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Interactions between the Werner Syndrome Helicase and DNA Polymerase δ Specifically Facilitate Copying of Tetraplex and Hairpin Structures of the d(CGG)$_n$ Trinucleotide Repeat Sequence*

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Werner syndrome (WS) is an inherited disorder characterized by premature aging and genomic instability. The protein encoded by the WS gene, WRN, possesses intrinsic 3′ → 5′ DNA helicase and 3′ → 5′ DNA exonuclease activities. WRN helicase resolves alternate DNA structures including tetraplex and triplex DNA, and Holliday junctions. Thus, one function of WRN may be to unwind secondary structures that impede cellular DNA transactions. We report here that hairpin and G′2 bimolecular tetraplex structures of the fragile X expanded sequence, d(CGG)$_n$, effectively impede synthesis by three eukaryotic replicative DNA polymerases (pol): pol α, pol δ, and pol ε. The constraints imposed on pol δ-catalyzed synthesis are relieved, however, by WRN; WRN facilitates pol δ to traverse these template secondary structures to synthesize full-length DNA products. The alleviatory effect of WRN is limited to pol δ; neither pol α nor pol ε can traverse template d(CGG)$_n$ hairpin and tetraplex structures in the presence of WRN. Alleviation of pausing by pol δ is observed with *Escherichia coli* RecQ but not with UvrD helicase, suggesting a concerted action of RecQ helicases and pol δ. Our findings suggest a possible role of WRN in rescuing pol δ-mediated replication at forks stalled by unusual DNA secondary structures.

Werner Syndrome (WS),[1] characterized by premature aging and genomic instability (1), is a result of mutations in the WS gene. The polypeptide encoded by the WS gene, WRN, contains a central seven-motif domain shared by DNA helicases of the RecQ family (2). This family of DNA helicases is represented by *Escherichia coli* RecQ (3), *Saccharomyces cerevisiae* Sgs-1 (4), *Schizosaccharomyces pombe* Rqh1 (5), *Xenopus laevis* FFA-1 (6), and human RecQL (7), BLM (8), and RecQ4 and RecQ5 proteins (9). Multiple RecQ DNA helicases have also been identified in *Drosophila melanogaster* (10) and *Arabidopsis thaliana* (11). WRN is distinct from other members of the RecQ helicase family in that it also includes an N-terminal exonuclease domain (12–14). Indeed, recombinant WRN protein has been shown to possess, in addition to an ATP-dependent 3′ → 5′ DNA helicase activity, an intrinsic 3′ → 5′ DNA exonuclease activity (15, 16).

WRN helicase exhibits several characteristic features. 1) Unwinding of double-stranded DNA requires a 3′ single-stranded DNA tail, which presumably serves as a helicase loading DNA stretch (17, 18). 2) WRN exhibits low processivity such that the enzyme is capable of unwinding only short duplex regions <25 nt in length. 3) The processivity of WRN can be increased by the single-stranded DNA-binding protein, human replication protein A (19); in its presence, WRN unwinds duplex DNA tracts as long as 800 nt (20). 4) WRN can unwind alternate DNA structures, including DNA tetraplexes (21), four-way Holliday junctions (22), and triplex DNA (23).

A large body of evidence implicates WRN and its family members in replication. The prolonged S-phase of WS cells (24, 25), their sensitivity to the S-phase-specific topoisomerase I inhibitor camptothecin (26), and the more recent demonstrations of a physical and functional interaction between WRN and the major replicative DNA polymerase, pol δ (27, 28), all support the notion that WRN is involved in some aspects of DNA replication. If this is the case, a principle function of WRN helicase may be to resolve alternate DNA structures ahead of the replication fork that would normally impede the progression of DNA polymerases, analogous to the function of the dda helicase in bacteriophage T4 (29).

Guanine-rich DNA sequences readily form tetraplex structures *in vitro* under physiological-like conditions (30–32). Tetraplex formations of DNA are maintained by guanine quartets that are held together by Hoogsteen hydrogen bonds and stabilized by monovalent alkali cations. A direct demonstration for the existence of tetraplex DNA structures in cells is still lacking. However, their formation *in vitro* by biologically important G-rich sequences, such as telomeric DNA and the immunoglobulin class switch region, has led to speculations on their involvement in telomere transactions (32, 33) and in homologous recombination (30, 31) *in vivo*. Of interest is the formation of hairpin (34–37) and tetraplex structures (38–40) by the d(CGG) trinucleotide repeat sequence whose expansion in the *FMR1* gene leads to fragile X syndrome. Hairpin and tetraplex

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[1] The abbreviations used are: WS, Werner syndrome; WRN, Werner syndrome protein; pol δ, DNA polymerase δ; pol α, DNA polymerase α; pol ε, DNA polymerase ε; nt, nucleotide(s).

structures of this sequence have been shown to perturb movement of DNA polymerases during *in vitro* DNA synthesis (40–43). Stalling of replicative DNA polymerases could result in polymerase slippage and expansion of the repeat sequence.

Here we report that DNA synthesis by several eukaryotic DNA polymerases is blocked by hairpin and bimolecular G′2 tetraplex structures of a d(CGG)$_7$ tract in template DNA. Addition of WRN helicase, however, allows pol δ to traverse these template secondary structures and to synthesize full-length DNA. Further, we demonstrate that the ability of WRN to alleviate polymerase stalling at these secondary structures is specific and limited to pol δ.

EXPERIMENTAL PROCEDURES

Materials and Enzymes

[γ-^{32}P]ATP (~3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. High performance liquid chromatography-purified and crude oligodeoxynucleotide primer and template, respectively, were synthesized by Operon Technologies. Ultrapure deoxyribonucleoside triphosphates (dNTPs) were purchased from Promega Corp. Bacteriophage T4 polynucleotide kinase was supplied by New England Biolabs.

Recombinant hexa-His-tagged WRN protein was purified to >90% homogeneity by the protocol published by Shen *et al*. (16). Approximate concentrations of WRN protein were determined from Coomassie-stained SDS-polyacrylamide gels using bovine serum albumin as a standard. Molar amounts of WRN were calculated based on its being a monomer (~165 kDa). RecQ helicase was kindly provided by Dr. Stephen Kowalczykowski (University of California, Davis, CA), and UvrD helicase was a gift from Dr. Lawrence Grossman (Johns Hopkins University, Baltimore, MD). *S. cerevisiae* DNA pol δ and pol δ* were purified to homogeneity as described (44); concentrations of pol δ and pol δ* were determined spectrophotometrically at A_{280}. Human DNA polymerase α-primase complex (pol α) and human DNA polymerase ϵ (pol ϵ) were the generous gifts of Dr. Teresa Wang (Stanford University, Stanford, CA) and Dr. Stuart Linn (University of California, Berkeley, CA), respectively.

Preparation of Tetraplex DNA

High performance liquid chromatography-purified 18-mer primer (5′-d(GCCGGGGCCGGCCGCCGC)-3′) was 5′-end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase as described (45) and boiled to inactivate the kinase. Unincorporated [γ-^{32}P]ATP was removed from the reaction mixture by precipitating the labeled primer DNA with ethanol. Complementary 61-mer template (5′-d(TATGCCGGCGGCGG-CGGCGGCGGCGGATGTAATGCCTCGTCTTGCGGCGGCCGGCCCC-GGC)-3′) was purified by electrophoresis through a denaturing 7 M urea, 8% polyacrylamide gel (45).

The labeled primer (500 pmol) was mixed with an equivalent amount of unlabeled template DNA in 50 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$. The mixture was boiled for 5 min at 100 °C, and the denatured oligomers were allowed to anneal by slow cooling to room temperature. Unlabeled primer was hybridized in parallel to unlabeled template DNA in an identical manner. The labeled primer-template was mixed with unlabeled primer-template to a final DNA concentration of 60 μM in the presence of 300 mM KCl in a volume of 16 μl. The mixture was incubated at 4 °C for 15–18 h to allow formation of tetraplex DNA. Thereafter, the concentration of KCl was lowered to 30 mM by the addition of 25 mM Tris-HCl, pH 8.0, 20% glycerol.

Approximately 30-μl aliquots of the DNA mixture were loaded in individual lanes of a non-denaturing 6% polyacrylamide gel in TBE buffer (45 mM Tris borate buffer, pH 8.3, 1.25 mM EDTA) containing 30 mM KCl. The samples were electrophoresed at 4 °C at a constant current of 35 mA to resolve tetraplex forms of the oligomer from residual duplex and single-stranded DNA. Electrophoretically retarded tetraplex DNA was visualized by autoradiography and cut out from the gel. The excised gel slices were suspended in cold TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 100 mM KCl and vortexed at 4 °C overnight. Following separation of gel residue by centrifugation, the extracted DNA was precipitated with ethanol and resuspended in TE buffer, 20 mM KCl. Aliquots of the recovered DNA were stored frozen at −80 °C until use. Concentrations of the isolated tetraplex DNA were estimated from the amount of radioactivity recovered.

Preparation of Duplex Hairpin-containing DNA

^{32}P-5′-End-labeled 18-mer primer was hybridized to a 2-fold molar excess of gel-purified, unlabeled 61-mer template, as described above. The primed hairpin template was used without further purification in primer extension assays.

Assays

DNA Polymerase-catalyzed Primer Extension—Hairpin or tetraplex-containing DNA template (0.5 pmol) was copied by indicated concentrations of DNA polymerases in the absence or presence of known amounts of DNA helicases. DNA synthesis was carried out in reaction mixtures that contained, in a final volume of 10 μl: 40 mM Tris-HCl buffer, pH 7.5, 20 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 0.2 mM each of dATP, dGTP, dCTP, and dTTP. Reaction mixtures for the extension of primed hairpin-containing template did not include KCl. Following incubation at 37 °C for 15 min, the primer extension reactions were terminated by rapid cooling on ice and addition of denaturing loading buffer (45). The samples were boiled for 5 min, and aliquots were electrophoresed through 14% polyacrylamide-urea gels. The gels were dried and primer extension products were visualized by autoradiography or quantitated by PhosphorImager analysis (Molecular Dynamics).

DNA Helicase Activity—Helicase activity was measured in primer extension reaction mixtures except that 1 mM ATP was present in place of the four dNTP substrates. Radiolabeled tetraplex DNA substrate (0.3–0.5 pmol) was incubated with known amounts of WRN, *E. coli* RecQ, or *E. coli* UvrD at 37 °C for 15 min. The unwinding reaction was terminated by the addition of 2.5 μl of a solution containing 40% glycerol, 50 mM EDTA, 2% SDS, and 3% each bromphenol blue and xylene cyanol. Unwinding of tetraplex DNA was monitored by electrophoresis of reaction aliquots through a non-denaturing 12% polyacrylamide gel in 0.5× TBE, 20 mM KCl at 4 °C under a constant current of 35 mA, followed by autoradiography, as described (21).

RESULTS

The d(CGG)$_7$-containing Synthetic Template Forms a Bimolecular Tetraplex Structure—DNA tracts containing repeats of the d(CGG) trinucleotide fold into hairpin structures (34–37), which, in the presence of alkali cations, assume tetrahelical conformations (38–40). Both the hairpin and tetraplex structures of d(CGG)$_n$ DNA stretches have been shown to block synthesis by DNA polymerases *in vitro* (41–43) and *in vivo* (46).

We characterized the requirements for the formation of a tetraplex structure by the d(CGG)$_7$-containing template and determined its stoichiometry. A 15-h incubation of 60 μM ^{32}P-5′-labeled d(CGG)$_n$-containing primer-template in the presence of 300 mM KCl at 4 °C resulted in the formation of tetraplex structures, as evidenced by the appearance of a band with retarded electrophoretic mobility relative to that of the DNA duplex on non-denaturing gels (data not shown). To determine the stoichiometry of the tetraplex generated under these conditions, we used two oligomers of different length, each containing seven d(CGG) repeats. The ^{32}P-5′-labeled d(CGG)$_7$-containing 61-mer template annealed at its 3′ terminus to a complementary unlabeled 18-mer primer, ^{32}P-5′-d(CGG)$_7$ oligomer or a 1:1 equimolar mixture thereof, were incubated at 4 °C for 15 h in the presence of 300 mM KCl to promote tetraplex formation. Following incubation, half of each reaction mixture was denatured while the other half was maintained at 4 °C. Aliquots of each mixture were diluted to 20 mM KCl and electrophoresed through non-denaturing polyacrylamide gels to resolve single-stranded DNA from duplex and tetraplex complexes. As shown in Fig. 1*A*, electrophoretically retarded bands, representing respective multi-molecular complexes, were generated by the primer-d(CGG)$_7$ template and the d(CGG)$_7$ oligomer. Previous CD measurements (47) and dimethyl sulfate protection analyses (38) demonstrated that these complexes were DNA tetraplexes. Most notably, however, an additional band with mobility intermediate to the tetraplex complexes formed by each individual oligomer was observed in the 1:1 equimolar mixture of the two oligomers. As a result of their different stabilities, different amounts of tetraplex complexes were formed in the 1:1 mixture of d(CGG)$_7$ and primed

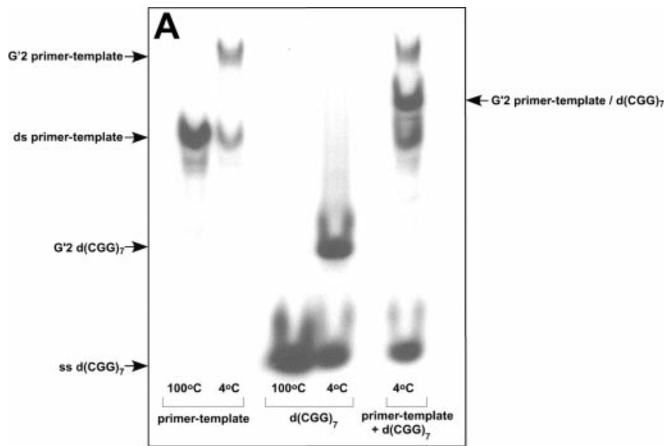

FIG. 1. **Stoichiometry and structure of d(CGG)$_7$ tetraplex-containing primer-template DNA.** *A*, unlabeled 18-mer primer was annealed to complementary ^{32}P-5′-labeled d(CGG)$_7$-containing 61-mer template. The annealed primer ^{32}P-5′-template, ^{32}P-5′-d(CGG)$_7$, or a 1:1 mixture thereof, was incubated at 4 °C for 15 h in 10-μl reaction mixtures that contained 9 μM DNA and 300 mM KCl. At the end of the incubation period, half of each reaction mixture was boiled for 10 min to denature formed tetraplex complexes and half was kept at 4 °C. Aliquots of the DNA samples were diluted to 20 mM KCl, and tetraplex complexes were resolved from double-stranded (*ds*) primer-template by electrophoresis at 4 °C through a 12% polyacrylamide gel in 0.5× TBE buffer, 20 mM KCl. Boiling of G′2 primer-^{32}P-template resulted in complete denaturation of the electrophoretically retarded tetraplex complex (*lane 1*). However, heat treatment left the double-stranded primer-template intact. As revealed by boiling of G′2 ^{32}P-primer-template, only a minor amount of labeled primer was released, whereas the major fraction of DNA remained as an intact double strand (results not shown). The heat resistance of the primer-template is presumably due to the high melting temperature of the G-C rich 18-mer primer and/or its rapid reannealing following denaturation. *ss*, single-stranded. *B*, schematic of the bimolecular tetraplex primer-template complex. Downstream to the primer-template stem of each of the two coupled DNA molecules is a 17-nucleotide-long single-stranded template tract followed by a tetraplex domain formed by two joined d(CGG)$_7$ template hairpins and a 5-nucleotide-long single-stranded tail. Four guanine quartets in a d(GGCGG) tract schematically represent the template G′2 d(CGG)$_7$ tetraplex structure.

d(CGG)$_7$-containing template (Fig. 1*A*). The single hybrid complex is a tetraplex composed of one molecule each of the primer-d(CGG)$_7$-containing template and the d(CGG)$_7$ oligomer. Hence, mixtures containing only primer-d(CGG)$_7$ template generate a bimolecular tetraplex of this DNA. The bimolecular tetraplex complex formed between two molecules of primed-^{32}P-template, schematically illustrated in Fig. 1*B* and henceforth designated as G′2 primer-template, was extracted from the gel (see "Experimental Procedures") and served as a substrate for WRN helicase as well as a template for DNA synthesis by DNA polymerases.

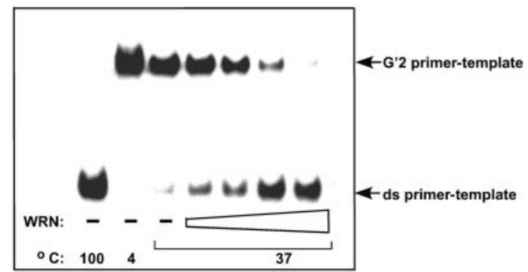

FIG. 2. **WRN unwinds tetraplex primer-template DNA.** WRN helicase assay mixtures (see "Experimental Procedures") contained 300 fmol G′2 tetraplex structures of primer-^{32}P-5′-template and increasing amounts (4–40 fmol) of WRN helicase. The mixtures were incubated at 37 °C for 15 min, and, after termination of the unwinding reaction, tetraplex DNA was resolved from double-stranded (*ds*) DNA by electrophoresis at 4 °C through a 12% polyacrylamide gel in 0.5× TBE buffer, 20 mM KCl. Control reactions that did not contain WRN were either boiled for 10 min prior to electrophoresis to denature the G′2 tetraplex DNA or were incubated at 4 °C or 37 °C to monitor spontaneous destabilization of the tetraplex primer-template. Heat denaturation of G′2 tetraplex primer-template resulted in its complete conversion to double-stranded primer-template rather than to single-stranded template (*lane 1*). As noted in the legend to Fig. 1*A*, this is most likely due to the high melting temperature of the primer and/or its reannealing to the template following denaturation.

The Bimolecular d(CGG)$_7$-containing Primer-Template Tetraplex Is Unwound by WRN—Purified G′2 primer-^{32}P-5′-labeled template was incubated at 37 °C with increasing amounts of WRN and electrophoresed through a non-denaturing gel to monitor unwinding of the DNA tetraplex. As illustrated in Fig. 2, WRN efficiently unwound the tetraplex structure in the presence of Mg^{2+} and ATP, as previously reported for different d(CGG)$_n$ tetraplex substrates (21). Similar unwinding results were obtained using G′2 primer-template radiolabeled at the primer stem (data not shown). Unwinding was also observed when ATP in the reaction mixture was substituted with 0.2 mM each of the four dNTPs, as used in subsequent polymerase-catalyzed primer extension reactions (results not presented).

WRN Enables Polymerase δ to Traverse past a Template d(CGG)$_7$ Tetraplex Structure—We monitored the ability of polymerase δ to copy a G′2 d(CGG)$_7$ tetraplex-containing template strand. The primer-tetraplex template complex was isolated as described under "Experimental Procedures" and incubated with pol δ in the presence of all four dNTPs at 37 °C. Products of the extension reaction were electrophoresed through denaturing polyacrylamide gels and visualized by autoradiography. We observed that pol δ was able to incorporate dNTPs up to the start of the tetraplex structure (Fig. 3, *lane 4*; data not shown). Notably, pol δ alone failed to generate full-length DNA product chains; strong pause sites were observed within the first of the seven repeat sequences at template nucleotide positions 37 and 38. The inability of pol δ to extend the primer beyond the pause sites indicates that the G′2 d(CGG)$_7$ tetraplex structure effectively blocked progression of pol δ along the template strand.

We next asked whether WRN, by virtue of its ability to unwind d(CGG)$_n$ tetraplex structures, would allow pol δ to traverse the template tetraplex domain. Primer extension reactions were carried out as described above with the exception that two different amounts of WRN were added to the mixtures along with pol δ. As seen in Fig. 3 (*lanes 5* and *6*), WRN allowed pol δ to extend the primer beyond the pause sites adjacent to the tetraplex structure and generate full-length DNA product. Using end-labeled single-stranded template DNA as a molecular size marker, we determined that the lower of the two bands indicated in Fig. 3 by *arrowheads*, corresponds to the 61-nt product. The upper band with retarded mobility is most likely to represent an altered conformation of the full-length product

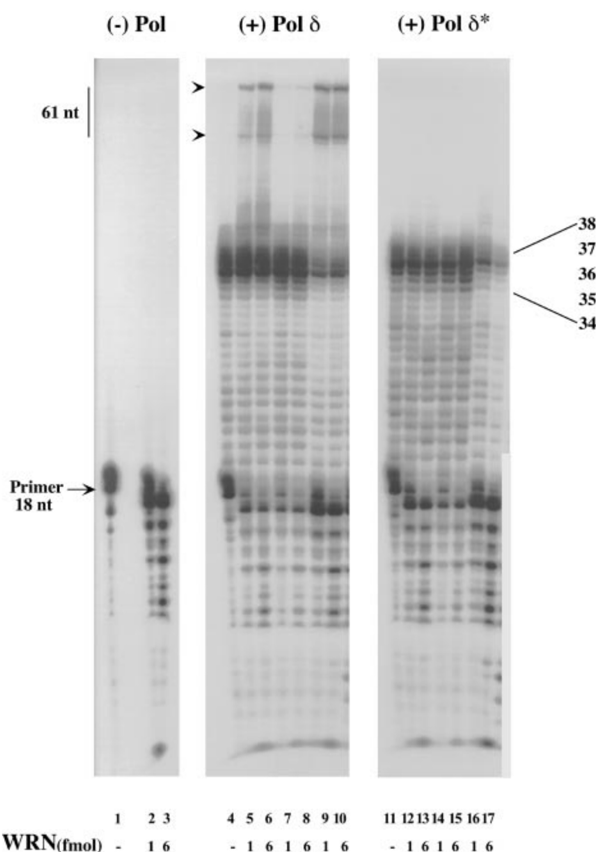

FIG. 3. **WRN enables pol δ to traverse a template d(CGG)$_7$ tetraplex structure.** Gel-purified G′2 d(CGG)$_7$ tetraplex containing ^{32}P-5′-primer-template DNA (0.5 pmol) was copied by pol δ (~0.7 fmol) or pol δ* (~0.9 fmol) in the absence or presence of WRN helicase (1 and 6 fmol). Following incubation at 37 °C for a total length of 20 min, the reactions were terminated by the addition of denaturing loading buffer. The samples were boiled, and aliquots were electrophoresed through denaturing polyacrylamide gels as described (45). *Lane 1*, ^{32}P-5′-primer-template; *lanes 2* and *3*, WRN without added DNA polymerase; *lanes 4* and *11*, pol δ and pol δ*, respectively, minus WRN; *lanes 5*, *6*, *12*, and *13*, WRN added together with polymerase; *lanes 7*, *8*, *14*, and *15*, reactions pre-incubated with polymerase for 10 min prior to the addition of WRN for another 10 min; *lanes 9*, *10*, *16*, and *17*, reactions pre-incubated with WRN for 10 min before incubation with polymerase for 10 min. *Arrowheads* correspond to the full-length product of 61 nt. Positions of primer and template pause sites are as indicated.

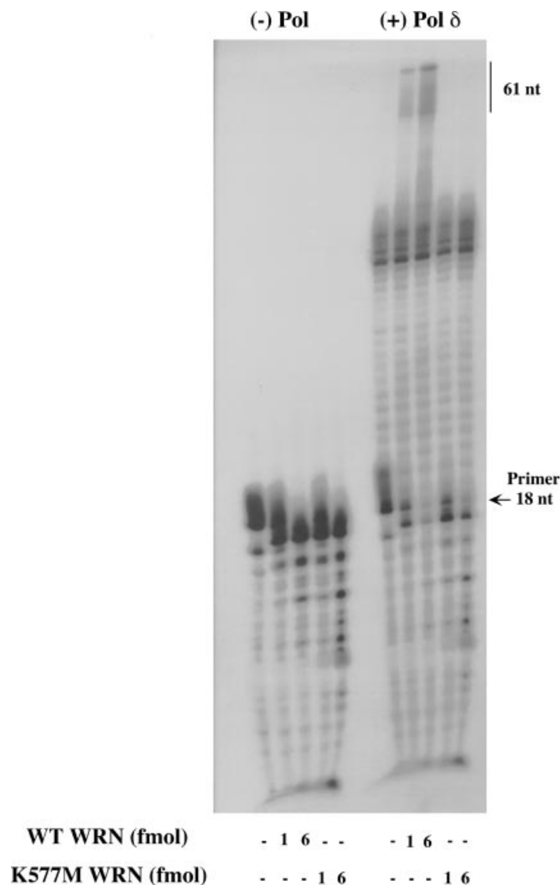

FIG. 4. **A helicase-deficient WRN protein does not enable pol δ to traverse a template d(CGG)$_7$ tetraplex structure.** End-labeled primed d(CGG)$_7$ tetraplex-containing template DNA was extended by pol δ (~0.7 fmol) in the absence or presence of equivalent amounts (1 or 6 fmol) of wild-type (*WT*) or K577M mutant WRN protein. The reactions were incubated at 37 °C for 15 min and processed as described in the legend to Fig. 3. *Lanes* under the − *Pol* designation correspond to ^{32}P-5′-primer-template DNA incubated in the absence of pol δ with or without WRN.

since electrophoresis of reaction products through a denaturing urea-formamide gel resulted in the appearance of only a single 61-nt band (data not shown). The ladder of bands observed below the primer corresponds to degradation products generated by the 3′ → 5′ exonucleolytic activity of WRN as evidenced by their accumulation in reaction mixtures that contained WRN without polymerase (Fig. 3, *lanes 2* and *3*).

We also inquired whether the order of addition of pol δ and WRN affects the ability of pol δ to traverse the template tetraplex structure. When DNA synthesis was carried out first by pol δ for 10 min at 37 °C and WRN was added subsequently, no full-length product was observed; the profile of extension products resembled that obtained with pol δ alone (Fig. 3, *lanes 7* and *8*). These results suggest that binding of the 3′-primer terminus and synthesis up to the start of the repeat sequence by pol δ may have prevented binding and unwinding of the tetraplex by WRN helicase. On the other hand, when the primer-template was pre-incubated with WRN followed by the addition of pol δ, synthesis past the tetraplex structure was as robust as in reactions where WRN and pol δ were incubated simultaneously (Fig. 3, *lanes 9* and *10*). However, the amount of paused products was less in these reactions relative to those in which WRN and pol δ were added simultaneously. This is most likely due to binding and degradation of the 3′ primer terminus by WRN during the pre-incubation step (note the predominant −1 products of degradations in *lanes 9* and *10*) preventing pol δ from binding and extending the primer.

WRN Helicase Activity Is Essential to Alleviate Pausing by Polymerase δ at d(CGG)$_7$ Template Tetraplex Region—Results presented above demonstrated that WRN can unwind the tetraplex structure assumed by a d(CGG)$_n$ repeat-containing DNA. We therefore determined whether the DNA helicase activity of WRN was essential to allow pol δ to traverse the template tetraplex tract and to synthesize full-length product chains. To address this question, we copied the tetraplex-containing template DNA by pol δ in the presence of K577M mutant WRN protein. Substitution of the lysine residue in the Walker A motif of the ATPase domain with methionine eliminates NTP/dNTP hydrolysis by WRN to generate a functional helicase-minus protein (16, 17). We confirmed that in fact, K577M WRN failed to unwind the tetraplex substrate (data not shown), although it retained in full its exonuclease activity (Fig. 4, *lanes 4* and *5*). We show that, in contrast to wild-type WRN (Fig. 4, *lanes 7* and *8*), K577M WRN, at molar concentrations equivalent to those of wild-type WRN, had no effect on the ability of pol δ to extend the primer stem beyond the pause site (Fig. 4, *lanes 9* and *10*). Although the primer was depleted, much of it was degraded by K577M WRN exonuclease rather than extended by pol δ. Thus, in order to enable pol δ to

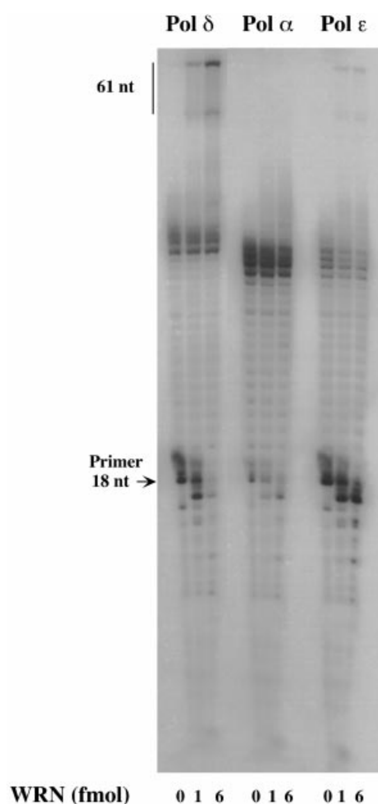

FIG. 5. **WRN does not allow DNA pol α or pol ε to fully traverse the template d(CGG)$_7$ tetraplex structure.** ^{32}P-5′-Primer-G′2 d(CGG)$_7$ template was extended by pol δ, pol α, or pol ε without or with equivalent amounts of WRN at 37 °C for 15 min as described. Primer extension products were visualized by autoradiography following electrophoresis through a denaturing 14% polyacrylamide gel.

synthesize DNA past template tetraplex-induced pause sites, WRN must maintain an active tetraplex DNA unwinding function.

WRN Does Not Alleviate Pausing by DNA Polymerases Other than pol δ at a d(CGG)$_7$ Tetraplex Structure—We recently demonstrated that WRN uniquely stimulates DNA synthesis by pol δ using a template that did not require unwinding by WRN. By contrast, evidence presented above indicates that copying of a template containing a d(CGG)$_n$ tetraplex domain by pol δ necessitated disruption of the tetraplex by WRN helicase. We inquired, therefore, if the permissive effect of WRN on tetraplex traversal by pol δ is also observed with other DNA polymerases.

To address this question, we initially carried out primer extension assays, identical to those presented in Fig. 3, with pol δ*, the two-subunit pol δ complex lacking Pol32p (44, 48). Like pol δ, pol δ* also strongly paused at template nucleotide position 37, with little extension beyond this point (Fig. 3, *lane 11*). However, neither pre-incubation of the reaction mixture with WRN (Fig. 3, *lanes 16* and *17*) nor its addition simultaneously with the polymerase (Fig. 3, *lanes 12* and *13*) allowed pol δ* to traverse the template d(CGG)$_7$ G′2 tetraplex. These results contrast strikingly with the data obtained with pol δ and WRN, and are consistent with our original report that Pol32p is an essential component in mediating the functional interaction between WRN and pol δ.

We next examined the ability of WRN to enable two other major replicative DNA polymerases, pol α and pol ε, to traverse the template tetraplex structure. As observed with pol δ, the d(CGG)$_7$ tetraplex was an effective barrier to synthesis by both pol α and pol ε (Fig. 5). Notably, however, these two polymerases stalled before the start of the tetraplex. This was evident by the appearance of major pause sites at nucleotide positions 34–37 corresponding to DNA sequences just before and within the first trinucleotide repeat. When pol α was incubated with WRN, at concentrations identical to those used with pol δ, no alleviation of pausing was discernible. Although a few faint read-through product DNA chains were present, no full-length products could be observed. Similar results were obtained with lower concentrations of pol α where only 10–20% of the primer was extended (results not shown). Although essentially similar, results obtained with pol ε differed slightly from those of pol α, in that WRN did allow pol ε to synthesize a small amount of full-length product. However, the proportion of full-length DNA chains was only ~20% of the amount synthesized by pol δ in the presence of WRN. Thus, to a first approximation, it appears that the ability of WRN to allow polymerases to traverse the tetraplex DNA structure may be limited to pol δ.

Not All Helicases Alleviate Pausing by pol δ at a Template d(CGG)$_7$ Tetraplex Region—To determine whether helicases other than WRN are also capable of allowing pol δ to traverse the template tetraplex region, we used two *E. coli* helicases: RecQ, the prototypical RecQ family member homologue of WRN, and UvrD. First, we investigated whether these two helicases could unwind tetraplex DNA. We observed that RecQ, at molar concentrations comparable to those of WRN, resolved the template G′2 d(CGG)$_7$ tetraplex to generate duplex primer-template DNA (data not shown). Likewise, UvrD also unwound this tetraplex. However, unwinding was very inefficient requiring 100–500-fold higher molar amounts of protein relative to RecQ or WRN to attain complete unwinding (data not shown).

Next, we carried out primer extension assays with pol δ as described, with either RecQ or UvrD substituting WRN. As demonstrated in Fig. 6, RecQ allowed pol δ to traverse the template d(CGG)$_7$ G′2 tetraplex domain and to generate full-length product DNA chains, characteristic of reactions containing WRN. By contrast, UvrD did not alleviate stalling by pol δ. Thus, it appears that at least some RecQ family members that efficiently unwind d(CGG)$_n$ tetraplex DNA structures preferentially allow pol δ to synthesize DNA beyond tetraplex-induced pause sites.

WRN Alleviates Pausing by DNA Polymerase δ at a Template d(CGG)$_7$ Hairpin Structure—DNA containing d(CGG)$_n$ repeats has been shown to fold spontaneously into hairpin structures (34–37) that also block the progression of synthesis by DNA polymerases (43). Extension of the ^{32}P-5′-end-labeled 18-mer primer hybridized to the hairpin-containing template was carried out with all three replicative DNA polymerases as described under “Experimental Procedures.” Results of such an experiment, shown in Fig. 7, demonstrated that the d(CGG)$_7$ hairpin impeded progression of synthesis by pol α, δ, and ε. The major product chains terminated before and within the first trinucleotide repeat sequence (pol α and pol ε), or immediately after the first repeat (pol δ). Trace amounts of full-length product chains were observed with the two processive DNA polymerases, pol δ and pol ε.

Simultaneous addition of WRN to reactions containing pol α had no significant effect on the extension profile. Notably, no full-length product chains accumulated in the presence of WRN. WRN did have a minimal effect on reactions containing pol ε, as evidenced by a slight increase in the amount of full-length 61-nt product with 6 fmol of WRN. By far, however, WRN had the most significant effect on reactions carried out with pol δ, allowing a larger fraction of the extension products to reach the full-length size.

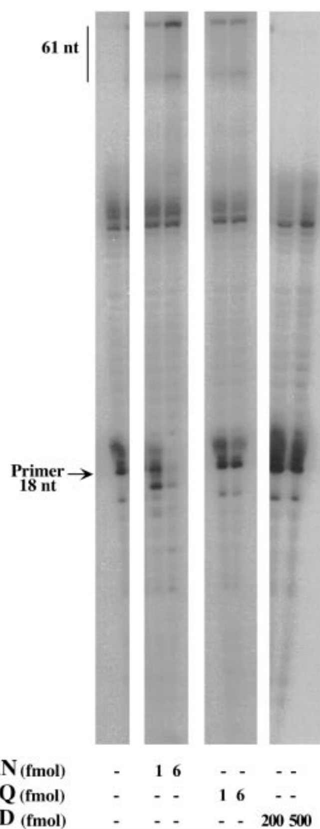

FIG. 6. **UvrD helicase can not substitute for WRN in extending tetraplex-containing template DNA by pol δ.** ^{32}P-5′-Primer-G′2 d(CGG)$_7$ tetraplex template was extended by pol δ (~0.7 fmol) in the absence or presence of indicated amounts of WRN, RecQ, or UvrD DNA helicases. Reaction conditions were as described under "Experimental Procedures." Aliquots of the reaction mixtures were electrophoresed through a 14% polyacrylamide urea gel and subjected to autoradiography to visualize primer extension products.

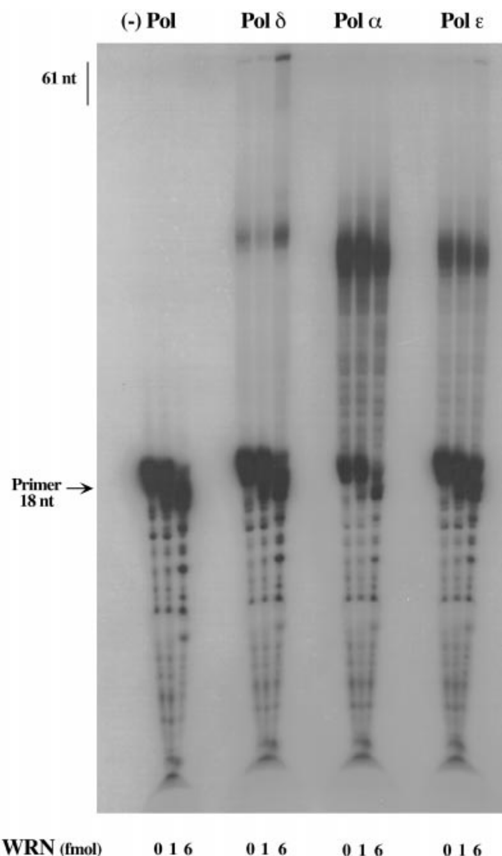

FIG. 7. **WRN allows pol δ to traverse a template d(CGG)$_7$ hairpin structure.** ^{32}P-5′-End-labeled 18-mer primer was hybridized to the d(CGG)$_7$ hairpin-containing template DNA. The primer-template (0.5 pmol) was extended by pol α, pol δ, or pol ϵ in the absence or presence of indicated amounts of WRN as described under "Experimental Procedures." Reaction aliquots were electrophoresed through a denaturing 14% polyacrylamide gel, and primer extension products were visualized by autoradiography of the dried gel.

DISCUSSION

The DNA metabolic processes that WRN participates in are still not clear. However, several lines of evidence point to its involvement in DNA replication. In particular, WS cells exhibit S-phase defects, including a decreased frequency of DNA initiations and a reduced rate of chain elongation (24, 49). Furthermore, these cells are sensitive to the S-phase-specific topoisomerase I inhibitor, camptothecin (26). The functional and physical interaction of WRN with a major replicative DNA polymerase, pol δ (27, 28) lends additional support for a role of WRN in replication. However, the finding that stimulation of pol δ activity by WRN occurs in the absence of the pol δ accessory factor, proliferating cell nuclear antigen (28), suggested that WRN may not participate in processive DNA replication. This observation, together with the finding that WRN can unwind alternate DNA structures (21–23), has led us to hypothesize that WRN may be involved in proliferating cell nuclear antigen-independent replication restart at forks blocked by DNA damage or stalled by DNA secondary structures. In this report we tested this hypothesis in part, by monitoring the effect of WRN on the progression of synthesis by pol δ through replication-impeding hairpin and tetraplex DNA structures.

We used bimolecular tetraplex or hairpin formations of the trinucleotide repeat sequence d(CGG)$_n$ as model template secondary structures. A d(CGG)$_n$ trinucleotide was first identified in the 5′-untranslated region of the *FMR1* gene (50–52). The ability of d(CGG)$_n$ tracts to fold into hairpins (34–37) and to assemble into quadruplex structures (38–40) was implicated in the expansion of this sequence that leads to fragile X syndrome. Hairpin and tetraplex structures of d(CGG)$_n$ have also been shown to block the progression of several DNA polymerases both *in vitro* (41–43) and *in vivo* (46).

We constructed a synthetic d(CGG)$_7$-containing primed DNA template that folds spontaneously into a hairpin structure, or forms a bimolecular G′2 tetraplex structure in the presence of K^+ ions (Fig. 1). In line with previous reports, we too demonstrate that the template G′2 d(CGG)$_7$ hairpin and tetraplex structures impose a strong barrier to DNA synthesis by three eukaryotic replicative DNA polymerases: α, δ, and ϵ (Figs. 3 and 4). Extension of a primer by all three DNA polymerases stalls either just before or within the first repeat of the trinucleotide sequence with no product DNA chains discernible beyond this point. Even when the concentration of polymerase is increased such that >90% of the primer is utilized, the initiated DNA chains pause near the start of the tetraplex region (data not shown).

Addition of WRN markedly alleviates pausing by pol δ at the tetraplex domain (Fig. 3); a significant fraction of the product constitutes 61-nt-long full-length DNA chains. Several lines of evidence indicate that alleviation of pol δ pausing is a result of the tetraplex d(CGG)$_n$ unwinding activity of WRN. First, WRN is able to efficiently unwind the template d(CGG)$_7$ G′2 tetraplex in the presence of dNTPs under conditions employed in primer extension reactions (data not shown). This is consistent with our previous results demonstrating that dNTPs can substitute for ATP in WRN-catalyzed unwinding reactions (19).

Second, the helicase deficient K577M mutant WRN protein that is unable to unwind DNA (16, 17), also fails to relieve tetraplex-induced stalling of pol δ (Fig. 4). Third, by changing the order of addition of WRN and pol δ, we demonstrate that alleviation of polymerase pausing requires that unwinding of the tetraplex precedes synthesis or occurs simultaneously with DNA synthesis by pol δ (Fig. 3).

The ability to complete synthesis past the G′2 tetraplex $d(CGG)_7$ replicative barrier and to generate full-length product DNA chains in the presence of WRN appears, by far, to be limited to pol δ. WRN does not allow pol α or the two-subunit pol δ enzyme, pol δ*, to traverse the template tetraplex structure (Figs. 3 and 5), and only a trace amount of full-length DNA products is observed in reactions containing WRN and pol ϵ (Fig. 5).

The specificity of alleviating tetraplex DNA-induced stalling of DNA polymerase is not only limited to the polymerase used, but also to the helicase utilized for unwinding. Our data show that, similarly to WRN, *E. coli* RecQ can unwind G′2 $d(CGG)_7$ and allow pol δ to synthesize past the tetraplex, albeit less efficiently than WRN (Fig. 6). This is not totally unexpected since RecQ and WRN belong to the same family of DNA helicases (2). Further, these results are consistent with the finding that replicative bypass of hairpin structures in *E. coli* can occur via a RecQ helicase-dependent pathway (53). In contrast to RecQ and WRN, *E. coli* UvrD that can also unwind the $d(CGG)_7$ tetraplex does not alleviate pol δ stalling at this secondary structure. Based on these results, we propose that DNA helicases of the RecQ family may serve to resolve tetraplex secondary structures in DNA templates copied by pol δ.

Data presented in Figs. 5 and 6 indicate that unwinding of the tetraplex structure by itself is not sufficient to allow traversal of the tetraplex domain by DNA polymerases. Instead, the results suggest a requirement for a concerted action of DNA unwinding by WRN and DNA synthesis by polymerase. The two processes may be coupled through a direct interaction of these proteins. Indeed, a physical interaction between WRN and human pol δ has been reported recently (27). The lack of a permissive effect of WRN on replication of tetraplex DNA template by pol δ* indicates that the Pol32p subunit of pol δ is required to couple synthesis with unwinding. These results extend our previous work that implicated Pol32p as an essential component in the functional interaction between WRN and pol δ (28). It should be noted that the human pol δ subunit (p50) that has been shown to interact physically with WRN (27) is not the same subunit that is required for stimulation of pol δ activity by WRN. Although seemingly discrepant, these findings are not mutually exclusive. It is conceivable that WRN physically associates with the p50 subunit of human pol δ but requires the p66 subunit (homologue of *S. cerevisiae* Pol32p) (54) for mediating its stimulatory effect on pol δ activity.

Unwinding of the tetraplex structure by WRN generates double-stranded DNA primer-template (Fig. 2). If unwinding of the tetraplex is not coupled to extension of the primer, the single-stranded $d(CGG)_n$ tract would refold spontaneously into a hairpin structure. Such hairpin structures effectively impede progression of synthesis by pol α and pol ϵ (Fig. 7). Therefore, we surmise that, although WRN can unwind the tetraplex structure, synthesis by pol α and pol ϵ cannot keep pace with unwinding, allowing formation of hairpin structures that block the progression of these polymerases. In contrast, synthesis by pol δ, tightly coupled to unwinding of the hairpin (Fig. 7) or tetraplex structure (Fig. 3) by WRN, results in traversal of the DNA secondary structures and syntheses of full-length DNA product chains.

The capacity of WRN to resolve tetraplex structures of $d(CGG)_n$ and to alleviate pausing by pol δ at these template formations might be of biological significance. $d(CGG)_n$ trinucleotide repeats are not restricted to the untranslated region of the *FMR1* gene. Computational analyses have revealed a statistical over-representation of $d(CGG)_n$ tracts in the human genome in genes other than *FMR1* (55, 56). Therefore, it is conceivable that hairpins and tetraplexes formed by such $d(CGG)_n$-rich DNA may be the preferred target of WRN helicase. Exposure of single-stranded regions of DNA during replication of $d(CGG)_n$ tracts may result in the formation of hairpins and tetraplexes. If these structures cannot be resolved by WRN helicase, they would impede fork progression, induce polymerase stalling and prolong the S-phase (25), as observed in WS cells lacking WRN protein (57). Further, stalled forks could usher the collapse of the replisome triggering recombination pathways that could result in the generation of large DNA deletions that are also characteristic of WS cells (58).

In conclusion, we have shown that DNA templates containing $d(CGG)_n$ hairpin or tetraplex structures impede DNA synthesis by three major replicative DNA polymerases: pol α, pol δ, and pol ϵ. The constraints imposed on DNA synthesis can be relieved by the action of a DNA helicase that can displace tetraplex structures. However, we demonstrate that alleviation of polymerase stalling shows specificity with respect to both the polymerase and the helicase. The combination of a RecQ helicase that can unwind $d(CGG)_n$ hairpin and tetraplex structures, and pol δ allows for synthesis of full-length reaction products.

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