose gel electrophoresis. Native agarose gel electrophoresis was performed in 1% agarose with TBE (89 mM Tris borate/1 mM EDTA) containing 0.5 mg of ethidium bromide per ml as the electrode buffer at 2.5 V/cm for 12 hr. Denaturing agarose gel electrophoresis was carried out in 1% agarose with 30 mM NaOH/1 mM EDTA as the electrode buffer at 1.5 V/cm for 12 hr. After electrophoresis, the gels were dried and autoradiographed with an intensifying screen.

RESULTS

Rolling Circle DNA Replication by Insect Cell Extracts Containing HSV-1-Encoded Enzymes. Extracts of insect cells multiply infected with baculoviruses recombinant for the seven HSV-1-encoded gene products required for HSV-1 DNA replication promoted the replication of a plasmid containing a HSV-1 origin (pUO7) as template. The rate and extent of DNA replication was ≈5-fold greater than that observed with extracts of mock-infected cells (Fig. 1). To determine whether this synthesis represents semiconservative rather than repair replication, the DNA product was treated with the *Dpn* I restriction endonuclease and subjected to agarose gel electrophoresis. The template pUO7 was propagated in *Escherichia coli*; hence, it is fully methylated and sensitive to *Dpn* I. The products of semiconservative DNA replication should be hemimethylated and/or unmethylated and thus resistant to the action of *Dpn* I.

As shown in Fig. 2 (lanes 1 and 4), both the replicative forms RFI and RFII of pUO7 were labeled after incubation with extracts of recombinant baculovirus-infected as well as mock-infected cells. However, upon treatment with *Dpn* I both were degraded to DNA molecules smaller than RFIII (lanes 2 and 5), indicating that they were products of repair replication. However, extracts of cells infected with the recombinant baculoviruses produced a heterogeneous mixture of DNA molecules larger than the parental plasmid and resistant to the action of *Dpn* I (lane 5). The heterogeneous mixture was resolved into unit length molecules by further digestion with the restriction endonuclease *Hin*dIII, which cleaves the plasmid once (lane 6).

When the high molecular weight products were digested with *Dpn* I, alkali-denatured, and subjected to agarose gel electrophoresis under denaturing conditions, high molecular weight *Dpn* I-resistant DNA molecules were still observed (Fig. 3, lane 2). When the high molecular weight products were digested with *Dpn* I and *Hin*dIII and then denatured, they were resolved into unit length single strands, indicating that at least one strand of newly synthesized DNA was intact (lane 3).

The Products of the *UL30/UL42*, *ICP8*, and *UL52/UL5/UL8* Genes but Not the *UL9* Gene Are Essential for Rolling Circle DNA Replication. To determine which of the seven HSV-1-encoded gene products that are essential for HSV-1 DNA replication *in vivo* (14) are required for rolling circle replication *in vitro*, recombinant viruses expressing *UL30/UL42*, *ICP8*, *UL52/UL5/UL8*, and *UL9* were each omitted

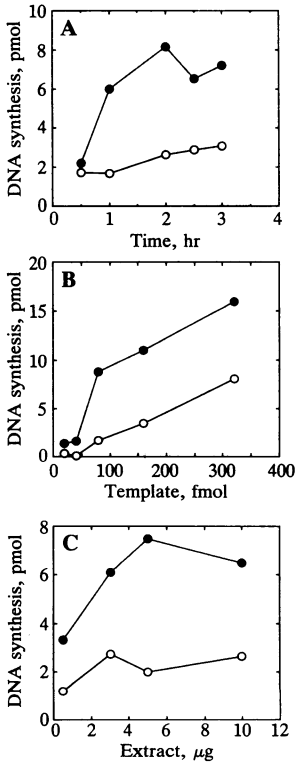

FIG. 1. DNA synthesis by extracts of insect cells multiply infected with baculoviruses recombinant for the seven essential genes. DNA synthesis was determined by measuring incorporation of [^{32}P]dCMP into acid-insoluble material with pUO7 as template. ○, Extract from mock-infected cells; ●, extract from cells infected with the seven recombinant baculoviruses.

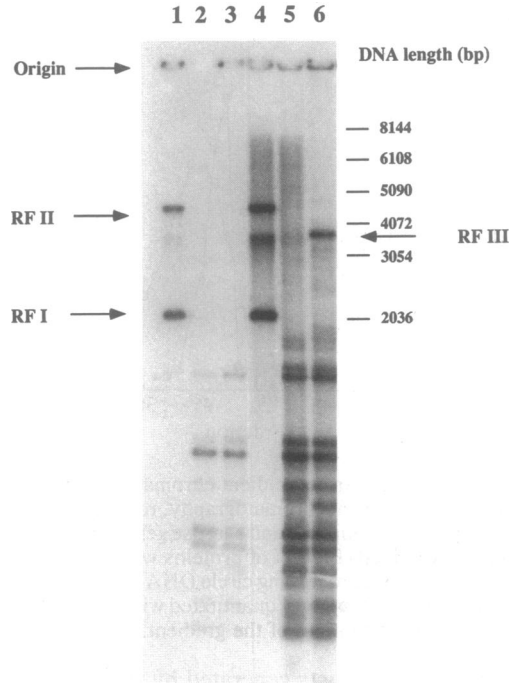

FIG. 2. Analysis of DNA products by agarose gel electrophoresis. Rolling circle DNA replication and agarose gel electrophoresis were performed as described with plasmid pUO7 as template. Lanes: 1–3, extract from mock-infected cells; 4–6, extract from multiply infected cells; 1 and 4, untreated products; 2 and 5, products digested with *Dpn* I; 3 and 6, products digested with *Dpn* I and *Hin*dIII.

from the infection. As shown in Fig. 4, the high molecular weight products of rolling circle DNA replication appeared only when extracts from cells expressing all seven gene products (lanes 1, 6, and 11) or extracts lacking the UL9 protein (lanes 2, 7, and 12) were used. When the recombinant

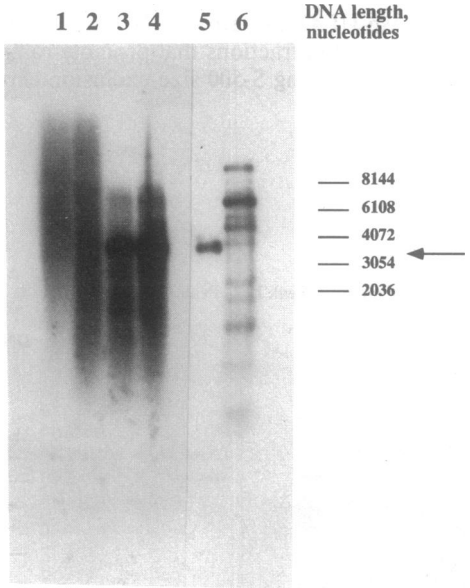

FIG. 3. Analysis of DNA products by alkaline agarose gel electrophoresis. Rolling circle DNA replication with pUO7 as template using an extract from multiply infected cells and alkaline gel electrophoresis of the products were performed as described. The products were digested with *Dpn* I and/or *Hin*dIII and denatured prior to electrophoresis. Lanes: 1, untreated products; 2, products digested with *Dpn* I; 3, products digested with *Dpn* I and *Hin*dIII; 4, products digested with *Hin*dIII; 5 and 6, DNA size markers. Arrow indicates unit length single strands.

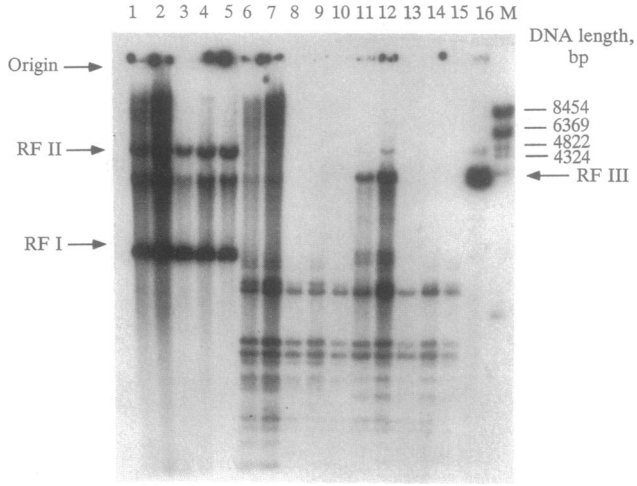

FIG. 4. Requirement for HSV-1-encoded enzymes for rolling circle DNA replication. Products synthesized by extracts from cells infected with the indicated recombinant baculoviruses with pUO7 as template were subjected to agarose gel electrophoresis as described. Lanes: 1–5, undigested products; 6–10, *Dpn* I digest; 11–15, *Dpn* I and *Hin*dIII digest; 16, ^{32}P-end-labeled RFIII; M, DNA size markers; 1, 6, and 11, extract from cells infected with all seven recombinant baculoviruses; 2, 7, and 12, extract lacking UL9; 3, 8, and 13, extract lacking UL30 and UL42; 4, 9, and 14, extract lacking ICP8; 5, 10, and 15, extract lacking UL52, UL5, and UL8.

baculoviruses expressing *UL30/UL42* (lanes 3, 9, and 13), *ICP8* (lanes 4, 9, and 14), and *UL52/UL5/UL8* (lanes 5, 10, and 15) were omitted, neither high molecular weight products nor the unit length product of *Dpn* I and *Hin*dIII digestion appeared. When the recombinant baculoviruses expressing the *UL52*, *UL5*, or *UL8* genes individually were omitted from the infection, rolling circle DNA replication was abolished. However, omission of *UL42* produced a limited amount of replication activity (data not shown). All the extracts had similar repair replication activity, indicating that the absence of rolling circle replication was not a result of differences in preparation of the various extracts (lanes 1–5).

Rolling Circle DNA Replication Is Independent of HSV-1 ori$_s$ and Is Inhibited by UL9 When the Plasmid Contains ori$_s$. The replication of plasmids *in vivo* by insect cells multiply infected with baculoviruses recombinant for the essential HSV-1-encoded replication enzymes requires ori$_s$ as well as a functional UL9 protein (5). In contrast, rolling circle DNA replication by extracts of insect cells in which the HSV-1-encoded replication enzymes were expressed was independent of ori$_s$. Plasmid pUC18, which lacks a HSV-1 origin, could serve as a template for rolling circle replication. Thus, high molecular weight products were formed, which were converted to unit length molecules upon treatment with *Dpn* I and *Hin*dIII (Fig. 5, lanes 1 and 3). Furthermore, expression of the UL9 protein significantly reduced the amount of unit length product formed after treatment of the high molecular weight products formed with an ori$_s$-containing template (pUO7) with *Dpn* I and *Hin*dIII. This decrease was dependent on ori$_s$ (lanes 2 and 4).

Isolation of an Enzyme Complex That Can Promote Rolling Circle DNA Replication. When an extract of multiply infected cells (lacking the UL9 protein) was subjected to S-500 size-exclusion chromatography, three peaks of protein appeared (Fig. 6*A*). Of the three, only the peak eluting at a mass of $\approx 2 \times 10^6$ Da (peak II) promoted rolling circle DNA replication as judged by the production of high molecular weight, *Dpn* I-resistant forms that could be resolved to unit length DNA molecules upon digestion with *Hin*dIII (Fig. 6*B*, lanes 8 and 9, respectively). When peak II (fractions 37–42) was subjected to DEAE-Sepharose gradient chromatogra-

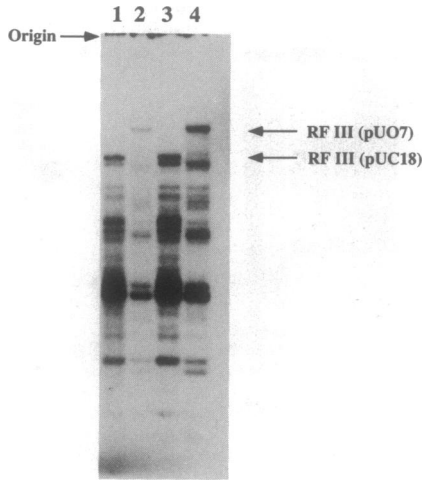

FIG. 5. Inhibition of rolling circle DNA replication of ori_s-containing plasmid by UL9 protein. Rolling circle DNA replication was performed with an extract from cells infected with all seven recombinant baculoviruses (lanes 1 and 2) or with an extract lacking UL9 (lanes 3 and 4). The products were digested with *Dpn* I and *Hin*dIII and subjected to agarose gel electrophoresis. Lanes: 1 and 3, plasmid lacking ori_s (pUC18); 2 and 4, plasmid containing ori_s (pUO7).

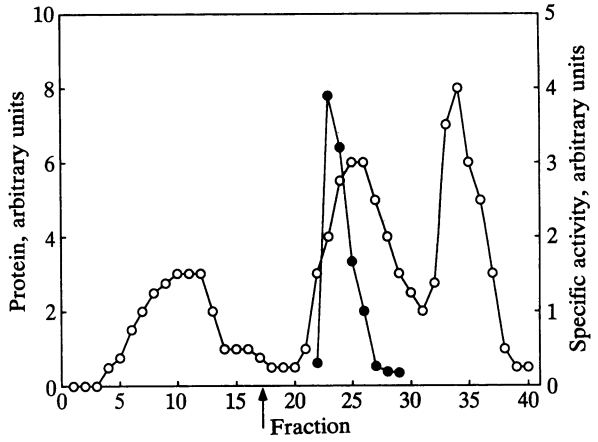

FIG. 7. DEAE-Sepharose gradient chromatography of complex. DEAE-Sepharose gradient chromatography, rolling circle DNA replication with pUO7 as template, and agarose gel electrophoresis were performed as described. Elution of proteins was monitored by A_{280} (○) (OD 1 = 50). Products of rolling circle DNA replication (after *Dpn* I and *Hin*dIII digestion) (●) were quantitated with a PhosphorImager. Arrow indicates starting point of the gradient.

phy, rolling circle DNA replication activity appeared as a sharp peak approximately midway through the gradient (Fig. 7).

When peak II was subjected to step elution from DEAE-Sepharose, rolling circle DNA replication was found exclusively in the 0.25 M KCl eluate (data not shown). Treatment of the products of replication generated by the DEAE-Sepharose step eluate with *Dpn* I followed by partial digestion with *Hin*dIII yielded DNA molecules ranging from 1 to 4 monomers in length (Fig. 8). Thus, the complex can promote rolling circle replication to generate multimeric DNA molecules.

Fig. 9 compares the ability of the crude extract, the S-500 fraction, and the 0.25 M DEAE-Sepharose step eluate to promote rolling circle DNA replication. Although the high molecular weight products generated by the extract and the S-500 fraction were heterogeneous in size, the product of rolling circle replication by the DEAE-Sepharose fraction was relatively homogeneous, yielding DNA molecules 3–5 times unit length (also see Fig. 8). The crude extract contains a nuclease that fails to bind to DEAE-Sepharose. This nuclease may be responsible for the disappearance of some of the DNA fragments (smaller than RFIII) produced by digestion of the *Dpn* I-sensitive DNA molecules (Fig. 9, lane 3). Removal of this nuclease may prevent random degradation of the high molecular weight products observed in the extract and S-500 fractions.

As judged by Western analysis, each of the HSV-1-encoded replication enzymes was present in the extract. DNA polymerase-UL42 protein, ICP8, and the helicase-primase were present in fractions that promote rolling circle replication obtained during S-500 size-exclusion chromatog-

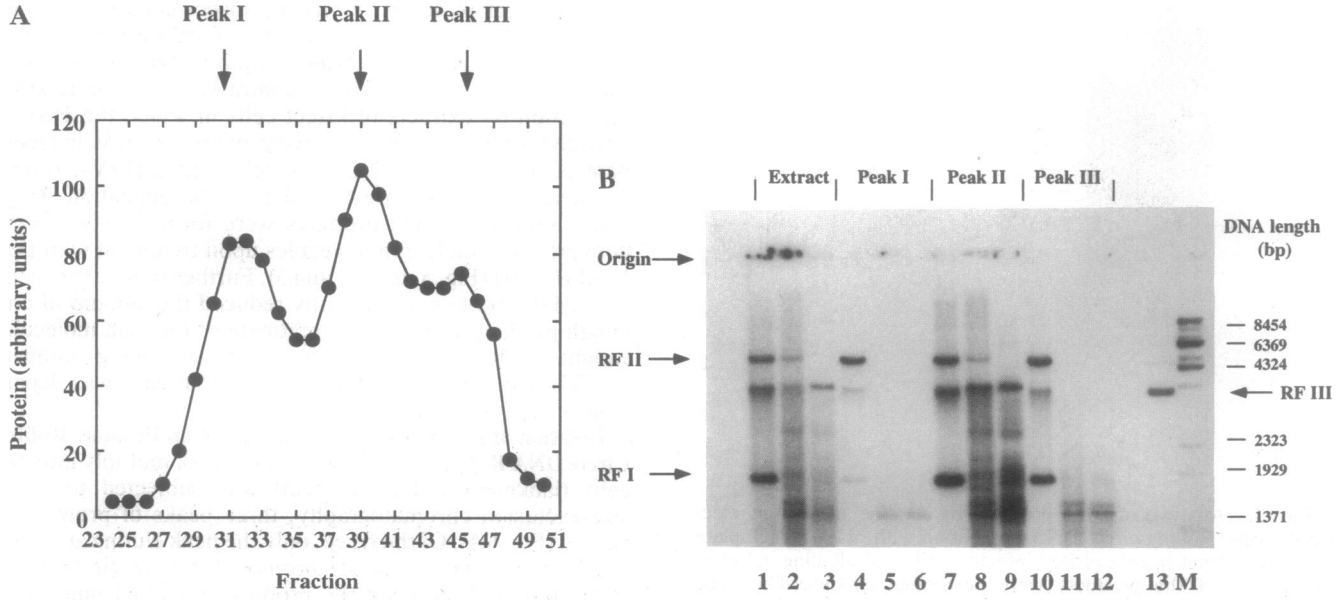

FIG. 6. S-500 size-exclusion chromatography of complex. (*A*) Elution of proteins from the S-500 column was monitored by A_{280} (OD 1 = 100). (*B*) Rolling circle DNA replication with pUO7 as template and agarose gel electrophoresis were performed as described. Lanes: 1–3, 4–6, 7–9, and 10–12, products obtained with crude extract, peak I, peak II, and peak III, respectively; 1, 4, 7, and 10, untreated products; 2, 5, 8, and 11, products digested with *Dpn* I; 3, 6, 9, and 12, products digested with *Dpn* I and *Hin*dIII.

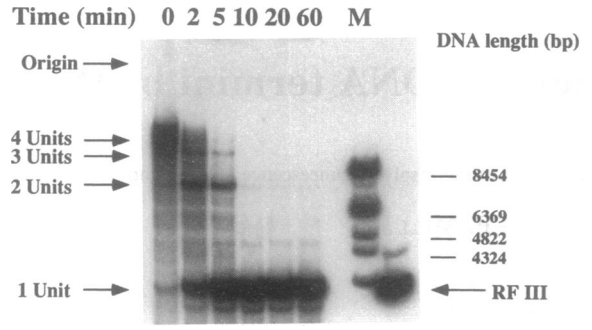

FIG. 8. Analysis of high molecular weight products of rolling circle DNA replication by partial digestion. Rolling circle DNA replication with pUO7 as template was performed with the 0.25 M DEAE-Sepharose eluate. Products were treated with *Dpn* I and then digested for the indicated time periods with *Hin*dIII. Agarose gel (0.7%) electrophoresis was performed as described. DNA products 1–4 monomer units in length are indicated by arrows.

raphy as well as during gradient and step elution from DEAE-Sepharose (data not shown). These proteins were also present in DEAE-Sepharose fractions that lacked replication activity, suggesting that elution of rolling circle DNA replication activity in a sharp peak was not the result of coincidental coelution of the HSV-1-encoded replication enzymes but is rather a consequence of their existence in the form of a tight complex. The 0.25 M KCl DEAE-Sepharose step eluate was essentially free of nucleic acids as judged by its absorption spectrum ($A_{280/260}$ = 1.4) and agarose gel electrophoresis. The complex is therefore unlikely to be a result of nucleic acid-induced aggregation of the replication enzymes.

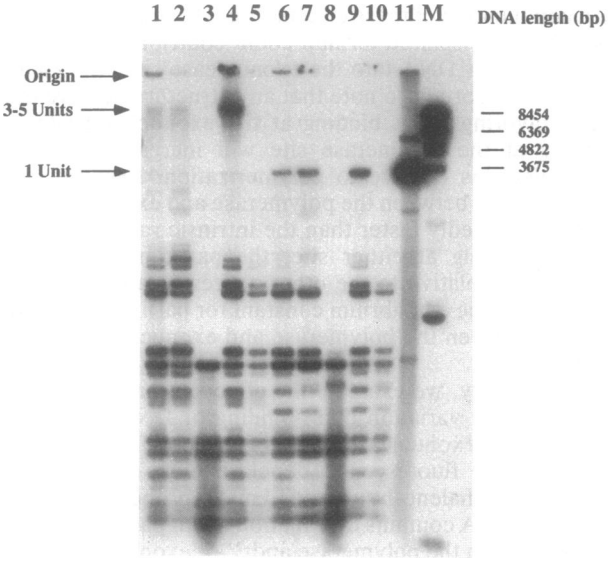

FIG. 9. Analysis of rolling circle DNA replication by fractions obtained during S-500 and DEAE-Sepharose step chromatography. Fractions obtained during S-500 and DEAE-Sepharose step chromatography were analyzed for their ability to promote rolling circle DNA replication with pUO7 as template. Lanes: 1–5, products digested with *Dpn* I; 6–10, products digested with *Dpn* I and *Hin*dIII; 1 and 6, products obtained with extract of cells multiply infected with the essential HSV-1 replication genes with the exception of UL9; 2 and 7, products obtained with S-500 fraction; 3 and 8, products obtained with DEAE-Sepharose flow-through fraction; 4 and 9, products obtained with 0.25 M KCl DEAE-Sepharose eluate; 5 and 10, products obtained with 0.5 M KCl DEAE-Sepharose eluate; 11, end-labeled linearized parental plasmid; M, DNA size markers.

DISCUSSION

Stow (5) has reported that insect cells infected with baculoviruses recombinant for each of the seven HSV-1 genes required for viral replication can promote the replication *in vivo* of plasmids containing a HSV-1 origin. We have found that extracts prepared from such cells are capable of promoting rolling circle DNA replication of these plasmids *in vitro*. By omitting each of the seven gene products, we found that rolling circle DNA replication requires the HSV-1-encoded helicase-primase, the single-strand DNA binding protein ICP8, and the DNA polymerase-UL42 protein. Neither the UL9 protein nor a HSV-1 origin is required. In fact, the presence of ori_s significantly inhibits DNA replication. The inhibition is, however, not observed in the absence of UL9 protein.

Because of the limited extent of synthesis observed, only a fraction of the plasmid molecules appear to function as templates for rolling circle DNA replication. The structure of the functional template is presently unknown.

The enzymes that promote rolling circle DNA replication can be isolated as a complex free of nucleic acids that persists through size-exclusion and ion-exchange chromatography. A mixture of the purified baculovirus-expressed DNA polymerase-UL42 protein, ICP8, and helicase-primase fails to promote rolling circle DNA replication. However, addition of an extract of mock-infected insect cells did produce some rolling circle DNA replication (unpublished data). The complex may therefore contain one or more essential host factors.

The products of rolling circle replication promoted by the DEAE-Sepharose fraction are rather homogeneous, 1–4 times unit length. Since no effort was made to synchronize the reaction, the relative homogeneity of the product is surprising. Possibly it is a consequence of the ability of the complex to limit the size of the rolling circle product.

Note Added in Proof. The complex consisting of the HSV-1 DNA polymerase-UL42 protein, helicase-primase, and ICP8 can be immunoprecipitated by antiserum directed against ICP8.

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