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 contains 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...
structures of this sequence have been shown to perturb movement of DNA polymerases during \textit{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 G2 tetraplex structures of a d(CGG)$_7$ tract in template DNA. Addition of WRN helicase, however, allows pol $\delta$ 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 $\delta$.

**EXPERIMENTAL PROCEDURES**

**Materials and Enzymes**

[$\gamma$-$^{32}$P]ATP ($\sim$3000 Ci/mm mol) 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 $\geq$90% homogeneity by the protocol published by Shen et al. (16). Approximate concentrations of WRN protein were determined from Coomassie-stained SDS-polyacrylamide gels using bovine serum albumin as a standard. Molar amounts of WRN were calculated based on its being a monomer ($\sim$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 $\delta$ and pol $\delta^*$ were purified to homogeneity as described (44); concentrations of pol $\delta$ and pol $\delta^*$ were determined spectrophotometrically at $A_{260}$. Human DNA polymerase $\alpha$-primase complex (pol $\alpha$) and human DNA polymerase $\epsilon$ (pol $\epsilon$) 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'-dGCCGGGCGCGCGCGCGCGCG-3') was 5'-end-labeled with [$\gamma$-$^{32}$P]ATP by T4 polynucleotide kinase as described (45) and boiled to inactivate the kinase. Unincorporated [$\gamma$-$^{32}$P]ATP was removed from the reaction mixture by precipitating the labeled primer DNA with ethanol. Complementary 61-mer template (5'-dTATGCCGGCGCGCGCGCGCGCGCGCGCGCGCG-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 1500 mA for 16 h. 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. 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, \textit{E. coli} RecQ, or \textit{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 bромphenol 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 tetrahedral conformations (38–40). Both the hairpin and tetraplex structures of d(CGG)$_7$, DNA stretches have been shown to block synthesis by DNA polymerases \textit{in vitro} (41–43) and \textit{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 [$\gamma$-$^{32}$P]-labeled d(CGG)$_7$-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 3'2P-5'-labeled d(CGG)$_7$-containing 61-mer template annealed at its 3' terminus to a complementary unlabeled 18-mer primer, 32P-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. 1A, electrophoretically retarded bands, representing respective multi-molecular complexes, were generated at 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 tetraplex forms. 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
d(CGG)$_7$ tetraplex structure. Quartets in a d(GGCGG) tract schematically represent the template G$_2$ hairpins and a 5-nucleotide-long single-stranded tail. Four guanine nucleo-
sisted by DNA polymerases.

The Bimolecular d(CGG)$_7$-containing Primer-Template Tet-

pol$_d$ to copy a G$'2$ d(CGG)$_7$ tetraplex-containing tem-

WRN Enables Polymerase $\delta$ to Traverse past a Template d(CGG)$_7$ Tetraplex Structure—We measured the ability of polymerase $\delta$ 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 $\delta$ 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 $\delta$ was able to incorporate dNTPs up to the start of the tetraplex structure (Fig. 3, lane 4; data not shown). Notably, pol $\delta$ 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 $\delta$ to extend the primer beyond the pause sites indicates that the G$'2$ d(CGG)$_7$ tetraplex structure effectively blocked progression of pol $\delta$ along the template strand.

We next asked whether WRN, by virtue of its ability to unwind d(CGG)$_n$ tetraplex structures, would allow pol $\delta$ 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 $\delta$. As seen in Fig. 3 (lanes 5 and 6), WRN allowed pol $\delta$ 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.
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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 fmol) 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.

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)$_7$ 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
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Pol δ Pol α Pol ε

WRN (fmol) 0 1 6 0 1 6 0 1 6

FIG. 5. WRN does not allow DNA pol α or pol ε to fully traverse the template d(CGG)₇ tetraplex structure. ³²P-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)₇ 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 δ₇ 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 Escherichia 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)₇ 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)₇ 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 ³²P-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.
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 d (27, 28) lends additional support for a role of WRN in replication. However, the finding that stimulation of pol d activity by WRN occurs in the absence of the pol d 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 d through replication-impeding hairpin and tetraplex DNA structures.

We used bimolecular tetraplex or hairpin formations of the trinucleotide repeat sequence d(CGG)₇ as model template secondary structures. A d(CGG)₇ trinucleotide was first identified in the 5'-untranslated region of the FMR1 gene (50–52). The ability of d(CGG)₇ 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)₇ 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 G₂ tetraplex structure in the presence of K₁ ions (Fig. 1). In line with previous reports, we too demonstrated that the template G₂ 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)₇ unwinding activity of WRN. First, WRN is able to unwind the template d(CGG)₇ tetraplex in the presence of dNTPs under conditions employed in primer extension 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)₃ 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)₃ 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)₃ 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 pol δ together with DNA synthesis by polymerase and the helicase. The combination of a RecQ helicase that can unwind d(CGG)₃ hairpin and tetraplex structures, and pol δ allows for synthesis of full-length reaction products.

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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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