Role of Protein-Protein Interactions during Herpes Simplex Virus Type 1 Recombination-dependent Replication*

Received for publication, January 26, 2004, and in revised form, March 15, 2004
Published, JBC Papers in Press, March 16, 2004, DOI 10.1074/jbc.M400832200

Amitabh V. Nimmonkar and Paul E. Boehmer‡

From the Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101-6129

Recombination-dependent replication is an integral part of the process by which double-strand DNA breaks are repaired to maintain genomic integrity. It also serves as a means to replicate genomic termini. We reported previously on the reconstitution of a recombination-dependent replication system using purified herpes simplex virus type 1 proteins (Nimmonkar A. V., and Boehmer, P. E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10201–10206). In this system, homologous pairing by the viral single-strand DNA-binding protein (ICP8) is coupled to DNA synthesis by the viral DNA polymerase and helicase-primase in the presence of a DNA-relaxing enzyme. Here we show that DNA synthesis in this system is dependent on the viral polymerase processivity factor (UL42). Moreover, although DNA synthesis is strictly dependent on viral components, it is only stimulated by the viral helicase in a manner that requires the helicase-loading protein (UL8). Furthermore, we have examined the dependence of DNA synthesis in the viral system on species-specific protein-protein interactions. Optimal DNA synthesis was observed with the herpes simplex virus type 1 replication proteins, ICP8, DNA polymerase (UL30/UL42), and helicase-primase (UL5/UL52/UL8). Interestingly, substitution of each component with functional homologues from other systems for the most part did not drastically impede DNA synthesis. In contrast, recombination-dependent replication promoted by the bacteriophage T7 replisome was disrupted by substitution of the replication proteins from herpes simplex virus type 1. These results show that although DNA synthesis performed by the T7 replisome is dependent on cognate protein-protein interactions, such interactions are less important in the herpes simplex virus replisome.

Herpes simplex virus type 1 (HSV-1) is an ~152-kbp double-stranded DNA virus (1). Replication of the viral genome occurs in the nuclei of infected cells and requires at least seven virus-encoded proteins as well as several cellular factors (reviewed in Refs. 1 and 2). The viral genome possesses distinct origins of replication and encodes an origin binding protein (UL9), which is highly suggestive of an initial θ-mode of replication (1). Furthermore, the failure to detect genomic DNA ends shortly after infection hints at the possibility that the viral genome circularizes immediately upon infection and replicates via a θ-mode to generate circular intermediates (1). The observation of high molecular weight viral DNA later during the life cycle (3) prompted the suggestion that replication switches to a rolling circle or σ-mode to produce head-to-tail concatamers that are subsequently cleaved into unit length genomes and packaged (2). This strategy for replication, resembling that adopted by bacteriophages λ (4), has been considered the “dogma” for HSV-1 replication for more than 2 decades. However, recent evidence indicates that circularization of the genome is not a requisite for lytic viral replication and that the template for replication is in fact a linear genome (5). Thus, it seems likely that the strategy of HSV-1 replication resembles more that of bacteriophages T4 and T7 (6, 7). Replication of a linear genome possesses an intrinsic problem, replication of the genomic termini. In T4, this is overcome by using recombination-dependent replication (RDR) in which the end of one genome invades into a homologous region of another and utilizes it as a template to complete lagging strand synthesis (6). Such a mode of replication would generate highly branched replication intermediates that are commonly seen in HSV-1-infected cells (3).

During the later stages of HSV-1 replication, the genome recombines at a high frequency, with a rate estimated to be 0.6%/kb of genome (8). In addition to recombination acting to replicate the ends of the linear genome, it may also act to repair double-strand DNA breaks (DSB). The HSV-1 genome possesses sites called σ sequences that are recombination hotspots and arecleaved by endonuclease G to introduce DSB (9, 10). The σ sequence-mediated cleavage of the genome is thought to be involved in genome isomerization (10). DSB may also be generated as a consequence of oxidative damage induced upon infection (11, 12).

HSV-1 encodes its own replication machinery that consists of a single-strand DNA-binding protein (SSB) (ICP8), DNA helicase-primase (UL5/UL52 core enzyme and UL8 loading protein), and DNA polymerase (pol) (UL30 catalytic subunit and UL42 processivity factor) (2). These factors have been shown to associate into a replisome that is capable of long chain leading and lagging strand synthesis (13–15). We recently proposed a model for RDR in HSV-1 based on the ability of HSV-1-encoded factors to catalyze such reactions in vitro (16). In our model, processing of DSB allows ICP8 to pair single-stranded (ss) donor DNA with complementary duplex DNA resulting in the formation of displacement loops (D-loops). These D-loops nucleate the assembly of the viral replisome that promotes long

* This work was supported by Grant GM58264 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Miami School of Medicine, P. O. Box 016129, Miami, FL 33101-6129. Tel.: 305-243-2934; Fax: 305-243-3955; E-mail: pboehmer@molbio.med.miami.edu.

The abbreviations used are: HSV-1, herpes simplex virus, type 1; D-loop, displacement loop; E-SSB, E. coli single-strand DNA-binding protein; pol, DNA polymerase; RDR, recombination-dependent replication; SDS, strand displacement synthesis; SSB, single-strand DNA-binding protein; ss, single-stranded; SV40, simian virus 40; TAg, large T antigen; Tpo, topoisomerase; BSA, bovine serum albumin; nt, nucleotides; DTT, dithiothreitol; Dda, DNA-dependent ATPase; DSB, double-strand DNA breaks.
chain DNA synthesis in the presence of a DNA-relaxing enzyme (16). A schematic representation of the reaction is shown in Fig. 1A.

In this work, the ability of the HSV-1 replication proteins to promote DNA synthesis on D-loops (i.e., RDR) was examined in greater detail. Furthermore, we investigated the requirement for species-specific protein-protein interactions during HSV-1 RDR by replacing the HSV-1 replication proteins with their functional counterparts from other systems. Our results indicate that HSV-1 RDR exhibits a less stringent requirement for species-specific protein-protein interactions when compared with T7 RDR.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—Escherichia coli sss (E-SSB), T4 gene 32 protein (gp32), and calf thymus DNA topoisomerase I (Topo I) were purchased from U. S. Biochemical Corp. One unit of Topo I is that amount of enzyme that relaxes 0.5 μg of pBR322 in 30 min at 37 °C. The specific activity of Topo I was 16,949 units/mg. T4 polynucleotide kinase and proteinase K were purchased from New England Biolabs and Roche Applied Science, respectively. ICP8 (17), UL9 (18), UL5/UL52 core enzyme, and UL8 (19) were purified as described previously. Their concentrations, expressed in moles of monomeric protein, were determined using extinction coefficients of 82.720, 89.220, 171.380, and 130,390 M−1 cm−1 at 280 nm, respectively, calculated from their predicted amino acid sequences (20). UL30 and UL42 were purified as described, and their concentrations, in moles of monomeric protein, were determined by the method of Bradford using BSA as a standard (21). The T7 replication proteins, namely gene 2.5 protein (gp2.5), gene 5 protein/thioredoxin (gp5/Th), and gene 4 protein (gp4) were a kind gift from Dr. Charles C. Richardson (Harvard Medical School, Boston). Human RP-A (hRP-A), simian virus 40 (SV40), large T antigen (TAg), T4 DNA-dependent ATPase (Dda), and E. coli pol III holoenzyme (containing β subunit) were kind gifts from Drs. Patrick Sung (Yale University, New Haven, CT), James A. Borowiec (New York University School of Medicine, New York), Kevin Raney (University of Arkansas for Medical Sciences, Little Rock, AR), and Arthur Kornberg (Stanford University, Stanford, CA), respectively. ATP (disodium salt) and γ-32P-ATP (4,500 Ci/mmol) were purchased from Sigma and MP Biomedicals, respectively. Deoxyriboonucleoside triphosphates (disodium salts) were purchased from Amersham Biosciences.

Nucleic Acids—Oligodeoxyribonucleotide PB11 (100-mer) (22), complementary to residues 379–478 of the minus strand of pUC18, was synthesized and gel-purified by Sigma-Genosys. Its concentration was determined by using an extinction coefficient of 939,208.1 M−1 cm−1 at 260 nm. PB11 was 5-32P-labeled with T4 polynucleotide kinase and purified using Sephadex G-25 (fine) spin columns (Roche Applied Science, Indianapolis). PB11 was isolated from Brij-58-lysed cancer cells by anion exchange (Q high (Bio-Rad)) as described previously (23). The DNA was further treated with 0.1 M NaOH, followed by neutralization and extraction with the Promega Wizard Plus DNA purification system. All DNA concentrations are expressed in moles of molecules.

D-loop Formation—PB11 (10.5 nm) was preincubated with ICP8 or gp2.5 (250 nM) on ice for 5 min in a buffer containing 25 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 1 mM DTT, and 100 μg/ml BSA. The pairing reaction was initiated by adding pUC18 form I DNA (3.5 nm), and incubation was continued for 30 min at 30 °C. D-loops were purified by extraction by the Promega Wizard DNA cleanup system followed by removal of excess unannealed oligonucleotide by gel filtration through a Maxima Spin + TE-1000 column (BD Biosciences). DNA Synthesis Using Pre-formed D-loops—DNA synthesis reactions with purified D-loops were performed in a buffer containing 16.7 mM Tris acetate, pH 7.5, 6.7 mM magnesium acetate, 0.66 mM DTT, 2.5 mM ATP, 500 μM each of dATP, dCTP, dGTP, and TTP, and 66 μg/ml BSA. Unless otherwise stated, purified D-loops (0.6 nm) were supplemented with mixture A (40 nM ICP8, 5 nM UL5/UL52, 10 nM UL5/UL52, 30 nM UL8, and 2.5 units of Topo I) or mixture B (200 nM gp2.5, 5 nM T7 gp5/Th, 10 nM T7 gp4, and 2.5 units of Topo I). Reactions were incubated for 60 min at 30 °C and quenched by the addition of termination buffer (final concentration, 50 mM EDTA and 3 μg/ml proteinase K) followed by further incubation for 20 min. Reaction products were resolved through 10% agarose containing 50 mM NaOH and 1 mM EDTA at −2.25 V/cm for 10 h. Following electrophoresis the gels were dried onto DE81 chromatography paper (Whatman), analyzed, and quantitated by storage phosphor analysis with a Molecular Dynamics Storm 820 PhosphorImager (Amersham Biosciences).

RESULTS

Long Chain Synthesis during RDR Requires the UL42 Processivity Factor—We had demonstrated previously the requirement for the heterodimeric HSV-1 pol (UL30 and UL42) during RDR (16). Here we examined the requirement for the viral processivity factor, UL42. Thus, purified D-loops were supplemented with increasing concentrations of UL30 or UL30/UL42, along with ICP8, helicase-prime, and Topo I. As shown in Fig. 1B, UL30 failed to generate full-length products (2686 nt) at concentrations ranging from 1 to 10 nM (lanes 1–10). In contrast, UL42 by itself could promote primer extension with increasing concentrations (Fig. 1B, lanes 3–6), leading to the formation of intermediates up to ~1000 nt in length. On the other hand, the presence of equimolar UL42 enabled full-length synthesis (lanes 7–12), with complete products forming at UL30/UL42 concentrations as low as 5 nM (Fig. 1B, lane 9). Therefore, subsequent experiments were performed using UL30/UL42 at 5 nM.

RDR Is Topo I-dependent and Stimulated by Helicase—We stated previously (16) that efficient RDR is dependent on Topo I as well as helicase action. Here we examined the requirement for both proteins in more depth. Thus, purified D-loops were supplemented with increasing concentrations of helicase-prime in the absence and presence of Topo I, along with ICP8 and UL30/UL42. In the absence of Topo I, at helicase-prime concentrations ranging from 0 to 100 nm, only marginal DNA synthesis was observed (Fig. 2A, lanes 1–5). On the other hand, the presence of Topo I enabled full-length synthesis (Fig. 2A, lanes 6–10). Approximately 4% (~33% of maximum) of the primer within the D-loop was extended to full length even in the absence of helicase-prime (Fig. 2A, lane 6). The presence of helicase-prime nevertheless stimulated the reaction ~3-fold (Fig. 2B) and also increased the length of intermediates (Fig. 2A, compare lane 6 with lanes 7–10). Optimal RDR was observed with 2.5 units of Topo I (data not shown).

Fig. 3 examines the role of the helicase-prime loading factor, UL8, during RDR. The data show that synthesis of longer intermediates (~500 nt) in the presence of helicase-prime was dependent on UL8 (Fig. 3, lane 2). In its absence, only shorter intermediates (~500 nt), as observed with Topo I alone, were synthesized (Fig. 3, compare lanes 1 and 3). Similar observations were made at a variety of UL5/UL52 concentrations (data not shown). Based on these data, subsequent experiments were performed using 2.5 units of Topo I, 10 nM UL5/UL52, and 30 nM UL8.

DNA Synthesis Coupled to D-loop Formation—D-loops were formed using 250 nM ICP8 or gp2.5, 3.5 nM pUC18 form I DNA, and 10.5 nM PB11 in a buffer containing 16.7 mM Tris acetate, pH 7.5, 6.7 mM magnesium acetate, 0.66 mM DTT, 2.5 mM ATP, 500 μM each of dATP, dCTP, dGTP, and TTP, and 66 μg/ml BSA. Unless otherwise stated, D-loop reactions were supplemented with HSV-1 proteins (final concentration, 166 nM ICP8, 5 nM UL30/UL42, 10 nM UL5/UL52, 30 nM UL8 or T7 proteins (final concentration, 166 nM gp2.5, 5 nM gp5/Th, 10 nM gp4) and 2.5 units of Topo I, incubated for 15 min at 30 °C, and quenched by the addition of termination buffer (final concentration, 50 mM EDTA and 3 μg/ml proteinase K) followed by further incubation for 20 min. The final concentration of plasmid DNA in the reaction was 2.33 μM of which ~15% were D-loops. The reaction products were resolved by two-dimensional native-denaturing agarose gel electrophoresis (16). Each reaction was resolved in duplicate in the first dimension through 0.75% agarose-Tris acetate EDTA, pH 7.6, gels at 9 V/cm for 2 h. Under these conditions, free 100-mer migrates out of the gel. One of the lanes was used as a reference to visualize products resolved in the first dimension. The other lane was excised, soaked in buffer containing 50 mM NaOH and 1 mM EDTA, embedded in a second gel composed of 1% agarose in 50 mM NaOH and 1 mM EDTA, and electrophoresed at ~2 V/cm for 7 h. Following electrophoresis the gels were dried onto DE81 chromatography paper (Whatman), analyzed, and quantitated by storage phosphor analysis with a Molecular Dynamics Storm 820 PhosphorImager (Amersham Biosciences).
Importance of Species-specific Protein-Protein Interactions during RDR

The preceding experiments established the role of the various HSV-1 factors in our RDR system. To ascertain whether species-specific protein-protein interactions are important for efficient RDR, we performed a series of experiments wherein each of the HSV-1 replication components was replaced by its counterpart from a variety of systems. First, we substituted ICP8 with SSBs from other systems, namely hRP-A, E-SSB, T4 gp32, and T7 gp2.5. Fig. 4 depicts the results of this experiment. The concentrations of SSBs used in the experiment were twice that required to coat the D-loops, based on the site sizes of the SSBs on ssDNA (24–28). In the absence of SSB, most replication products accumulated as intermediates (<500 nt) and less than ~1% of the primer reached full length (Fig. 4, lane 1). In the presence of ICP8, ~15% of the primer was extended to full length (Fig. 4, lane 2). Substitution of ICP8 with hRP-A, E-SSB, and T4 gp32 generated full-length DNA molecules with little difference in efficiency (Fig. 4, lanes 3–5), except for gp32 that only promoted full-length synthesis to approximately half the level seen with ICP8 (Fig. 4, compare lanes 2 and 5). However, there was an abundance of small intermediates (100–500 nt) with the heterologous SSBs com-
Heterologous helicases with the exception of T4 Dda can support HSV-1 RDR. 

Reactions were performed with purified D-loops as described under “Experimental Procedures,” followed by one-dimensional denaturing agarose gel electrophoresis. Where indicated, UL5/UL52/UL8 was replaced with the following helicases: Dda, T7 gp4, TAg, and UL9. Lane 1, no helicase; lane 2, Dda; lane 3, T7 gp4; lane 4, UL5/UL52/UL8; lane 5, TAg; lane 6, UL9. The concentration of each helicase was 12.5 nM. The positions of 100-mer, full-length (FL) extension products (2686 nt), intermediates, and markers (HindIII-digested λ DNA) are as indicated. The concentration of the pols used was standardized by determining full-length synthesis and led to the accumulation of short (<250 nt) intermediates (Fig. 4, lane 6). It should be noted that gp2.5 was able to stimulate the extension of singly primed M13 ssDNA by the T7 pol (data not shown), indicating that the protein was active. Moreover, results similar to those described were obtained for the various SSBs at a variety of concentrations (data not shown).

We then tested the effect of substituting the 5′–3′ unwinding activity of the HSV-1 helicase-primase with other helicases of like and opposing polarities. The helicases used in this experiment were the 5′–3′ helicases T4 Dda and T7 gp4 and the 3′–5′ helicases SV40 TAg and HSV-1 UL9, all of which have known functions in replication (29–32). The concentrations of all the helicases used in this experiment exhibited the same activity in unwinding a 100-mer annealed to M13 ssDNA (data not shown).

Control reactions showed that this concentration of Dda efficiently unwound a 100-mer annealed to M13 ssDNA (data not shown). Consistent with the observation made in Figs. 2 and 3, full-length products were generated in the absence of helicase (Fig. 5, lane 1), albeit less efficiently. The most striking effect was obtained upon replacing helicase-primase with Dda, where substitution prevented full-length synthesis, i.e. inhibited the helicase-independent extension reaction, and only led to limited primer extension (Fig. 5, compare lanes 1 and 2). Control reactions showed that this concentration of Dda efficiently unwound a 100-mer annealed to M13 ssDNA (data not shown).

Fig. 5 also shows that substitution of helicase-primase (lanes 4, 5) decreased the efficiency of full-length DNA synthesis and only permitted synthesis of short (<500 nt) intermediates (Fig. 4, lane 6). It should be noted that gp2.5 was able to stimulate the extension of singly primed M13 ssDNA by the T7 pol (data not shown), indicating that the protein was active. Moreover, results similar to those described were obtained for the various SSBs at a variety of concentrations (data not shown).

Finally, we studied the effect of replacing the dimeric HSV-1 pol (UL30/UL42) with heterologous pols. The enzymes tested were the replicative pols from T7 (gp5/Th) and E. coli (pol III).
mining the amounts required to completely extend a 100-mer annealed to M13 ssDNA (in the presence of coating concentrations of T4 gp32 as SSB) (data not shown). In the absence of a pol, no extension was observed, thereby demonstrating the necessity for this activity during RDR (Fig. 6, lane 1). UL30/UL42 (Fig. 6, lane 2) as well as T7 gp5/Th (lane 3) were both capable of supporting RDR with comparable efficiency. E. coli pol III on the other hand only supported limited primer extension, generating products up to ~200 nt and failing to generate full-length products (Fig. 6, lane 4). Similar results were obtained with higher concentrations of the pols (data not shown).

Comparison of RDR Catalyzed by the HSV-1 and T7 Replicosomes—Thus far, we have shown that the HSV-1 set of replication proteins was the most efficient at promoting RDR and that substitution with heterologous proteins in some cases (gp2.5, Dda, and pol III) prevented completion of RDR. Next, we wished to examine whether a complete set of heterologous replication proteins, namely that of T7 (i.e., gp2.5, gp4, and gp5/Th) could support RDR either on purified D-loops or in a coupled manner, i.e., during ongoing D-loop reactions. Hence, we first compared the activities of the HSV-1 and T7 replicosomes on purified D-loops. Purified D-loops were supplemented with HSV-1 (ICP8, UL30/UL42, and UL5/UL52/UL8) or T7 (gp2.5, gp4, and gp5/Th) replication proteins along with Topo I. The reaction products were analyzed by one-dimensional denaturing agarose gel electrophoresis. Fig. 7 shows that both the HSV-1 and T7 replicosomes were capable of RDR using purified D-loops as template, albeit with significant differences. First, HSV-1 and T7 replisomes were capable of RDR using purified agarose gel electrophoresis. Where indicated, UL30/42 was replaced by T7 gp5/Th or pol III. Lane 1, no pol; lane 2, UL30/UL42; lane 3, T7 gp5/Th; lane 4, pol III. The concentration of each pol was 5 nM. The positions of 100-mer, full-length (FL) extension products (2886 nt), intermediates, and markers (HindIII-digested λ DNA) are as indicated.

min to generate full-length products as opposed to 1 min by the T7 replisome. Finally, the pattern of replication intermediates between the two systems was contrasting. The major pause sites for the HSV-1 replisome were at ~500 and ~1200 nt, which may be attributed to the stalling of the replication fork at sites of secondary structure. The T7 replisome, on the other hand, could overcome these barriers with the majority of products proceeding to full-length or beyond.

The experiments shown in Figs. 4–6 indicate that although there is some specificity in HSV-1 RDR because certain heterologous functions (gp2.5, Dda, and pol III) do not substitute for the HSV-1 proteins, the system is promiscuous in the sense that RDR could be observed, albeit less efficiently, when ICP8, helicase-primase, or the pol were substituted with certain functional homologues. Fig. 8 shows the results of an experiment in which we examined the specificity that governs RDR (i.e., SDS) promoted by the T7 replisome. The data show that SDS was only supported by the combination of T7 proteins that extended ~50% of the primer beyond full length (Fig. 8, lane 1). Consistent with the established role of gp2.5 in promoting SDS (33), its substitution with ICP8 supported full-length synthesis but not SDS (Fig. 8, lane 2). Substitution of the gp4 helicase-primase
products, and markers (HindIII-digested /H9261 (length products were observed, and only short intermediates enzyme had a much more pronounced effect on RDR; no full-length (/
/H11022 with the HSV-1 counterpart led to efficient primer extension (Fig. 8,
lane 3 gp4; gp5/Th, and 10 nM gp4; (lane 3, 200 nM gp2.5, 5 nM gp5/Th, and 10 nM gp4; lane 3, 200 nM gp2.5, 5 nM gp5/Th, and 10 nM UL30/UL42; lane 4, 200 nM gp2.5, 5 nM UL30/42, and 10 nM gp4; and lane 5, 400 nM ICP8, 5 nM UL30/42, and 10 nM UL5/UL2/UL8. The positions of 100-mer, full-length (FL) extension products (2686 nt), intermediates, SDS products, and markers (HindIII-digested λ DNA) are as indicated.

with the HSV-1 counterpart led to efficient primer extension (products >500 nt) but generated minimal full-length products (Fig. 8, lane 3). Substitution of the T7 pol with the HSV-1 enzyme had a much more pronounced effect on RDR; no full-length products were observed, and only short intermediates (~200 nt) were synthesized (Fig. 8, lane 4). The full-length products formed by the action of the HSV-1 replisome (Fig. 8, lane 5) indicate that the HSV-1 factors were active under these conditions and that the failure to observe SDS when they were used to replace the T7 factors was indeed due to disruption of protein-protein interactions.

Having performed experiments using purified D-loops, we wished to examine whether specific recognition of ICP8-formed D-loops was crucial for efficient RDR. We therefore tested the abilities of the HSV-1 and T7 replisomes to perform RDR in reactions in which D-loop formation was coupled to DNA synthesis. Thus, D-loops were generated by ICP8, and the ongoing reactions were supplemented with the HSV-1 or T7 replication proteins. The ability to perform RDR was determined by resolving the reaction products using two-dimensional native-denaturing agarose gel electrophoresis. Electrophoresis in the first dimension resolved two major species, D-loops (D) and extension products (E) (Fig. 9 and 10, upper panels). Electrophoresis in the second dimension under alkaline conditions resolved the species based on length. The arc is representative of extension reactions originating from the 100-mer assimilated within a D-loop and culminating in full-length products (Figs. 9 and 10, lower panels). Consistent with our earlier observation (16), Fig. 9A shows the extension of 100-mer assimilated in the D-loop culminating in full-length products by the HSV-1 replisome. Surprisingly, in the context of an ongoing reaction in which the D-loops are associated with ICP8, the T7 pol and helicase-primase could efficiently substitute for the HSV-1 proteins and generate full-length products (Fig. 9B). In both cases, ~15% of the 100-mer assimilated in the D-loop was extended to full length. The addition of gp2.5 protein did not affect the amount of full-length synthesis (~15%) but greatly increased the formation of D-loops presumably because of its strand annealing activity (34) (Fig. 9C). We then tested whether gp2.5 could form D-loops and whether there was any specificity in their utilization for DNA synthesis. Thus, D-loops were generated by the action of gp2.5 and the ongoing D-loop reactions supplemented with T7 or HSV-1 replication proteins. Most interesting, although the T7 replisome could utilize D-loops formed by gp2.5 in the context of an ongoing reaction, the HSV-1 pol and helicase-primase could not substitute for the T7 factors (Fig. 10, A and B). Addition of ICP8 to the HSV-1 pol and helicase-primase did not enable DNA synthesis (Fig. 10C). The lesser extent of SDS observed in this experiment compared with that in Fig. 8 is due to the shorter incubation time (15 min).

**DISCUSSION**

We had shown previously that recombination intermediates, i.e. D-loops, formed by the pairing activity of ICP8, served as a substrate for replication (16). Efficient DNA synthesis in this system is dependent on ICP8 as well as the HSV-1 pol (UL30/UL42) and the replicative helicase-primase (16). In addition, the reaction also requires a relaxing activity (Topo I or gyrase) (16). The current work is a continuation of our efforts to characterize this RDR reaction. We also examined the necessity for species-specific protein-protein interactions.

By using purified D-loops, we studied the requirement for the viral pol processivity factor (UL42), the relaxing enzyme (Topo I), and the replicative helicase-primase (UL5/52) including its associated loading factor (UL8). Our results show that full-length synthesis in our system is absolutely dependent on UL42. In its absence, at high concentrations of the catalytic subunit (UL30), only intermediates were synthesized. The dependence on UL42 in this regard is similar to that reported previously for the extension of singly primed M13 ssDNA, even under conditions that allow rebinding of the pol (35). The requirement for a processivity factor in our RDR system is not unexpected because it involves long chain synthesis (2686 nt full length). However, given that our reaction conditions ([pol] > [DNA]) should allow re-initiation of DNA synthesis even in the absence of a processivity factor, it is somewhat surprising that the catalytic subunit (UL30) alone could not complete DNA synthesis.

We had shown previously that RDR in our system requires a DNA-relaxing enzyme (16). Here we confirm that RDR is indeed dependent on Topo I. In contrast, RDR is apparently independent of helicase action because significant full-length synthesis was observed in the absence of the viral helicase-primase. However, addition of helicase-primase stimulated full-length synthesis and increased the length of intermediates. This effect was dependent on the helicase-primase loading factor, UL8, that is known to stimulate primer utilization and is required for optimal activity of the enzyme on ICP8-coated DNA templates (19, 37, 38). DnaB helicase-independent RDR has also been described for *E. coli* (39). In that system as in ours, replication fork progression is presumably driven by the relaxation activity of DNA gyrase (*E. coli* system) or Topo I (HSV-1 system). It should be noted that the dependence of RDR on Topo I is presumably a reflection of the use of a covalently
closed circular DNA template (pUC18). Considering that the HSV-1 genome is now believed to replicate as a linear molecule (5), we would not expect dependence on topoisomerase but rather a necessity for the helicase-primase. This notion is supported by the observation that the viral pol by itself is not capable of SDS (40).

The requirement for species-specific protein-protein interactions during DNA replication has been previously examined in several contexts. Replication of SV40 and polyoma virus is dependent on their cognate TAgS and extracts derived from permissive cells (primate for SV40 and murine for polyoma virus) (41). In addition, species-specific interactions between hRP-A and human pol α have been shown to be required for efficient initiation and elongation of SV40 DNA (42). Reconstitution of SV40 origin containing plasmid replication with highly purified Drosophila melanogaster replication proteins (pol α and RP-A) showed that replacement of Drosophila RP-A with hRP-A or E-SSB reduced replication efficiency (43). The importance of species-specific protein-protein interactions has also been highlighted by the requirement of a functional “coupling” between the pol and helicase during SDS in T4, where neither Klenow fragment of E. coli pol I nor T7 DNA pol could carry out SDS in conjunction with the T4 helicase (44).

Our experiments were aimed at examining the requirement for species-specific protein-protein interactions during HSV-1 RDR. In addition, because the majority of the experiments were performed using purified D-loops that resemble a replication bubble, the results may also reflect the situation during normal replication fork progression.

We had shown previously that ICP8 was not only active as the pairing protein during RDR but also required as an SSB in the DNA synthesis phase of the reaction (16).
D-loops as templates, we examined whether heterologous SSBs could substitute for ICP8 in the synthesis phase of the reaction. Surprisingly, E-SSB, T4 gp32, and RP-A all substituted for ICP8, albeit slightly less efficiently and with the accumulation of shorter intermediates. In contrast, T7 gp2.5, albeit capable of stimulating its cognate pol, failed to substitute as an SSB in our reaction. These results indicate that with the exception of gp2.5, heterologous SSBs can substitute for ICP8 at the replication fork, indicating that species-specific protein-protein interactions between the SSB and other replisome components are not crucial in HSV-1. This notion is also supported by the previous observation that E-SSB was capable of stimulating the HSV-1 pol during the extension of singly primed M13 ssDNA (45). However, it is contrary to previous observations that ICP8 specifically interacts with and stimulates the viral helicase-primase (19, 37, 38). Interestingly, none of the SSBs, even at supersaturating concentrations, were capable of eliminating the pause sites that presumably arise due to stalling of the replisome at sites of secondary structure (46).

Helicases provide an essential role at the replication fork by unwinding the DNA duplex ahead of the pol. In other systems, such as E. coli and T4, important interactions between helicase and pol have been described that optimize the rate of DNA synthesis and couple leading and lagging strand synthesis (47, 48). However, no appreciable effect of the helicase on pol action has been found in the HSV-1 system (49). Moreover, RDR in our system is helicase-independent, although its presence increased the overall efficiency and length of DNA synthesis. In this regard, it is not surprising that heterologous helicases (T7 gp4 and SV40 Tag) and the HSV-1 UL9 helicase could substitute for the HSV-1 helicase-primase, regardless of their polarities. It is noteworthy that UL9 has been shown to interact with the HSV-1 pol via the UL42 subunit, possibly to enable SDS (50).

In contrast to the aforementioned helicases, Dda from T4, a 5′−3′ helicase that performs roles in replication initiation and recombination, could not substitute for the HSV-1 helicase-primase. Its presence prevented primer extension. This observation may be due to the ability of Dda to destroy D-loops that are the templates for RDR. This is supported by the observation that Dda inhibits homologous pairing by unwinding heteroduplexes (51).

Pols fulfill a key role at the replication fork inasmuch as they perform the actual DNA synthesis. Interactions between the pol, SSB, and helicase are required for optimal and coordinated DNA synthesis at a replication fork (36, 47, 48). We examined whether the dimeric HSV-1 pol could be replaced with replicative pols from other systems. We found that although the T7 pol was just as efficient in promoting RDR in reactions with ICP8 and the HSV-1 helicase-primase, E. coli pol III was incapable of extensive primer extension in the same context. Xu and Marians (39) have observed that exonuclease-deficient T7 pol could substitute for the cognate pol (i.e. pol III) in the E. coli RDR system. The inability of pol III, even in the presence of its processivity factor (β subunit), to generate significant extension products in our system indicates the existence and requirement for specific protein-protein interactions. Collectively, these results indicate that although the optimal combination of proteins to perform RDR was that of the HSV-1 factors, the viral replication fork appears to be promiscuous because SSBs, helicases, and pols from other systems could substitute for the HSV-1 factors, albeit less efficiently.

We also examined whether a heterologous replisome, i.e. a complete set of replication proteins, in this case that of T7, could promote RDR both on preformed D-loops as well as in the context of an ongoing reaction in which replisome assembly and DNA synthesis occurred on D-loops formed by ICP8. We observed that RDR by the T7 replisome on preformed D-loops was faster than that performed by the HSV-1 replisome and also resulted in long chain SDS, consistent with previous reports (33) on the products of the T7 replisome. Moreover, the reaction performed by the T7 replisome was dependent on interactions between its cognate factors. Substitution of T7 SSB with ICP8 only led to the synthesis of unit length products, and substitution of the T7 helicase-primase and pol with their HSV-1 counterparts resulted in limited primer extension. The T7 replisome could also promote RDR in ongoing reactions during which D-loop formation by ICP8 was coupled to RDR. However, in the reverse experiment, in which D-loops were formed by the annealing activity of T7 gp2.5 (34), only the cognate combination of factors and not the HSV-1 replisome could promote RDR, further indicating a dependence on protein-protein interactions among the T7 factors. It should also be noted that our experiments with the T7 proteins show for the first time the ability of gp2.5 to form D-loops and for the T7 pol and helicase-primase to utilize these structures as templates for DNA synthesis.

In summary, RDR performed by the HSV-1 replication proteins is dependent on the viral processivity factor (UL42) and the relaxation of the topologically restrained template by Topo I. Although not strictly dependent on helicase action, RDR is stimulated by the viral helicase-primase in a manner that requires the helicase-primase loading protein UL8. We found that although the optimal combination of proteins for RDR was that of the HSV-1 proteins, with certain exceptions products were formed when the HSV-1 factors were replaced with functional counterparts from other systems. In contrast to the promiscuity exhibited by the HSV-1 system, RDR performed by the T7 replisome exhibited dependence on T7 proteins presumably due to the existence of specific protein-protein interactions among these factors.

REFERENCES
1. Boehmer, P. E., and Villani, G. (2003) Prog. Nucleic Acids Res. Mol. Biol. 75, 139–171
2. Boehmer, P. E., and Nimonkar, A. V. (2003) IUBMB Life 55, 13–22
3. Severini, A., Scrob, D. G., and Tyrrell, D. L. (1996) J. Virol. 70, 3169–3175
4. Enquist, L. W., and Skalka, A. (1973) J. Mol. Biol. 75, 185–212
5. Jackson, S. A., and DeLuca, N. A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7871–7876
6. Louder, A., and Mosig, G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1101–1105
7. Richardson, C. C. (1983) Cell 33, 315–317
8. Smolley, J. R., Wagner, M. J., Summers, W. P., and Summers, W. C. (1980) Virology 102, 83–93
9. Wohlrab, F., Chatterjee, S., and Wells, R. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6432–6436
10. Huang, K. P., Zinellman, B. V., and Lehrman, I. R. (2002) J. Biol. Chem. 277, 21071–21079
11. Valsly-Nagy, N., Olson, S. J., Valys-Nagy, K., Montine, T. J., and Dermody, T. S. (2000) Virology 278, 309–321
12. Milatovic, D., Zhang, Y., Olson, S. J., Montine, K. S., Roberts, L. J., H. Morrow, J. D., Montine, T. J., Dermody, T. S., and Valys-Nagy, T. (2002) J. Neurovirol. 8, 205–209
13. Skaliter, R., and Lehrman, I. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10665–10669
14. Bakkin, S. D., and Hanlon, B. (1990) J. Virol. 64, 4957–4967
15. Falkenberg, M., Lehrman, I. R., and Elias, P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3986–3990
16. Nimonkar A. V., and Boehmer, P. E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10201–10206
17. Boehmer, P. E., and Lehrman, I. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8444–8448
18. Sampson, D. A., Arana, M. E., and Boehmer, P. E. (2000) J. Biol. Chem. 275, 2931–2937
19. Tanguy Le Gac, N., Villani, G., Hoffmann, J. S., and Boehmer, P. E. (1996) J. Biol. Chem. 271, 21645–21651
20. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
21. Boehmer, P. E. (1996) Methods Enzymol. 275, 16–35
22. Boehmer, P. E., Dodson, M. S., and Lehrman, I. R. (1998) J. Biol. Chem. 273, 1220–1225
23. Nimonkar, A. V., and Boehmer, P. E. (2003) Nucleic Acids Res. 31, 5275–5281
24. Gourselles, A. S., Tanguy Le Gac, G., Villani, G., Boehmer, P. E., and Johnson, N. P. (2000) J. Biol. Chem. 275, 10894–10899
25. Kim, C., Paulus, B. F., and Wold, M. S. (1994) Biochemistry 33, 14197–14206
26. Bujalski, W., and Lehrman, T. M. (1986) Biochemistry 25, 7799–7802
27. Jensen, D. E., Kelly, R. C., and von Hippel, H. P. (1976) J. Biol. Chem. 251, 7215–7228
28. Kim, Y. T., Tabur, S., Bortner, C., Griffith, J. D., and Richardson, C. C. (1992)
29. Jongeneel, C. V., Bedinger, P., and Alberts, B. M. (1984) *J. Biol. Chem.* 259, 12933–12938
30. Park, K., Debyser, Z., Tabor, S., Richardson, C. C., and Griffith, J. D. (1998) *J. Biol. Chem.* 273, 5260–5270
31. Fairman, M., Prelich, G., Tsurimoto, T., and Stillman, B. (1988) *Biochem. Biophys. Acta* 951, 362–367
32. Makhov, A. M., Lee, S. S., Lehman, I. R., and Griffith, J. D. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 898–903
33. Nakai, H., and Richardson, C. C. (1988) *J. Biol. Chem.* 263, 9831–9839
34. Kong, D., and Richardson, C. C. (1998) *EMBO J.* 17, 2010–2019
35. Hernandez, T. R., and Lehman, I. R. (1990) *J. Biol. Chem.* 265, 11227–11232
36. Lee, J., Chastain, P. D., II, Griffith, J. D., and Richardson, C. C. (2002) *Mol. Biol.* 316, 19–34
37. Falkenberg, M., Bushnell, D. A., Elias, P., and Lehman, I. R. (1997) *J. Biol. Chem.* 272, 22766–22770
38. Hamatake, R. K., Bifano, M., Hurlburt, W. W., and Tenney, D. J. (1997) *J. Gen. Virol.* 78, 857–865
39. Xu, L., and Marians, K. J. (2002) *J. Biol. Chem.* 277, 14321–14328
40. Zhu, Y., Trego, K. S., Song, L., and Parris, D. S. (2003) *J. Virol.* 77, 10147–10153
41. Wehbe, C. R., Dean, F. B., Murakami, Y., Borowiec, J. A., Bullock, P., and Hurwitz, J. (1987) *Philos. Trans. R. Soc. Lond-Biol. Sci.* 317, 439–453
42. Brill, S. J., and Stillman, B. (1989) *Nature* 342, 92–95
43. Kamakaka, R. T., Kaufman, P. D., Stillman, B., Mitsu, P. G., and Kadonaga, J. T. (1994) *Mol. Cell. Biol.* 14, 5114–5122
44. Delagoutte, E., and von Hippel, P. H. (2001) *Biochemistry* 40, 4459–4477
45. O’Donnell, M. E., Elias, P., and Lehman, I. R. (1987) *J. Biol. Chem.* 262, 4252–4259
46. Fermosa, T., and Alberts, B. M. (1986) *Cell* 47, 793–806
47. Mariano, K. J. (1992) *Annu. Rev. Biochem.* 61, 673–719
48. Miller, E. S., Kutter, E., Mosig, G., Arisaka, F., Kuniawa, T., and Ruger, W. (2003) *Microbiol. Mol. Biol. Rev.* 67, 86–156
49. Falkenberg, M., Elias, P., and Lehman, I. R. (1998) *J. Biol. Chem.* 273, 32154–32157
50. Trego, K. S., and Parris, D. S. (2003) *J. Virol.* 77, 12646–12659
51. Kodadek, T. (1991) *J. Biol. Chem.* 266, 9712–9718