THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 48, Issue of November 27, pp. 32154–32157, 1998

Printed in U.S.A.

The Herpes Simplex Virus Type 1 Helicase-primase

ANALYSIS OF HELICASE ACTIVITY*

(Received for publication, July 10, 1998, and in revised form, September 11, 1998)

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The rate of unwinding of duplex DNA by the herpes simplex virus type 1 (HSV-1)-encoded helicase-primase (primosome) was determined by measuring the rate of appearance of single strands from a circular duplex DNA containing a 40-nucleotide 5′ single-stranded tail, *i.e.* a preformed replication fork, in the presence of the HSV-1 single strand DNA-binding protein, infected cell protein 8 (ICP8). With this substrate, the rate at low ionic strength was highly sensitive to Mg^{2+} concentration. The Mg^{2+} dependence was a reflection of both the requirement for ICP8 for helicase activity and the ability of ICP8 to reverse the helicase reaction as a consequence of its capacity to anneal homologous single strands at Mg^{2+} concentrations in excess of 3 mM. The rate of unwinding of duplex DNA by the HSV-1 primosome was also determined indirectly by measuring the rate of leading strand synthesis with a preformed replication fork as template in the presence of the T7 DNA polymerase. The value of 60–65 base pairs unwound/s by both methods is consistent with the rate of 50 base pairs/s estimated for the rate of fork movement *in vivo* during replication of pseudorabies virus, another herpesvirus. Interaction with the helicase-primase did not increase its helicase activity.

The 152-kb[1] genome of herpes simplex virus type 1 (HSV-1) encodes three enzymes, in addition to a single strand DNA-binding protein, that are required for its replication (1, 2). These include a heterodimeric, highly processive DNA polymerase (3–6), an origin-binding protein with 3′-5′-helicase activity (7–9), and a heterotrimeric primosome with both helicase and primase activities (10–13). Earlier studies of the helicase activity of the helicase-primase showed its rate of unwinding of duplex DNA substrates to be 2 bp/s (12), a rate far below the 50 bp/s estimated for the rate of replication of pseudorabies virus, a related herpesvirus (14).

The HSV-1 helicase-primase very likely exists in association with other HSV-1-encoded enzymes as part of a replisome (15, 16). Several structural and functional interactions between the components of the putative replisome have been described (1). For example, the single strand DNA-binding protein, infected cell protein 8 (ICP8) interacts with the UL8 subunit of the helicase-primase (17). It was recently demonstrated that the catalytic subunit of the HSV-1 DNA polymerase also interacts with the UL8 subunit (18). In light of these observations, we undertook an examination of the effect of the HSV-1-encoded DNA polymerase on the helicase rate. Studies of the *Escherichia coli* DnaB helicase have shown its rate to be strongly influenced by its association with its cognate DNA polymerase III holoenzyme (19). We show here that the helicase activity of the HSV-1 helicase-primase in the presence of the HSV-1 single strand DNA-binding protein ICP8 is extraordinarily sensitive to reaction conditions, and at the appropriate Mg^{2+} conditions and ionic strength, the rate approaches the rate of replication fork movement *in vivo*. However, this rate is not significantly influenced by its association with the HSV-1 DNA polymerase.

MATERIALS AND METHODS

Enzymes—The helicase-primase, DNA polymerase-UL42 protein, and ICP8 were purified by previously described procedures (17).

DNA Helicase Substrates—The formation of the 20-bp helicase substrate (Fig. 1, Structure *A*) has been described previously (17). The circular duplex DNA substrate containing a preformed replication fork was generated by annealing a 60-base oligonucleotide (15 pmol, 5′-ACATGATAAGATACATGGATGAGTTTGGACAAACCACAACGTAAAACGACGGCCAGTGCC-3′) to 5 pmol of M13mp18 single-stranded DNA (Biolabs) to generate a 20-bp double-stranded region with a 40-nucleotide unpaired 5′ tail. The single-stranded circle was converted to the duplex form (Fig. 1, Structure *B*) by incubating the tailed M13mp18 single stranded DNA (10 pmol) with ICP8 (1 nmol) and HSV-1 DNA polymerase (100 pmol) in a reaction mixture (500 μl) containing 20 mM Tris-HCl, pH 7.3, 10% glycerol, 4 mM dithiothreitol, 0.5 mM ATP, 200 μg/ml bovine serum albumin, 4.5 mM $MgCl_2$, 0.1 mM dATP, dCTP, dGTP, and dTTP, and 50 mM NaCl. Incubation was at 37 °C for 90 min, and the reaction was stopped by the addition of 125 μl of 2% SDS and 0.8 mg/ml proteinase K, followed by further incubation at 37 °C for 30 min.

Linear double stranded DNA was generated by cleaving plasmid pTZ18r (Amersham Pharmacia Biotech) with the *Eco*RI and *Sma*I restriction enzymes to yield a linear double-stranded DNA, 2860 bp in length, containing a 4-base overhang at the 5′ end and a 3′ blunt end. Both the circular duplex DNA with a preformed replication fork and the linear pTZ18r DNA were extracted first with phenol/chloroform and then with chloroform and precipitated by the addition of ammonium acetate to 2.5 M and 2.5 volumes of ethanol. The DNAs were kept at 4 °C for 30 min, centrifuged, washed with ice-cold 70% ethanol, dried, and dissolved in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.

Assay of DNA Helicase Activity—The reaction mixture (20 μl) contained either 0.1 μg (21 fmol) of circular duplex M13mp18 DNA with a preformed replication fork (Fig. 1, Structure *B*) or 0.1 μg (48 fmol) of linear pTZ18r, 20 mM Tris-HCl, pH 7.6, 10% glycerol, 3 mM dithiothreitol, 3 mM ATP, 100 μg/ml bovine serum albumin, and the indicated concentration of $MgCl_2$. Twenty pmol of ICP8 and 1 pmol of helicase-primase were added. Incubation was at 34 °C for the times indicated and stopped by addition of 4 μl of stop solution (90 mM EDTA, pH 8.0, 6% SDS, 30% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol). The products were separated by electrophoresis at 150 V for 3 h through

* This work was supported in part by National Institutes of Health Grant AI-26538 (to I.R.L.) and Swedish Cancer Society Grant 2552-B97-11XAC (to P.E.). 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.

¶ Supported by a grant from the Swedish Institute.

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[1] The abbreviations used are: kb, kilobase(s); bp, base pair(s); HSV-1, herpes simplex virus type 1; ICP8, infected cell protein 8.

an 0.8% agarose gel with Tris borate/EDTA (89 mM Tris borate, 1 mM EDTA) containing 1.0 μg/ml ethidium bromide. The DNA was denatured by submerging the gel in 900 ml of denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 30 min with gentle agitation. The gel was rinsed with water for 5 min and then neutralized by gentle agitation in 900 ml of neutralization buffer (1.5 M NaCl, 1 M Tris-HCl, pH 7.4) for 30 min. The DNA was transferred by a Hybond-N nylon membrane (Amersham Pharmacia Biotech) using 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.5) overnight. It was covalently bound to the filter by UV irradiation and then preincubated for 60 min at 65 °C with 50 ml of prehybridizing buffer (5 × SSC, 0.5% SDS, 0.1 g of bovine serum albumin, 0.1 g of Ficoll 400, 0.1 g of polyvinylpyrrolidone) before adding a randomly primed probe (Stratagene Prime-it II) for M13mp18 or pTZ18r. After incubation overnight, the filter was washed for 30 min with 0.1% SDS and l × SSC at 65 °C. The labeled hybrids were visualized by autoradiography. Measurement of helicase activity with Substrate A (Fig. 1) was performed as described previously (17).

Measurement of Leading Strand Synthesis—The reaction mixture (50 μl) contained 30 mM Hepes-NaOH, pH 7.6, 3 mM dithiothreitol, 8 mM magnesium acetate, 4 mM ATP, 100 μM dATP, dGTP, and dTTP, 10 μM dCTP, 5 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), 250 μM CTP, 250 μM GTP, 250 μM UTP, 40 mM creatine phosphate, 5 μg of creatine kinase, and 40 fmol of M13mp18 circular duplex DNA with a preformed replication fork (Fig. 1, Structure *B*). Where indicated, 1 pmol of helicase-primase, 10 pmol of ICP8, 500 fmol of HSV-1 DNA polymerase-UL42 protein, or 0.01 unit of T7 DNA polymerase was added. Incubation was at 37 °C for the times indicated and stopped by the addition of 5 μl of 8% SDS and 2.4 μg/ml proteinase K, followed by incubation for 30 min at 37 °C. Twelve μl of denaturing gel loading buffer (50 mM NaOH, 1 mM EDTA, 0.25% bromphenol blue, 0.25% xylene cyanol) and 1 μl of 0.5 M EDTA were added before loading half the reaction mixture onto a 0.7% denaturing agarose gel (50 mM NaOH, 1 mM EDTA). The gels were run at 1 V/cm for 20 h, dried, and autoradiographed with an intensifying screen.

Measurement of DNA Polymerase Activity—The reaction mixture (50 μl) contained 30 mM Hepes-NaOH, pH 7.6, 3 mM dithiothreitol, 8 mM magnesium acetate, 4 mM ATP, 100 μM dATP, dGTP, and dTTP, 10 μM dCTP, 5 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), and 20 fmol of tailed M13 mp18 single-stranded DNA (Fig. 1, Structure *A*). Ten pmol of ICP8 and 500 fmol of HSV-1 DNA polymerase-UL42 protein were added. Incubation was at 37 °C. Twelve μl of stop solution (90 mM EDTA, pH 8.0, 6% SDS, 30% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol) were then added to 25 μl of the reaction mixture, and the samples were analyzed gel electrophoresis in 0.8% agarose gels containing Tris borate/EDTA with 1.0 μg/ml ethidium bromide. The gels were run at 150 V for 3 h. The dried gels were autoradiographed with an intensifying screen.

RESULTS AND DISCUSSION

Effect of Mg^{2+} Concentration on the Helicase Activity of the HSV-1 Helicase-primase—To measure the helicase activity of the HSV-1 helicase-primase, a circular duplex DNA with a preformed replication fork was used (Fig. 1, Structure *B*). Our earlier studies had shown that ICP8 is essential for the unwinding of long stretches of duplex DNA by the helicase-primase, presumably as a consequence of its ability, as a helix-destabilizing protein, to prevent reannealing of the separated single strands (17). Saturating amounts of ICP8 (one ICP8 monomer/12 nucleotides of single-stranded DNA) were therefore included in all reactions with this substrate. Examination of the helicase activity at various ionic strengths showed 20 mM NaCl to be optimal (data not shown). However, as shown in Fig. 2*A*, in the presence of 3 mM ATP unwinding was observed only at a narrow range of Mg^{2+} concentrations, between 1.0 and 3.5 mM.

The narrow range of Mg^{2+} concentrations at which helicase activity could be observed prompted us to examine the effect of Mg^{2+} concentration on the other component of the reaction, ICP8. ICP8 can promote the annealing of homologous single strands of DNA (20, 21). Earlier studies had in fact shown this reaction to be sensitive to Mg^{2+} concentration (21). As substrate, the circular duplex DNA with a preformed replication fork, which had been heated to 100 °C for 2 min and then quickly chilled on ice, was used. The experimental conditions, including 3 mM ATP, were identical to those described for the previous experiment. As shown in Fig. 2*B*, the single strands reannealed in the presence of ICP8 at Mg^{2+} concentrations of ≥3.5 mM. At Mg^{2+} concentration <3.5 mM, no ICP8-mediated

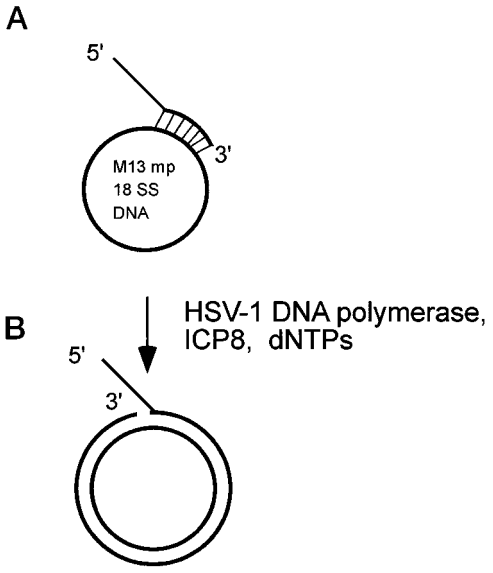

FIG. 1. **Helicase substrates.** The circular duplex DNA with a preformed replication fork was generated by annealing a 60-base oligonucleotide to single-stranded M13 mpl8 DNA (Structure *A*). The 20-bp duplex region was then extended by reaction with HSV-1 DNA polymerase-UL42 protein and ICP8 (Structure *B*). See "Materials and Methods" for details.

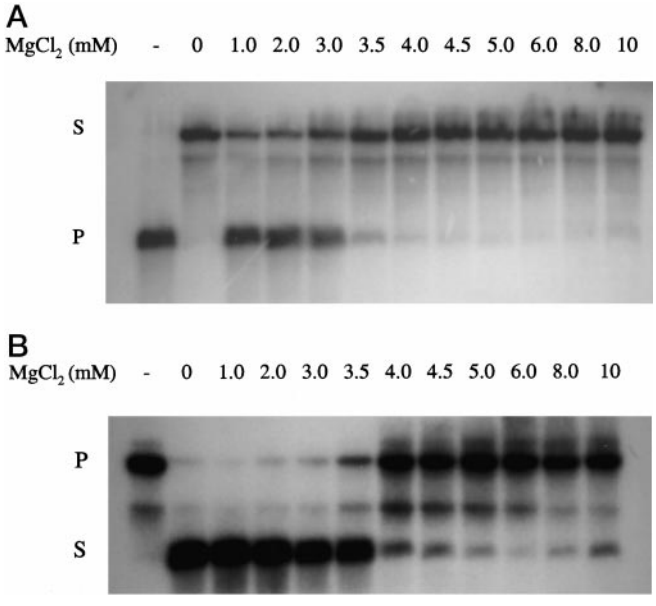

FIG. 2. **Effect of Mg^{2+} concentration on the helicase activity of the helicase-primase in the presence of ICP8.** Reaction mixtures were prepared as described under "Materials and Methods," except that increasing amounts of $MgCl^{2+}$ were added as indicated. *A*, helicase activity of the helicase-primase on the circular duplex DNA with a preformed replication fork (Fig. 1, Structure *B*). Twenty pmol of ICP8 and 1 pmol of helicase-primase were added to each reaction. *First lane*, substrate heated to 100 °C and then chilled. *B*, reannealing of separated single strands. Structure B was heated to 100 °C and then rapidly chilled. Twenty pmol of ICP8 were added to each reaction mixture. The products were subjected to 0.8% agarose gel electrophoresis, transferred to a nylon filter, and hybridized with a random probe as described under "Materials and Methods." *First lane*, untreated substrate. *S*, substrate; *P*, product.

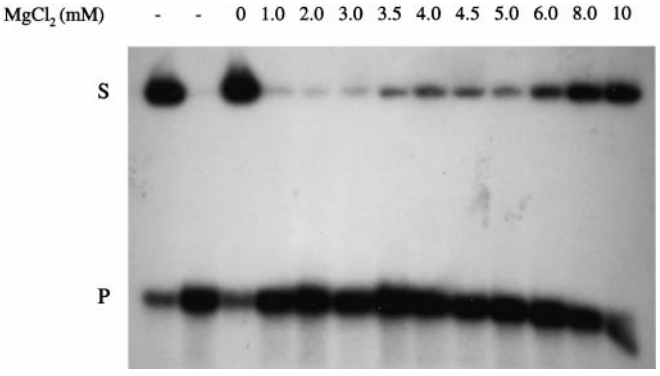

FIG. 3. **The helicase activity of helicase- primase in the absence of ICP8 is independent of Mg^{2+} concentration.** Reaction mixtures were prepared as described under "Materials and Methods," except that 500 fmol of helicase-primase were added and increasing amounts of $MgCl_2$ were added as indicated. The products were subjected to 12% polyacrylamide gel electrophoresis as described under "Materials and Methods." *S*, substrate; *P*, product. *First lane*, untreated substrate; *second lane*, substrate heated to 100 °C and then chilled.

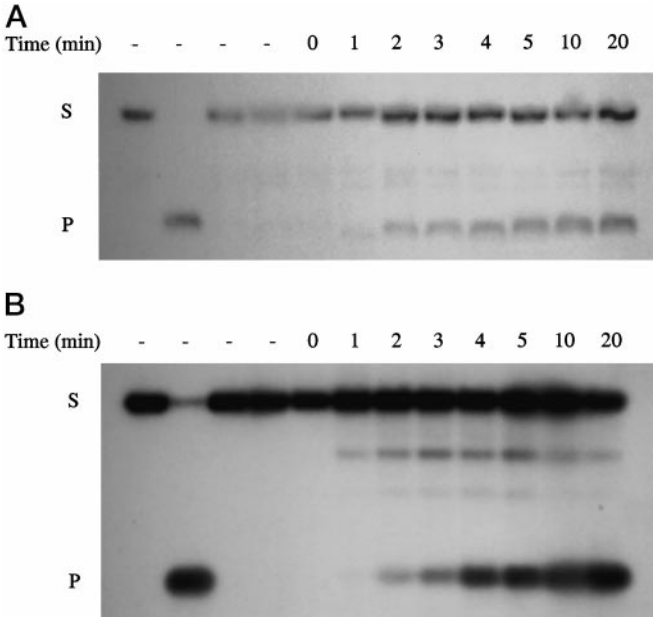

FIG. 4. **Measurement of helicase rate of the HSV-1 helicase-primase.** Reaction mixtures were prepared as described under "Materials and Methods." Unless otherwise indicated, all reactions contained 1 pmol of helicase-primase and 20 pmol of ICP8. *A*, helicase assay using circular duplex DNA with a preformed replication fork (Fig. 1, Structure *B*) as substrate. *B*, helicase assay using linear double-stranded DNA (pTz18r) as substrate. The products were subjected to 0,8% agarose gel electrophoresis, transferred to a nylon filter, and hybridized with a random probe as described under "Materials and Methods." *First lane*, untreated substrate; *second lane*, substrate heated to 100 °C and then chilled; *third lane*, helicase-primase incubated for 20 min without ICP8; *fourth lane*, ICP8 incubated for 20 min without helicase-primase. *S*, substrate; *P*, product.

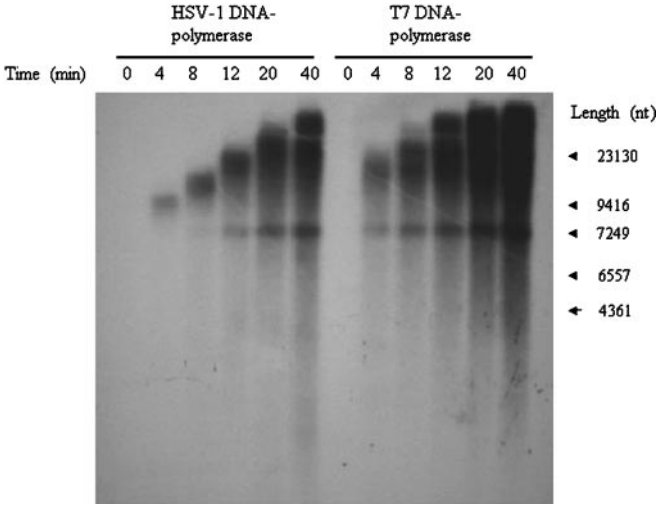

FIG. 5. **Leading strand DNA synthesis by T7 DNA polymerase and HSV-1 DNA polymerase-UL42 protein in the presence of helicase-primase.** Reaction mixtures were prepared as described under "Materials and Methods." Samples (50 μl) were removed at the times indicated and analyzed by 0.7% denaturing agarose gel electrophoresis as described under "Materials and Methods." Size markers were nonradioactive phage λ DNA cleaved with the *Hin*dIII or *Kpn*I restriction enzymes. *nt*, nucleotides.

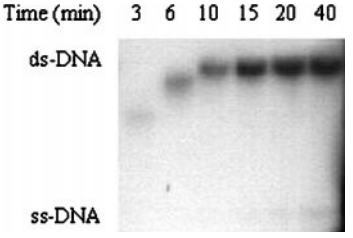

FIG. 6. **Rate of the HSV-1 DNA polymerase.** The reaction mixture was prepared as described under "Materials and Methods." Samples (50 μl) were removed at the times indicated and analyzed by 0.8% denaturing agarose gel electrophoresis as described under "Materials and Methods." *ssDNA*, position of single-stranded circular M13mp18 DNA; *dsDNA*, position of fully double-stranded circular M13mp18 DNA.

reannealing was observed. The reaction was dependent on ICP8, because no reannealing of the single strands was observed in an identical experiment performed in the absence of ICP8 (results not shown). The range of Mg^{2+} concentrations at which ICP8 could promote the reannealing of single strands coincided with the concentrations at which no helicase activity could be detected. It therefore appears that the apparent inhibition of helicase activity at Mg^{2+} concentrations >3.5 mM is simply a consequence of the reannealing of the product single strands under these conditions. In contrast, with the 20-bp helicase substrate (Fig. 1, Structure *A*) for which ICP8 is not required (17), helicase activity was observed at all Mg^{2+} concentrations tested (Fig. 3).

The HSV-1 Helicase-primase Can Unwind Duplex DNA at the Rate of 60 bp/s—To estimate the rate of DNA unwinding catalyzed by the HSV-1 helicase-primase, we performed the reaction under conditions that were found to be optimal for helicase activity (20 mM NaCl, 3 mM Mg^{2+}, 3 mM ATP, saturating ICP8). The molar ratio of enzyme to substrate was 40:1 for the circular substrate and 20:1 for the linear substrate. The complete unwinding of the circular duplex with a preformed replication fork (7240 bp) occurred in 2 min (Fig. 4*A*). The rate of unwinding was therefore 60 bp/s. This value is sufficient to support the rate of DNA replication observed *in vivo* with pseudorabies virus.

With a linear duplex substrate (linear pTZ18r DNA), the rate was 24 bp/s (Fig. 4*B*). The lower rate is presumably attributable to the lack of a sufficiently long single-stranded loading site for the helicase-primase.

As an alternative approach to measurement of the rate of unwinding of duplex DNA by the helicase-primase, the rate of leading strand DNA synthesis by the T7 DNA polymerase coupled to the helicase action of the helicase-primase in the absence of ICP8 was determined. This method depends on the inability of the T7 DNA polymerase to replicate through the regions of duplex DNA (22, 23) and has the advantage of being

independent of the reannealing reaction, because DNA synthesis and unwinding are closely coordinated.

Because a functional interaction between the HSV-1 helicase-primase and the T7 DNA polymerase is unlikely, the rate of the T7 DNA polymerase-catalyzed leading strand synthesis should reflect the rate of unwinding of the DNA duplex by the helicase-primase. We found that at 4 min the 7-kb substrate had been extended by the T7 DNA polymerase to yield products of ~23 kb. The rate of unwinding was calculated to be 65 bp/s, in excellent agreement with the direct measurements of helicase activity (60 bp/s) (Fig. 5).

The HSV-1 DNA Polymerase Does Not Stimulate the Helicase Activity of the Helicase-primase—Studies of the dnaB helicase of *E. coli* have shown it to be markedly stimulated by its interaction with the DNA polymerase III holoenzyme (19). We therefore wished to determine whether there was a comparable stimulation of the helicase-primase by the HSV-1 DNA polymerase. In a reaction with helicase-primase, the DNA polymerase-UL42 protein, and ICP8, leading strand synthesis was observed (Fig. 5). Under these conditions we found no evidence for lagging strand synthesis. With the HSV-1 DNA polymerase-UL42 protein, 12 min were required to extend the 7-kb substrate to a length of ~23 kb. The rate was therefore 20 bp/s. This rate was similar to the rate of DNA synthesis by the DNA polymerase-UL42 protein in the presence of ICP8 with a primed M13mp18 single stranded DNA template (Figs. 1, Structure *A*, and 6). In this instance the synthesis of the 7.2-kb duplex DNA was completed within 10 min. Thus, leading strand synthesis by the DNA polymerase does not enhance the rate of unwinding of a DNA duplex by the helicase-primase. Moreover, deoxynucleotide polymerization by the HSV-DNA polymerase appears to be rate-limiting during leading strand synthesis.

Thus far we have not found evidence for a coupled leading and lagging strand synthesis in these reactions. Possibly additional factors are required to promote synthesis of the lagging strand.

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