DNA Helicase E and DNA Polymerase ϵ Functionally Interact for Displacement Synthesis†

John J. Turchi,‡,§ Gregg Siegal,‡ and Robert A. Bambara*,‡,§

Department of Biochemistry and Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

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ABSTRACT: A functional interaction between DNA helicase E and DNA polymerase ϵ from calf thymus has been detected which results in the extension of an upstream 3′ OH through a downstream primer to the end of a synthetic template. DNA synthesis resulting in full-length extension products was dependent on the addition of DNA helicase E and hydrolysis of ATP, suggesting that displacement of the downstream primer was required. Identical reactions using DNA polymerases α and δ in place of DNA polymerase ϵ showed no full-length products dependent on helicase E, indicating that polymerases α and δ were incapable of functionally interacting with the helicase. The reaction leading to full-length extension products was time dependent and dependent on the concentration of added polymerase ϵ and helicase E. Exonucleolytic degradation of the downstream primer, or ligation of the downstream primer to the upstream 3′ OH, were not responsible for the full-length products observed. Displacement of the downstream primer by DNA helicase E was not affected by the addition of polymerase ϵ to the reactions. Template dilution experiments demonstrated that DNA polymerase ϵ and helicase E were acting in concert to perform displacement synthesis. Additional evidence for functional coordination was obtained by demonstration that DNA helicase E stimulated DNA polymerase ϵ in a standard DNA synthetic assay using dA_{3000}·dT_{16} as the template–primer. The results presented are consistent with the hypothesis that DNA helicase E and DNA polymerase ϵ are capable of coordinated activities that result in displacement synthesis. A functional interaction of this sort may be involved at the eukaryotic replication fork or in DNA repair.

DNA helicases are required in many facets of DNA and RNA metabolism including replication, repair, recombination, and termination of transcription (Thömmes & Hübscher, 1990a; Matson, 1991; Matson & Kaiser-Rogers, 1990). Eukaryotic chromosomal DNA replication requires a helicase activity at each replication fork to separate the duplex DNA strands prior to their replication. This yet unidentified eukaryotic helicase presumably interacts with the DNA polymerases responsible for DNA replication. It has recently been demonstrated in yeast that DNA polymerase α (pol α)[1] (Johnson et al., 1985), pol δ (Boulet et al., 1989), and pol ϵ (Morrison et al., 1990) are each encoded by separate genes, all of which are required for viability. Deletion mutants constructed for each DNA polymerase arrest in early S phase, suggesting that each polymerase, α (Sitney et al., 1989), δ (Boulet et al., 1989), and ϵ (Morrison et al., 1990), is required for chromosomal DNA replication. The helicase responsible for strand separation at the replication fork might, therefore, interact with one or more of these DNA polymerases.

SV40 DNA replication in vitro has been a useful system to identify and study proteins involved in chromosomal DNA replication, since the only viral protein required is the multifunctional large T antigen (Wobbe et al., 1987; Fairman et al., 1988). Dornreiter et al. (1990) have demonstrated a physical complex between DNA pol α and SV40 large T antigen utilizing ELISA and immunoprecipitation assays and have mapped the domain of contact to the N-terminal 83 amino acids of large T antigen. This suggests that the eukaryotic analog of large T antigen may also physically interact with pol α. The problem in identifying the helicases that participate at the replication fork using the SV40 in vitro replication system is that the intrinsic helicase activity of large T antigen is capable of performing the strand separation reaction required for fork movement and replication (Mastrangelo et al., 1989; Goetz et al., 1988; Dean et al., 1987). Consequently, there has been no systematic approach to identify these cellular helicases.

In DNA excision repair, displacement of a damaged DNA strand by a helicase is followed by DNA synthesis using the complementary strand as a template (Sancar & Sancar, 1988). A functional complex between a helicase and polymerase has been identified in *Escherichia coli*. Excision repair in *E. coli* requires DNA helicase II (UvrD) to displace the damaged DNA strands following nicking by UvrABC (Runyon et al., 1990). Pol I then fills in the resulting 12-base gap (Sibghat-Ullah et al., 1990). Interestingly, the UvrAB complex is also capable of displacing short DNA oligonucleotides (Oh & Grossman, 1989; Seeley & Grossman, 1990). Caron et al. (1985) suggested that a complex of UvrBCD and pol I exists as a "repairosome". Short-patch excision repair in eukaryotes has been modeled with pol β and exonuclease V (Mosbaugh & Linn, 1983; Randahl et al., 1988). It has been suggested that pol ϵ (Nishida et al., 1988) or pol α (Mosbaugh & Linn, 1984) may participate in long-patch repair. There was no requirement for helicase activity in either of these model systems.

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* Corresponding author: Department of Biochemistry, University of Rochester Medical Center, Box 607, 601 Elmwood Ave., Rochester, NY 14642.

‡ Department of Biochemistry.

§ Cancer Center.

[1] Abbreviations: pol, DNA polymerase; hel, DNA helicase; PCNA, proliferating cell nuclear antigen; RF-C, replication factor C; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; IPD, interprimer distance; ATP-γS, adenosine 5′-[γ-thio]triphosphate; ELISA, enzyme-linked immunosorbent assay.

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We recently have purified and partially characterized a DNA helicase (hel E) from calf thymus (Siegal et al., 1992b). Hel E is a 104-kDa monomer capable of displacing a deoxynucleotide primer from single-stranded M13mp18 DNA. It translocates in the 3′ to 5′ direction with respect to the template strand and preferentially utilizes (d)ATP as a source of energy. These physical and enzymological characteristics distinguish hel E from the other eukaryotic DNA helicases reported to date (Thömmes et al., 1992; Zhang & Grosse, 1991; Tuteja et al., 1990, 1991).

DNA hel E was initially identified as an activity present in preparations of DNA pol ϵ. The purification procedure employed for pol ϵ was based on the DNA pol III holoenzyme purification from *E. coli* (McHenry & Kornberg, 1977). This protocol was designed to minimize disruption of the protein–protein interactions between the subunits that make up the pol III holoenzyme. Our initial report on the purification of hel E demonstrated partial coelution of DNA pol ϵ with hel E over the chromatographic matrices used for the purification (Siegal et al., 1992b). These data suggest that hel E may form a loose physical complex with DNA pol ϵ. In this report we have characterized the functional coordination between hel E and pol ϵ, resulting in DNA synthesis through a downstream primer dependent on helicase activity, that appears to be specific for pol ϵ. The results presented provide evidence that hel E may participate with pol ϵ at the eukaryotic replication fork or in DNA repair.

EXPERIMENTAL PROCEDURES

Materials. Unlabeled nucleotides were from Pharmacia (Piscataway, NJ) and radiolabeled nucleotides were from New England Nuclear (Boston, MA). ATP-γS was from Boehringer Mannheim (Indianapolis, IN). Synthetic DNA oligonucleotides were purchased, gel purified, from Genosys (Woodlands, TX). DNA-modifying enzymes and Sequenase (version 2.0) were from U.S. Biochemicals (Cleveland, OH). dA_{3000} and dT_{16} were from Midland Scientific (Midland, TX). All other reagents were purchased from standard suppliers.

Enzymes. DNA hel E was purified from calf thymus essentially as previously described to a specific activity of 1500 units/mg (Siegal et al., 1992b). One unit of helicase activity will displace 50% of a 21-oligonucleotide DNA primer from M13mp18 in a 30-min reaction at 37 °C in a reaction containing 10 fmol of template–primer. Pol ϵ was purified from calf thymus to a specific activity of 2000 units/mg (Siegal et al., 1992a). The pol ϵ used in this study had an active-site subunit molecular mass of 145 kDa. This preparation has similar structural and enzymological characteristics of calf thymus pol ϵ described by Focher et al. (1989) and Kesti and Syväoja (1991). DNA pol α was immunoaffinity-purified using an SJK-287 column according to Nasheuer and Grosse (1987) to a specific activity of 400 units/mg. DNA pol δ was purified from calf thymus to 250 units/mg as described by Lee et al. (1984) through step 3, except Bio-Rex-70 chromatography was used as the first column as previously described (Myers & Bambara, 1990). Calf thymus PCNA was kindly supplied by Drs. Antero So and Kathleen Downey (University of Miami). All reactions using pol δ contained 10 μg/mL PCNA. dA_{3000}·dT_{16} was used as the template–primer for pol ϵ and pol δ/PCNA, and activated calf thymus DNA was used for pol α. One unit of polymerase activity incorporated 1 nmol of nucleotide in a 60-min reaction at 37 °C.

DNA Substrates. DNA oligonucleotides were 5′ phosphorylated using [γ-^{32}P]ATP (100 μCi, 3000 Ci/mmol) and

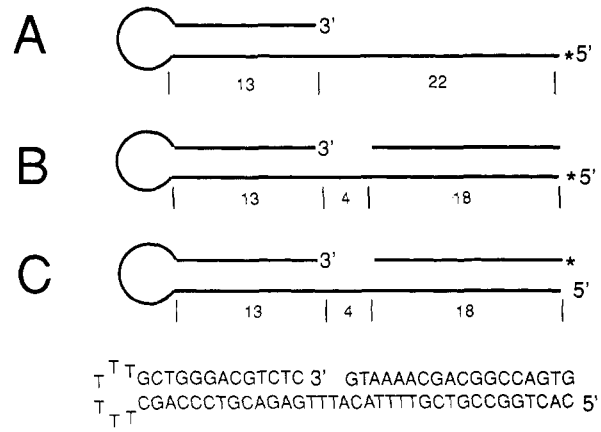

FIGURE 1: DNA substrates used in extension and displacement reactions. The templates consist of a 54-mer oligonucleotide that forms a snapback template–primer molecule with a 13-bp duplex region (template A). Annealing a specific 18-mer to the 5′ end region results in a 4-base gap between the 3′ OH of the snapback and 5′ terminus of the downstream primer (template B). Extension of the 3′ OH up to the downstream primer results in a product 58 bases in length, while extension through the downstream primer to the end of the synthetic template results in a product 76 bases in length. The substrate is labeled on either the 3′ terminus of the downstream primer (template C) or the 5′ terminus of the snapback DNA (templates A and B) as denoted by the asterisk.

T4 polynucleotide kinase. The labeled oligonucleotides were purified using NENsorb columns (NEN, Boston, MA) according to the manufacturer's protocol. dT_{16} was annealed to dA_{3000} in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA at 37 °C for 30 min and cooled to 25 °C over 1 h. The ratio of primer to template was varied to give the appropriate interprimer distance (IPD).

Helicase/Polymerase Interaction Template. To detect extension of the upstream 3′ OH, the 54-mer was 5′ labeled as described above. This was used directly as a snapback template–primer (Figure 1, template A) or an 18-mer, of identical sequence to the (–20) 17-mer universal sequencing primer except for an additional G residue at the 3′ end, was annealed (Figure 1, template B). To detect displacement of the downstream primer, the 3′ terminus of the primer was labeled. The (–20) 17-mer universal sequencing primer was annealed to the 54-mer synthetic snapback DNA at a 2:1 molar ratio at 65 °C for 10 min and then cooled to room temperature over 3 h. The DNA was extended by one base using [α-^{32}P]dGTP (100 μCi, 3000 Ci/mmol) and 5 units of Sequenase at 37 °C for 15 min. The reactions were stopped by the addition of EDTA to a final concentration of 10 mM, and the resulting product was purified by sedimenting through Sephadex G-50 spin columns (Penefsky, 1977) (Figure 1, template C).

Enzyme Reactions. Reactions combining hel E and pol ϵ were run in 20 mM Hepes, pH 7.0, 2 mM $MgCl_2$, 1 mM DTT, and 0.1 pmol of template–primer in a final volume of 20 μL. The reactions were incubated 5 min on ice, initiated by the additions of ATP to 5 mM and each dNTP to 100 μM, and incubated at 37 °C for the times indicated in the figure legends. Preliminary experiments, involving titrating the concentration of ATP in the reactions, demonstrated that 5 mM resulted in optimum displacement of DNA oligonucleotides by hel E. Extension reactions were terminated by the addition of 40 μL of 50% formamide, 6 M urea, 20 mM EDTA, and 0.025% bromophenol blue and xylene cyanol FF. The samples were heated at 95 °C for 5 min and electrophoresed on 8% polyacrylamide/7 M urea sequencing gels according to Sambrook et al. (1989). The gels were dried and subjected

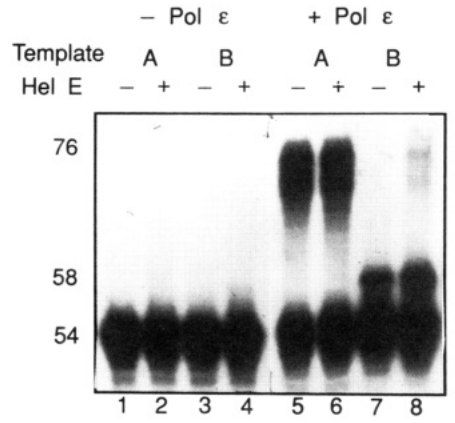

FIGURE 2: Effect of hel E on pol ϵ extension of templates with and without the downstream primer. Extension reactions were performed as described under Experimental Procedures and incubated for 10 min using 0.1 unit of pol ϵ, 0.3 unit of hel E, and the template denoted in the figure. Reaction products were separated on 8% polyacrylamide/7 M urea sequencing gels and visualized by autoradiography.

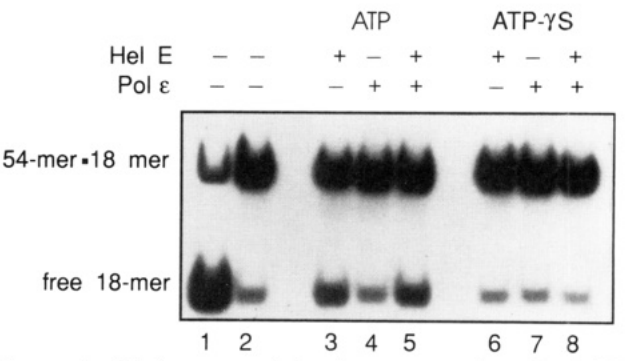

FIGURE 3: Displacement of the downstream primer by hel E. Displacement reactions were performed as described under Experimental Procedures. Reactions contained 0.32 unit of pol ϵ and 0.3 unit of hel E. Reactions were initiated by the addition of dNTP's and ATP (lanes 3–5) or dNTP's and ATP-γS (lanes 6–8) and were incubated for 15 min at 37 °C. After the reactions were stopped, products were separated on a 15% native polyacrylamide gel and visualized by autoradiography. Lane 1 is a control that was heated to 95 °C for 5 min prior to being loaded on the gel. Lane 2 is a control without added enzyme.

to autoradiography using X-OMAT XAR film (Kodak, Rochester, NY) and a DuPont Cronex lightning-plus intensifying screen at –70 °C. Displacement reactions were stopped by the addition of EDTA to 20 mM, SDS to 0.4%, 0.1% orange G dye, 10 μg of proteinase K, and sucrose to 10%. The reactions were incubated an additional 10 min at 37 °C and products were separated on 15% native polyacrylamide gels. The gels were dried and autoradiography performed as described above.

DNA Synthesis Assay. DNA synthesis assays using dA_{3000}·dT_{16} as the template–primer were performed in the reaction buffer described above but using [^{3}H]dTTP (4 Ci/mmol) as the deoxynucleotide substrate. Products were purified using Sephadex G-50 spin columns (Penefsky, 1977) and quantified by liquid scintillation counting.

RESULTS

Hel E and Pol ϵ Can Functionally Interact. A DNA substrate was designed that would allow synthesis from an upstream 3′ OH through a downstream duplex to determine whether pol ϵ and hel E could functionally interact. Either the 54-mer template–primer was 5′ labeled (Figure 1, templates A and B) or the downstream 17-mer primer was 3′ labeled with [α-^{32}P]dGTP by the addition of one nucleotide (Figure 1, template C). Reactions measuring extension and displacement were performed under conditions of primer–template excess. Preliminary experiments confirmed that 100% of the input DNA was capable of being utilized by polymerase in extension reactions or displaced by helicase in primer displacement reactions. Densitometric scans of the reaction products indicate that 10% or less of the input DNA substrate was utilized during the course of the reaction, unless noted otherwise.

Using the 5′ labeled 54-mer template–primer, with and without the downstream primer annealed (templates A and B), we monitored DNA synthesis in the presence of pol ϵ and hel E (Figure 2). Lanes 1–4 show control reactions on each of the templates without pol ϵ added. No extension of the 3′ OH is detected, demonstrating that the hel E preparation is essentially free from contaminating DNA polymerase activity. Lane 5 demonstrates that pol ϵ is capable of extending the 54-mer template–primer, resulting in the full-length 76-mer product. Pol ϵ is processive for the length of synthesis observed as evident by no intermediate products observed. The addition of hel E to reactions containing pol ϵ (lane 6) resulted in a slight increase in synthetic activity, as shown by the increased usage of template and greater intensity of full-length products observed. Using the 54-mer template–primer with the downstream primer annealed (lane 7), pol ϵ is capable of filling in the 4-base gap. Despite the efficient gap-filling synthesis by pol ϵ, no full-length products are observed, indicating that pol ϵ is not capable of strand displacement synthesis. Full-length products are observed upon the addition of hel E to the reactions (lane 8), demonstrating that displacement of the downstream primer by the hel E results in full-length synthesis by pol ϵ. The results suggest that concerted actions of pol ϵ and hel E generate full-length extension products.

Control experiments using template C described in Figure 1, containing the downstream primer labeled and annealed, were performed. Results demonstrated that the downstream primer was not degraded into progressively smaller fragments and did not become ligated to the 54-mer template–primer following extension by pol ϵ (data not shown). These experiments rule out the possibility that full-length products are the result of either ligation of the two fragments following gap-filling synthesis by pol ϵ or 5′ to 3′ exonuclease degradation of the downstream primer followed by extension by pol ϵ. The inefficiency of the extension reaction by pol ϵ, which leads to full-length products in the presence of hel E, is most likely due to the random distribution of pol ϵ and hel E on the DNA templates. Apparently, only the templates that contain both hel E and pol ϵ are capable of generating the full-length extension products.

Displacement of the Downstream Primer by Hel E. The results presented above suggest that hel E can functionally interact with pol ϵ to perform displacement synthesis. To determine whether pol ϵ had any effect on hel E activity we assayed displacement of the downstream primer from the 54-mer template. These reactions were identical to those described above except the standard helicase reaction stop mix was used and the downstream primer was annealed and 3′ labeled as described under Experimental Procedures. Figure 3 shows the results from a typical experiment. Lane 1 is the boiled template control indicating that approximately 95% of the 18-mer is displaced. The control reaction without added pol ϵ or hel E is shown in lane 2 and a small level of unannealed labeled 18-mer is detected. Hel E added alone is capable of displacing the downstream primer (lane 3). Densitometric scans of a lighter exposure of the gel revealed that less than

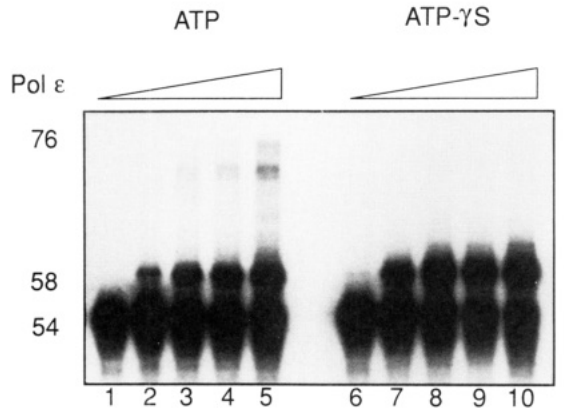

FIGURE 4: ATP dependence of the extension reaction catalyzed by pol ε/hel E. Extension reactions were performed as described under Experimental Procedures. Reactions were initiated by the addition of dNTP's and ATP (lanes 1–5) or dNTP's and ATP-γS (lanes 6–10) and were incubated for 10 min at 37 °C. Hel E was kept constant at 0.3 unit/reaction and pol ε was titrated as follows: lanes 1 and 6, no pol ε; lanes 2 and 7, 0.1 unit; lanes 3 and 8, 0.2 unit; lanes 4 and 9, 0.3 unit; lanes 5 and 10, 0.4 unit. Reaction products were separated on 8% polyacrylamide/7 M urea sequencing gels and visualized by autoradiography.

10% of the primer was displaced in the 15-min reaction time. Pol ε added alone to the reactions is incapable of displacing the downstream primer (lane 4). When pol ε and hel E are added together in the reaction (lane 5), the level of displacement of the downstream primer observed is similar to that obtained with hel E alone. These results demonstrate that pol ε does not alter the ability of hel E to displace the downstream primer. The addition of higher concentrations of hel E resulted in a greater level of displacement, while titration of pol ε to 5-fold higher concentrations also had no effect on the level of displacement (data not shown). Lanes 6–8 show the results obtained by substituting ATP-γS for ATP in the reactions. In each case, pol ε, hel E, or both together, no displacement of the downstream primer was observed. These results demonstrate that ATP hydrolysis is required for the displacement activity of hel E and that pol ε has no effect on the ability of hel E to perform a strand displacement reaction.

The amount of full-length extension products synthesized by pol ε is small in proportion to the total extension by pol ε, the latter including both 4-nucleotide gap filling and full-length extension (Figure 2). Likewise, the molar amount of primer displaced by hel E is greater than the molar amount of full-length extension by pol ε in the presence of hel E (Figure 3). These results suggest that a portion of the pol ε molecules are acting independently of hel E, a portion of the hel E molecules are acting independently at pol ε, and a portion of each are acting together. We propose that the latter portion is responsible for the displacement synthesis observed in Figure 2.

ATP Requirement for Displacement Synthesis. Since the preparations of pol ε and hel E used in these experiments were not purified to homogeneity, a series of control reactions were performed to verify that increased primer extension was promoted by helicase activity. The results in Figure 3 demonstrate the requirement for ATP hydrolysis for strand displacement. Therefore, we would expect that ATP-γS would inhibit displacement synthesis through the downstream primer by pol ε/hel E. Extension through the downstream primer by pol ε/hel E was analyzed in reactions initiated with dNTP's but using either ATP (Figure 4, lanes 1–5) or ATP-γS (lanes 6–10) as the nucleotide substrate for displacement. In the presence of ATP, full-length products are observed when both

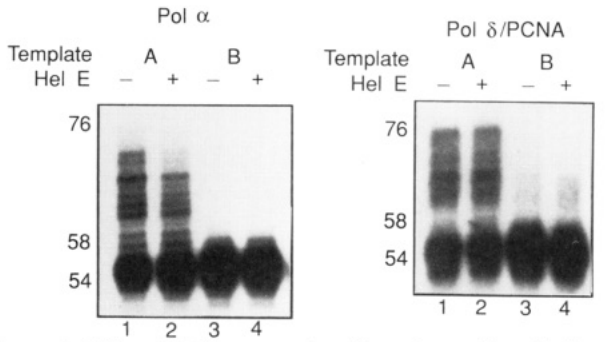

FIGURE 5: Effect of hel E on extension of templates with and without the downstream primer by pol α and pol δ/PCNA. Extension reactions were performed as described under Experimental Procedures and were incubated for 10 min using 0.3 unit of hel E and 0.1 unit of pol α (left panel) or 0.1 unit of pol δ/PCNA (right panel). Reaction products were separated on 8% polyacrylamide/7 M urea sequencing gels and visualized by autoradiography.

hel E and pol ε are present. In contrast, using ATP-γS as the substrate, pol ε is capable of gap-filling synthesis but no full-length products are observed. The reaction leading to displacement synthesis by pol ε, therefore, requires ATP hydrolysis. The dependence on ATP hydrolysis supports the hypothesis that the DNA displacement by hel E is enzymatic activity required for synthesis of full-length products by pol ε.

Specificity of the Hel E Interaction. DNA pol α–primase is thought to be responsible for priming and extension of RNA primers for leading- and lagging-strand synthesis (Kaguni & Lehman, 1988). A complex of PCNA and replication factor C (RF-C) is then thought to displace pol α on the leading strand, and pol δ then joins to complete leading-strand synthesis (Tsurimoto & Stillman, 1991). In order to determine whether hel E can interact with other DNA polymerases, identical extension reactions were performed substituting pol α and δ for pol ε (Figure 5). Pol α (left panel) is capable of extending the synthetic template–primer that does not have the downstream primer annealed (lane 1). Pol α is less processive than pol ε, as evident by the virual absence of full-length products. The majority of products appear to result from 4–5-nucleotide extensions. The addition of hel E has a minimal effect on the amount of synthesis by pol α, actually slightly decreasing the amount of extension products observed (lane 2). Pol α is essentially inactive on the 54-mer template–primer containing the downstream primer (lane 3), and the addition of hel E has no effect on the ability of pol α to utilize this gapped template (lane 4). In oligonucleotide displacement assays, addition of pol α has no effect on the displacement activity of hel E (data not shown). Overall, these results suggest that there is an incompatibility between pol α and hel E that only prevents synthesis on template–primers which require simultaneous function of these two enzymes.

The results obtained with DNA pol δ/PCNA (Figure 5, right panel) are similar to those observed with pol α. Pol δ/PCNA was capable of utilizing the 54-mer template–primer without the downstream primer, generating full-length products in addition to products of intermediate length (lane 1). The addition of hel E had no effect on synthesis by pol δ/PCNA (lane 2). Pol δ/PCNA utilized the 54-mer template–primer containing the downstream primer very inefficiently (lane 3). The products observed using the 54-mer template–primer with the downstream primer were similar to those obtained without the downstream primer but were generated at a much lower level. This suggests that pol δ/PCNA is capable of displacement synthesis in the absence of added helicase, consistent

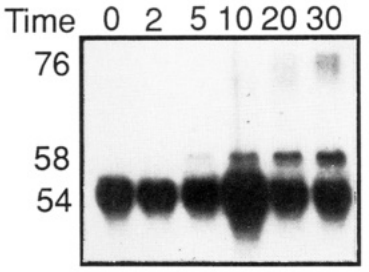

FIGURE 6: Kinetics of displacement synthesis by pol ϵ/hel E. Pol ϵ (0.03 unit) and hel E (0.1 unit) were assayed in extension reactions as described under Experiental Procedures. Aliquots were removed at the times indicated in the figure. Reaction products were separated on 8% polyacrylamide/7 M urea sequencing gels and visualized by autoradiography.

with a previous report (Downey et al, 1988). The addition of hel E had no effect on the ability of pol δ/PCNA to extend the 54-mer substrate. These results demonstrate that pol δ/PCNA is also incompatible with hel E and suggest that functional coordination with hel E appears to be specific for pol ϵ.

Kinetics of Full-Length Synthesis. The relatively high concentration of hel E, compared to pol ϵ, and the inefficiency of the reaction leading to full-length extension products suggested that the reaction leading to full-length extension products may not be catalytic. In order to determine whether hel E/pol ϵ was capable of acting catalytically, the time dependence of the interaction was analyzed in a time course reaction (Figure 6). Pol ϵ alone was capable of efficient gap-filling synthesis up to, but not into, the downstream primer, and hel E alone showed no full-length extension products even after a 30-min incubation (data not shown). The addition of pol ϵ and hel E together (Figure 6) demonstrated displacement synthesis through the downstream primer in a time-dependent manner. These results provide evidence that pol ϵ and hel E coordinate activities in a catalytic manner, involving displacement of the downstream primer by hel E and concurrent extension by pol ϵ.

Titration of Pol ϵ and Hel E. To further analyze the interaction between pol ϵ and hel E we performed a titration of each enzyme to assess their dependence on the synthesis of full-length products. Extension through the downstream primer was dependent on the concentration of added hel E (Figure 7). Lanes 1–3 show the results obtained without added pol ϵ. A small but detectable level of extension is observed with the highest concentration of the hel E used (lane 3). In the presence of added pol ϵ (lanes 4–6), full-length synthesis is observed, dependent on the concentration of added hel E. When the pol ϵ concentration was increased, extension through the downstream primer, independent of added hel E, was observed (lane 7). This result suggests that at high levels of pol ϵ displacement synthesis can occur, or alternatively, the polymerase preparation used in this experiment is slightly contaminated with hel E. Even at higher concentrations of pol ϵ, the addition of hel E increased the amount of full-length extension products observed (lanes 7–9). The percent of template utilized during the course of the reaction was greater than 10% in this experiment with respect to extension by pol ϵ.

Open Primer Template Dilution Experiment. One explanation for displacement synthesis by pol ϵ/hel E is that hel E could displace the downstream primer from a portion of the templates and pol ϵ could then arrive later to extend the upstream primer. Further evidence that hel E and pol ϵ were coordinately functioning on one DNA template and not independently on separate templates was obtained in a template

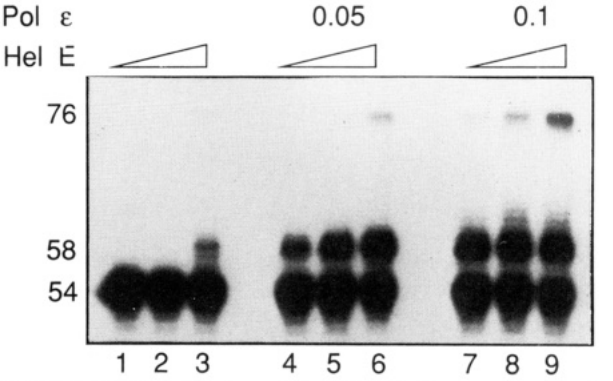

FIGURE 7: Titration of pol ϵ and hel E in displacement synthesis reactions. Extension reactions were performed as described under Experimental Procedures and incubated for 10 min at 37 °C. The units of pol ϵ are given in the Figure. Hel E was titrated as follows: lanes 1, 4, and 7, no hel E; lanes 2, 5, and 8, 0.1 unit; lanes 3, 6, and 9, 0.2 unit. Reaction products were separated on 8% polyacrylamide/7 M urea sequencing gels and visualized by autoradiography.

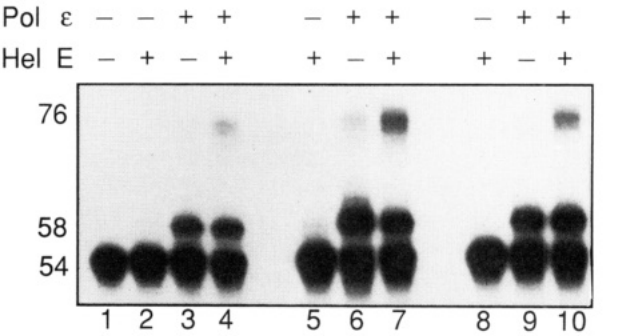

FIGURE 8: Template dilution does not affect displacement synthesis. Extension reactions were performed as described under Experimental Procedures using 0.4 unit of pol ϵ and 0.3 unit of hel E as denoted in the figure. Reactions contained 0.1 pmol of ^{32}P-labeled template B and 1 pmol of unlabeled template A (lanes 1–4). Enzymes were then added and reactions were initiated with ATP and dNTP's and incubated for 10 min at 37 °C. Lanes 5–7 contained only ^{32}P-labeled template B and reactions were intiiated with ATP and dNTP's. Lanes 8–10 contained 0.1 pmol of ^{32}P-labeled template B, enzymes were added, and reactions were initiated with ATP, dNTP's, and 1 pmol of unlabeled template A. Reactions were incubated for 10 min at 37 °C, and reaction products were separated on 8% polyacrylamide/7 M urea sequencing gels and visualized by autoradiography.

dilution experiment. In these reactions the hel E and pol ϵ were allowed to bind the labeled 54-mer template–primer, containing the downstream primer annealed (template B), on ice. The reactions were initiated with ATP and dNTP's, as in previous experiments, and 10-fold excess unlabeled DNA template–primer without the downstream primer annealed (template A). This serves to dilute the product resulting from hel E displacing the downstream primer. These conditions would result in a decreased level of full-length extension products if pol ϵ and hel E were acting independently on separate templates. Figure 8 depicts this experiment. Lanes 5–7 are standard extension reactions where pol ϵ and hel E can only distribute among the labeled 54-mer template–primers. Initiation with ATP and dNTP's resulted in gap-filling synthesis by pol ϵ and full-length extension products upon the addition of hel E to the reactions, as expected. Identical reactions were performed except the initiation mix contained excess unlabeled template A (lanes 8–10). The ability of pol ϵ to fill the 4-base gap in not inhibited by the addition of the unlabeled 54-mer template–primer. Furthermore, the ability of hel E/pol ϵ to generate full-length products is only slightly diminished. Lanes 2–4 show the results obtained if the unlabeled DNA is added prior to the initiation

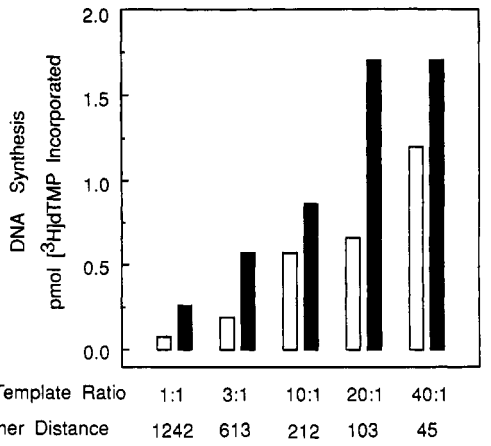

FIGURE 9: Hel E stimulation of pol ϵ independent of displacement activity. DNA synthetic activity was measured on dA_{3000}·dT_{16} at various IPD's. Reactions contained pol ϵ, 0.2 unit (open bars), or pol ϵ plus 0.3 unit of hel E (filled bars). The reactions were initiated with 5 mM ATP and 25 μm [^{3}H]dTTP and incubated for 10 min at 37 °C. The reaction products were separated and quantified as described under Experimental Procedures. Background counts were subtracted and reactions containing hel E alone gave no counts above background.

of the reaction with ATP and dNTP's. Pol ϵ and hel E could distribute between these templates in addition to the labeled 54-mer template–primer containing the downstream primer. Products of these reactions demonstrated that pol ϵ will fill the 4-base gap and in the presence of hel E will generate full-length extension products, albeit at a reduced efficiency, presumably due to the initial distribution between the labeled and unlabeled templates. If hel E and pol ϵ were acting completely independently, we would expect a large decrease in the level of full-length extension products upon the addition of unlabeled 54-mer template–primer at the initiation of the reaction. The control reactions (lanes 2–4) where pol ϵ and hel E distribute among the labeled and unlabeled template–primers result in a small proportion of the reaction products representing full-length extension. Additional experiments were performed, demonstrating that pol ϵ, following extension of a template–primer, repeatedly dissociates and binds new template–primers during the reaction (data not shown). Furthermore, hel E repeatedly dissociates from the template following displacement of the downstream primer (data not shown). Continual redistribution of these enzymes is evident in the time course experiment (Figure 4), in which the reaction leading to full-length extension products is nearly linear with time. Taken together, these results confirm that pol ϵ and hel E are acting together to generate the full-length extension products.

Hel E Stimulation of Pol ϵ Synthetic Activity. Examination of Figure 2 revealed that the addition of hel E to pol ϵ resulted in full-length extension products. The results also demonstrated that addition of hel E results in an increased level of synthesis as determined by an increase in the total amount of extension products (Figure 2, lanes 7 and 8). We therefore determined whether hel E could stimulate pol ϵ in a standard DNA synthetic assay using dA_{3000}·dT_{16} as the template–primer over a range of IPDs. Increasing concentrations of dT_{16} were annealed to a constant amount of dA_{3000} and these template–primers were used in a standard DNA synthetic assay. The results shown in Figure 9 demonstrate that hel E is capable of stimulating the synthetic activity of pol ϵ independent of IPD. This suggests that hel E is interacting with pol ϵ and stimulating synthetic activity that does not appear to require DNA displacement by hel E.

DISCUSSION

In this report we demonstrated a functional interaction between hel E and pol ϵ that results in DNA synthesis through a downstream primer dependent on helicase activity. Evidence that pol ϵ and hel E are carrying out a coordinated reaction resulting in displacement synthesis was obtained in an open template dilution experiment (Figure 8). The addition of 10-fold excess unlabeled template–primer without the downstream primer, at the initiation of the reaction, results in essentially the same level of full-length synthesis observed without the addition of the unlabeled template–primer. The 10-fold template–primer excess was calculated in relation to the amount of input DNA. In terms of the actual hel E reaction products, 5–10% of the input DNA, the dilution is on the order of 100–200-fold. The fact that we observe only a minor decrease in the intensity of the full-length products when the open template DNA is added supports the conclusion the pol ϵ and hel E are acting together on a single template–primer to generate the full-length extension products.

Additional evidence for a functional interaction between pol ϵ and hel E was obtained by demonstrating a 2-fold increase in DNA synthetic activity of pol ϵ by the addition of hel E to synthetic reactions. The template–primer used in these experiments was dA_{3000}·dT_{16} at a variety of IPDs. This homopolymeric template has essentially no secondary structure and, at the largest IPD used, there is only one primer per template. A 2-fold increase in activity was observed even under these conditions and suggests that hel E can stimulate pol ϵ in a mode that is independent of primer displacement. Experiments are currently being performed to further investigate this mode of polymerase stimulation.

The reaction leading to full-length products is an efficient process and we suggest that both hel E and pol ϵ distribute randomly between the template–primers prior to the initiation of the reaction. Upon initiation of the reaction, only the small proportion of templates that contain both pol ϵ and hel E are capable of generating full-length extension products. This conclusion suggests that the increased activity of pol ϵ on homopolymeric templates by hel E may be due to a large activation of a small proportion of pol ϵ molecules as opposed to all the pol ϵ molecules being stimulated to the same extent, i.e., 2-fold.

DNA pol α is capable of extending the 54-mer template–primer without the downstream primer independent of added hel E. In the presence of the downstream primer pol α was essentially inactive, and the addition of hel E had no effect on the reaction. The fact that pol α was inactive on the template–primer containing the 4-base gap is not surprising in view of the limited capacity of pol α to synthesize on templates with gaps less then 15 bases in length (Mosbaugh & Linn, 1984). The inability of pol α to extend the 54-mer in the presence of the downstream primer in reaction containing hel E suggests that hel E and pol α are functionally incompatible. We have demonstrated that pol α does not inhibit the displacement activity of hel E; therefore, 54-mer template–primers were generated that are devoid of the downstream primer (data not shown). We have also demonstrated that hel E does not inhibit polymerization activity of pol α on activated calf thymus DNA (data not shown). It appears that pol α and hel E cannot functionally interact when they both occupy the same template–primer, and coordinated activity is necessary for synthesis by pol α on the template–primer containing the downstream primer due to the inefficiency of filling a 4-nucleotide gap.

In addition, the inability of pol α to extend the 54-mer template–primer containing the downstream primer in the presence of hel E suggests that there is a negligible steady-state amount of 54-mer template–primer without the downstream primer from which hel E has departed. This should serve as a substrate for pol α but must be present at such a low level that extension products are not observed. Clearly, the fact that negligible amounts of 54-mer template–primer from which hel E has departed are available for synthesis by pol ϵ supports the conclusion that pol ϵ and hel E are acting in concert to generate full-length extension products.

Results obtained with pol δ/PCNA were similar to those of pol α. Pol δ/PCNA demonstrates almost no gap-filling synthesis and the addition of hel E does not affect the ability of pol δ/PCNA to utilize the 54-mer template–primer containing the downstream primer. These results demonstrate a specificity of hel E for pol ϵ. Preliminary experiments demonstrated the 215-kDa pol ϵ from calf was capable of generating full-length extension products in the presence of hel E (data not shown). Therefore, the specificity of hel E appears to be for the ϵ class of polymerases. In light of recent reports it will be of interest to determine whether other helicases can functionally interact uniquely with pol ϵ (Thömmes et al., 1992).

Pol ϵ was initially purified from cultured cells as an activity able to complement repair-deficient tissue culture cell extracts (Nishida et al., 1988). This suggests that pol ϵ may also participate in DNA repair. The functional coordination between pol ϵ and hel E suggests that these proteins may be involved in DNA repair. The functional interaction between polymerase and helicase may also be required in an excision repair system, similar to the UV repair pathway in *E. coli* (Sancar & Sancar, 1988). A number of eukaryotic helicases have been implicated in DNA repair. Weeda et al. (1990a) have recently cloned a gene (ERCC-3) based on complementation of UV-sensitive rodent mutant cell lines. On the basis of sequence analysis, ERCC-3 codes for a putative helicase with a predicted molecular mass of 89 kDa. This gene is required for DNA repair in that it complements cells from xeroderma pigmentosum B patients, and therefore is suspected to participate in an excision repair pathway (Weeda et al., 1990b). The Rad3 protein from *Saccharomyces cerevisiae* is a helicase thought to be involved in DNA repair on the basis of genetic and enzymological studies (Sibghat-Ullah et al., 1990). Rad3 helicase translocates in the 5′ to 3′ direction and is thought to be the analog of the human ERCC-2 gene (Weber et al., 1990), which complements excision repair mutant Chinese hamster ovary cells (Weber et al., 1988). It has been suggested that a 3′ to 5′ helicase is also required for UV repair (Weeda et al., 1990b). These helicases may be similar to the UvrAB complex that scans the DNA helix for structural abnormalities to be repaired (Oh & Grossman, 1989; Seeley & Grossman, 1990). Experiments are currently underway to determine the effect of damaged DNA on the DNA displacement activity of hel E to more directly address the possibility that hel E is involved in DNA repair.

In addition to DNA repair, DNA pol ϵ is thought to be involved in DNA replication. Reactions involving strand separation and concurrent polymerization are required for leading-strand DNA synthesis. The specific role of pol ϵ in DNA replication is still unresolved. Morrison et al. (1990) and Araki et al. (1992) suggest that pol ϵ may be required for leading-strand synthesis, but do not rule out the possibility that pol ϵ may be involved in lagging-strand DNA synthesis. Reconstitution experiments (Tsurimoto & Stillman, 1991; Lee et al., 1991) indicate that pol δ is required for leading-strand synthesis and show no requirement for pol ϵ. Interestingly, a 100-kDa helicase, RIP 100, has been isolated from eukaryotic cells on the basis of its association with a 60-kDa protein, RIP 60, that preferentially binds to "bent" DNA and yeast autonomous replication sequences (Dailey et al., 1990). The authors suggest that RIP 60 and RIP 100 maybe involved in replication initiation, responsible for the initial binding and subsequent melting of origins of replication. The helicase activity of RIP 100 was demonstrated using an oligonucleotide displacement assay. Preliminary results suggest that the helicase translocates in the 3′ to 5′ direction. These physical and enzymological characteristics are similar to those of hel E, suggesting that these two proteins may be homologous. The finding that hel E functionally interacts with pol ϵ suggest that these two proteins may be present in the chromosomal DNA replication machinery.

The 3′ to 5′ directionality of hel E could enable it to track along the leading strand at the replication fork ahead of the leading-strand DNA polymerase. If hel E is acting in DNA replication, the finding that pol α and δ will not functionally associate with hel E suggest that other factors may be required to form a functional complex. RF-C is a possible candidate since it participates as an accessory factor in SV40 replication by binding to 3′ termini in a complex with PCNA to displace pol α. This complex then transfers the 3′ termini to pol δ for leading-strand synthesis (Tsurimoto & Stillman, 1991). Initial results suggest that RF-C may also be involved at another level beyond polymerase transfer since elongation of leading-strand synthesis by pol δ and PCNA appears to require RF-C and ATP hydrolysis beyond the initial transfer to pol δ. This raises the interesting possibility that RF-C may function, following the exchange to pol δ, as a structural component in the replication fork complex required for leading-strand elongation. Experiments are in progress to determine whether other factors are required for pol α and δ to functionally interact with hel E or to make coordination with pol ϵ more efficient.

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