Biochemical Characterization of the WRN−FEN-1 Functional Interaction

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ABSTRACT: Werner Syndrome is a premature aging disorder characterized by chromosomal instability. Recently we reported a novel interaction of the *WRN* gene product with human 5′ flap endonuclease/5′-3′ exonuclease (FEN-1), a DNA structure-specific nuclease implicated in pathways of DNA metabolism that are important for genomic stability. To characterize the mechanism for WRN stimulation of FEN-1 cleavage, we have determined the effect of WRN on the kinetic parameters of the FEN-1 cleavage reaction. WRN enhanced the efficiency of FEN-1 cleavage rather than DNA substrate binding. WRN effectively stimulated FEN-1 cleavage on a flap DNA substrate with streptavidin bound to the terminal 3′ nucleotide at the end of the upstream duplex, indicating that WRN does not require a free upstream end to stimulate FEN-1 cleavage of the 5′ flap substrate. These results indicate that the mechanism whereby WRN stimulates FEN-1 cleavage is distinct from that proposed for the functional interaction between proliferating cell nuclear antigen and FEN-1. To understand the potential importance of the WRN−FEN-1[1] interaction in DNA replication, we have tested the effect of WRN on FEN-1 cleavage of several DNA substrate intermediates that may arise during Okazaki fragment processing. WRN stimulated FEN-1 cleavage of flap substrates with a terminal monoribonucleotide, a long 5′ ssDNA tract, and a pseudo-Y structure. The ability of WRN to facilitate FEN-1 cleavage of DNA replication/repair intermediates may be important for the role of WRN in the maintenance of genomic stability.

Understanding the cellular and molecular pathways that genes of the RecQ family participate in has generated considerable interest because of the genomic instability and predisposition to clinical features of cancer and aging in three human hereditary disorders of this gene family, Werner Syndrome (WS), Bloom Syndrome, and Rothmund-Thomson Syndrome (for review, see ref *1*). The genes defective in these diseases all possess the seven conserved motifs found in the RecQ family of DNA helicases. A number of studies suggest that underlying defects in the processes of DNA replication, repair, recombination, or a combination of these pathways are responsible for the genomic instability characteristic of the disorders (for review, see ref *1*).

Patients suffering from WS display characteristics of premature aging after adolescence (*2*). Clinical features of WS include the early onset of age-related symptoms such as gray hair, wrinkled skin, cataracts, atherosclerosis, diabetes, osteoporosis, and cancer. WS cells grown in culture display marked chromosomal instability (*3*−*5*), replication defects (*4*, *6*−*9*), aberrant recombination (*10*−*11*), altered telomere dynamics (*12*), and hypersensitivity to DNA damaging agents (*13*−*17*).

The gene defective in WS, designated *WRN*, encodes a 1432 amino acid protein (*18*) that resides primarily in the nucleus (*19*). The WRN protein is a DNA-dependent ATPase and helicase (*20*−*21*) that unwinds dsDNA with a 3′ to 5′ polarity with respect to the single strand that it is inferred to bind (*22*). WRN helicase is not a very processive DNA unwinding enzyme, as judged by its inability to unwind duplex DNA substrates as short as 69 bp (*23*); however, in the presence of the single-stranded DNA binding protein replication protein A (RPA), WRN can unwind long DNA duplexes up to 851 bp (*23*). Biochemical characterization of the DNA substrate specificity of WRN helicase indicates that the enzyme preferentially unwinds forked duplex substrates with noncomplementary single-stranded or double-stranded DNA tails by recognizing elements of fork structure to initiate DNA unwinding (*24*). The enzyme unwinds a number of alternate DNA structures including triplexes (*25*), tetraplexes (*26*−*27*), and Holliday junctions (*27*). WRN is also a 3′ to 5′ exonuclease (*22*, *28*, *29*), consistent with the presence of three conserved exonuclease motifs homologous to the exonuclease domain of *E. coli* DNA polymerase I and RNaseD in the protein sequence (*30*). A role of WRN in some aspect of DNA metabolism is also suggested by a number of its protein interactions (for review, see ref *31*). Precisely how these protein interactions are important in vivo remains to be established.

The replication defects and hypersensitivity of WS cells to certain DNA damaging agents might suggest a direct role of the WRN protein in the processes of replication and/or repair; however, these defects may be secondary to the primary defect(s) in WS cells. The possibility that WRN resides in a replication complex (*32*) supports the idea that WRN modulates, or is modulated by, other proteins within

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[1] Abbreviations: dsDNA, double-stranded DNA; FEN-1, flap endonuclease I; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; ssDNA, single-stranded DNA; WRN, Werner Syndrome protein; WS, Werner Syndrome.

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the complex. A strong candidate for interaction with WRN is the DNA structure-specific nuclease FEN-1, an enzyme that has been implicated in the processes of both DNA replication and repair. FEN-1 catalytically cleaves 5′ flap single-stranded (ss)DNA at the single strand-double strand junction (reviewed in ref *33*). FEN-1 is active as a 5′ to 3′ exonuclease at nicks in duplex DNA and also endonucleolytically removes the 5′ terminal RNA mononucleotide; the latter process thought to be important in Okazaki fragment processing (*34*−*35*). FEN-1 is also required during long patch base excision repair (BER) (*36*−*37*). Genetic evidence has implicated a role of the yeast FEN-1 homologue *RAD27* in pathways to maintain genome stability (*38*−*45*) and respond to DNA damage (*39*−*46*).

The collective evidence implicating FEN-1 and WRN in the maintenance of genome stability is consistent with the idea that the two proteins might interact with each other in vivo. However, the two proteins could also act in separate pathways. In support of the notion that they are associated, a physical interaction between WRN and FEN-1 was demonstrated by their co-immunoprecipitation from human cell lysate and affinity pull-down experiments using a recombinant C-terminal fragment of WRN (*47*). Importantly, WRN protein was shown to dramatically stimulate the rate of FEN-1 cleavage of short (≤5 nt) 5′ flap DNA substrates (*47*). On a per mole basis, WRN is a significantly more effective stimulator of FEN-1 cleavage on a 1 nt 5′ flap substrate than either human PCNA or RPA. The WRN−FEN-1 interaction is independent of WRN catalytic function and mediated by a 144 amino acid domain of WRN that shares homology with RecQ DNA helicases. The robust stimulation of FEN-1 cleavage by a protein interaction domain of WRN suggests that the mechanism for stimulation of FEN-1 by WRN may be different from that of PCNA or RPA and important under certain circumstances during DNA replication or repair in vivo.

In this study, we have investigated mechanistic aspects of the WRN−FEN-1 functional interaction and tested a number of biologically relevant substrates of DNA replication for cleavage by FEN-1 in the presence of WRN. WRN stimulates FEN-1 cleavage of a terminal RNA-DNA junction ribonucleotide, a proposed intermediate of DNA replication, and flap structures with long 5′ ssDNA tracts that may arise during strand displacement. Kinetic analyses indicate that WRN improves the efficiency of the FEN-1 cleavage reaction by a mechanism distinct from that proposed for PCNA (*48*). WRN also stimulated FEN-1 cleavage of DNA substrates resistant to FEN-1 nuclease activity including pseudo-Y structures. From our work, we have determined that WRN helicase activity on the 5′ flap substrate alters the ability of WRN to stimulate FEN-1 cleavage of the structure. The ability of WRN to target important DNA intermediates of replication and repair for cleavage by FEN-1 may be relevant to its role in the maintenance of genomic stability.

MATERIALS AND METHODS

Proteins. Histidine-tagged FEN-1, overexpressed in *E. coli* from a plasmid kindly provided by Dr. M. Lieber, was purified on Ni^{2+}-charged His-Bind resin (Novagen) as recommended by the manufacturer (*47*). Recombinant histidine-tagged WRN protein was overexpressed using a baculovirus/*Sf9* insect system and purified as previously described (*47*−*49*). A recombinant histidine-tagged WRN protein fragment corresponding to residues 940−1432 of the WRN sequence (His−$WRN_{940-1432}$), overexpressed in *E. coli*, was purified as previously described (*47*−*50*).

Table 1: Oligonucleotide Sequences for DNA Substrates (5′ to 3′)

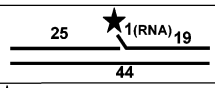
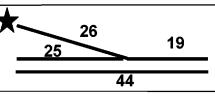
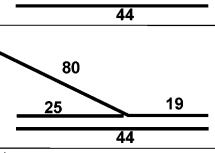
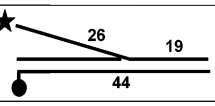
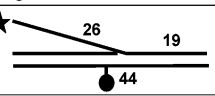
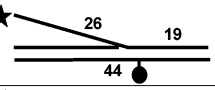
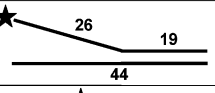
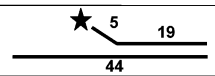
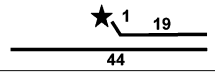
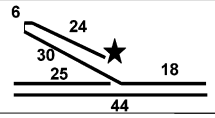
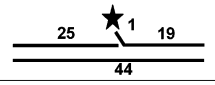
Template	Length (nt)	Sequence
$T_{STEM}25$	44	GCACTGGCCGTCGTTTTACGGTCGTGACTGGGA AAACCCTGGCG
Downstream Primer		
FLAP01	20	AGTAAAACGACGGCCAGTGC
FLAP01RNA	20	A*GTAAAACGACGGCCAGTGC
FLAP05	24	TCCAAGTAAAACGACGGCCAGTGC
FLAP26	45	TTTTTTTTTTTTTTTTTTTTTTCCAAGTAAAA CGACGGCCAGTGC
FLAP60FB	78	AGGTCTCGAGGCCTGCTCCTGCTCTATTATG AGCAGGAGCAGGCCTCGAGACCTTTCCAAG TAAAACGACGGCCAGTG
FLAP80	99	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTCCAAGTAAAACGACGGCCAGTGC
Upstream Primer		
U25	25	CGCCAGGGTTTTCCCAGTCACGACC
Biotinylated Oligonucleotides		
$T_{STEM}25B3$	44	GCACTGGCCGTCGTTTTACGGTCGTGACT GGGAAAACCCTGGC(G-BIOTIN)
$T_{STEM}25BUP$	44	GCACTGGCCGTCGTTTTACGGTC G(T-BIOTIN)GACTGGGAAAACCCTGGCG
TSTEM25BDOWN	44	GCACTGGCCGTCGTT(T-BIOTIN)TACGGTC GTGACTGGGAAAACCCTGGCG

* indicates that the preceding nucleotide is in the ribonucleic form

Oligonucleotide Substrates. PAGE-purified oligonucleotides (Midland Certified Reagent Co.) (Table 1) were used for preparation of substrates. Substrates were prepared as described previously (*48*) and are shown in Table 2.

FEN-1 Incision Assay. Reactions (20 μL) contained 10 fmol DNA substrate (except where indicated), and the indicated amounts of WRN and/or FEN-1 in 30 mM HEPES (pH 7.6), 5% glycerol, 40 mM KCl, 0.1 mg/mL BSA, and 8 mM $MgCl_2$. For incision reactions containing nucleoside triphosphate, the form and concentration are noted in the figure legend. For incision reactions containing streptavidin, streptavidin (15 nM) was preincubated with the DNA substrate with all reaction components except WRN and FEN-1 for 10 min at 37 °C. Streptavidin was effectively bound to the DNA substrate throughout the time course of the experiment, as detected by a gel-shifted species when streptavidin was present (data not shown). WRN was mixed with the substrate and buffer on ice prior to the addition of FEN-1. Reactions were incubated at 37 °C for 15 min (unless indicated otherwise), terminated with the addition of 10 μL of Formamide Stop Solution (80% formamide (v/v), 0.1% Bromophenol Blue, and 0.1% Xylene Cyanol), and heated to 95 °C for 5 min. Products were resolved on 20% polyacrylamide, 7 M urea denaturing gels. A PhosphorImager was used for detection, and the ImageQuant software (Molecular Dynamics) was used for quantitation of the

Table 2: DNA Substrates Used in this Study

1		FLAP01RNA $T_{STEM}25$ U25
2		FLAP26 $T_{STEM}25$ U25
3		FLAP80 $T_{STEM}25$ U25
4		FLAP26 $T_{STEM}25B3$ U25
5		FLAP26 $T_{STEM}25BUP$ U25
6		FLAP26 $T_{STEM}25BDOWN$ U25
7		FLAP26 $T_{STEM}25$
8		FLAP05 $T_{STEM}25$
9		FLAP01 $T_{STEM}25$
10		FLAP60FB $T_{STEM}25$ U25
11		FLAP01 $T_{STEM}25$ U25

reaction products. Percent incision was calculated from the equation: % incision = $(P/(S + P)) \times 100$, where P was the sum of the intensity of the bands representing incision products and S was the intensity of the band representing the intact oligonucleotide. Background values from the no enzyme controls were subtracted out. In WRN controls that lacked FEN-1, the percent of incision product was 0.5% or less at the highest level of WRN tested (100 fmol) on all substrates. Cleavage data represent the mean of at least three independent experiments, with standard deviations shown by error bars.

FEN-1 reactions for Michaelis−Menton kinetic studies contained 5 fmol FEN-1, 100 fmol WRN, and the indicated DNA substrate (0, 1.2, 2.5, 5, 10, 20, 40 nM) in the FEN-1 incision buffer. All other conditions for the assay were the same as those used for the general FEN-1 incision assays described above. Double reciprocal plots (1/ product formation velocity (nM/s)) versus (1/substrate concentration (nM)) were used to determine the kinetic parameters K_m and V_{max} by linear regression analysis.

WRN Helicase Assay. For those reactions in which the products were analyzed for WRN-catalyzed DNA unwinding and FEN-1 cleavage, the reaction mixtures (as described above) (20 μL) were split into two 9 μL aliquots, one for helicase activity analysis on native polyacrylamide gels and one for cleavage activity analysis on denaturing polyacrylamide gels as described above. For analysis of helicase activity, 9 μL reaction volumes were quenched with 9 μL of Helicase Stop Solution (50 mM EDTA, 40% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol) containing 10-fold excess of unlabeled oligonucleotide with the same sequence as the labeled strand. The products of the helicase reactions were resolved on nondenaturing 12% polyacrylamide gels. Radiolabeled DNA species in polyacrylamide gels were visualized using a PhosphorImager and quantitated using the ImageQuant software (Molecular Dynamics).

The percent helicase substrate unwound was calculated by the following formula: % unwinding = $100 \times (P/(S + P))$. P is the product and S is the substrate. The values of P and S have been corrected after subtracting background values in the no enzyme and heat-denatured substrate controls, respectively. Helicase data represent the mean of at least three independent experiments with standard deviations shown by error bars.

RESULTS

WRN Stimulates FEN-1 Cleavage of Terminal RNA-DNA Junction Monoribonucleotide. A required step of Okazaki fragment processing to complete lagging strand DNA replication is the removal of the initiator RNA primer. Genetic and biochemical evidence from yeast suggests that Dna2 and FEN-1 act sequentially to facilitate the complete removal of the primer RNA (*51*). On the basis of biochemical studies using mammalian cell extracts that support simian virus 40 DNA replication (*35*), RNase H1 has also been implicated in mammalian Okazaki fragment processing. RNase H1 is competent to remove the intact RNA primer but leaves one ribonucleotide at the RNA-DNA junction, which must be subsequently removed by FEN-1. We previously demonstrated that WRN stimulates FEN-1 cleavage of a DNA flap substrate with a single 5′ unannealed deoxynucleotide to yield the 1 and 2 nt products (*45*). To further characterize the effect of WRN on FEN-1 cleavage, we tested a 19 bp DNA substrate with an unannealed 5′ monoribonucleotide A adjacent to an upstream 25 bp DNA duplex. The 1 ribonucleotide 5′ flap substrate was susceptible to FEN-1 cleavage that generated the 1 ribonucleotide product (Figure 1A, lane 2), as previously published (*52*). A limiting amount of FEN-1 (5 fmol) was used such that cleavage of the monoribonucleotide 5′ flap is very low (~5%) (Figure 1B). We analyzed FEN-1 cleavage as a function of WRN concentration and observed dose-dependent stimulation of FEN-1 cleavage as a function of WRN amount. In the presence of 40 fmol of WRN, FEN-1 cleavage increased to approximately 27% incision, a 5.5-fold increase (Figure 1A, lane 7, and Figure 1B). At 80 fmol WRN, product formation began to plateau at 30% of the substrate cleaved (Figure 1A, lane 8, and Figure 1B). Only the single ribonucleotide product was observed, a result consistent with previously published results that FEN-1 cleaves primarily directly behind the ribonucleotide at the RNA-DNA junction (*52*). In the presence of only WRN (80 fmol), no product was detected (Figure 1A, lane 9), indicating that WRN alone does not cleave the structure.

WRN Stimulates FEN-1 Cleavage of a 26 nt 5′ ssDNA Flap Substrate. The ability of WRN to stimulate the FEN-1 cleavage reaction on 5′ flap structures may play an important

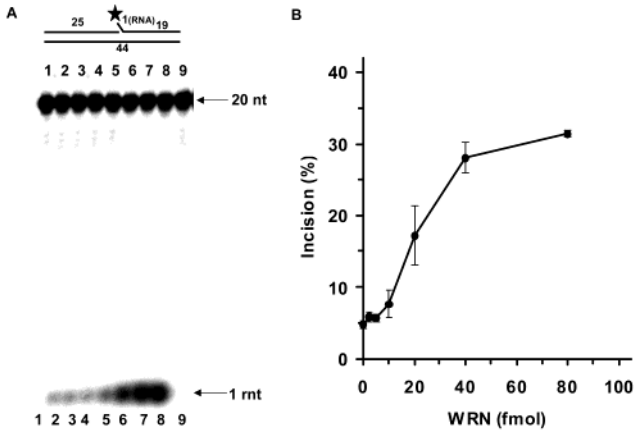


FIGURE 1: WRN stimulates FEN-1 cleavage activity on a ribonucleotide 5′ flap substrate. Panel A: Reaction mixtures (20 μL) containing 10 fmol of a 1 ribonucleotide 5′ flap DNA substrate, 5 fmol of FEN-1, and the indicated amounts of WRN were incubated at 37 °C for 15 min under standard conditions as described in Materials and Methods. A phosphorimage of a typical gel is shown. Lane 1, no enzyme; lane 2, FEN-1; lane 3, 2.5 fmol WRN + FEN-1; lane 4, 5 fmol WRN + FEN-1; lane 5, 10 fmol WRN + FEN-1; lane 6, 20 fmol WRN + FEN-1; lane 7, 40 fmol WRN + FEN-1; lane 8, 80 fmol WRN + FEN-1; lane 9, 80 fmol WRN. Panel B: % incision from panel A (mean value of three experiments), with standard deviation (SD) indicated by error bars.

role in Okazaki fragment processing since FEN-1 is implicated in the process of replication (*34*−*35*). It is currently understood that FEN-1 slides from the 5′ end of the ssDNA flap to the DNA duplex junction (*53*−*54*). Recently, it was reported that FEN-1 cleavage efficiency decreases substantially with increasing length of the 5′ ssDNA flap (*55*). The average size of an Okazaki fragment (100−150 nt) (*51*) may require that additional proteins facilitate cleavage of longer flap structures by FEN-1 or other nucleases. We examined the ability of WRN to stimulate FEN-1 cleavage of a 26 nt 5′ flap substrate, a structure with a significantly longer 5′ tail than the 5 nt 5′ flap substrate which we previously showed that WRN was able to stimulate FEN-1 cleavage (*47*). In the presence of 10 fmol of FEN-1, 4% of the substrate was incised (Figure 2A, lane 2, and Figure 2B). In the presence of WRN (80 fmol), FEN-1 incised 61% of the 5′ flap substrate molecules (Figure 2A, lane 3, and Figure 2B), a 15-fold stimulation. Stimulation of the FEN-1 cleavage reaction by WRN was also observed when 40 fmol of FEN-1 was used, although the increase (5−fold) was not quite as great (data not shown). Importantly, WRN alone did not yield the 26 and 27 nt products (Figure 2A, lane 4). Under these conditions, the Ku86/70 heterodimer, another DNA binding protein, did not stimulate FEN-1 cleavage at a range of Ku concentrations (10−500 fmol) (data not shown), providing evidence that stimulation of FEN-1 cleavage by WRN was not due to nonspecific protein or DNA interactions. These results demonstrate that WRN can readily stimulate FEN-1 cleavage of the 26 nt 5′ flap substrate.

We previously demonstrated that a C-terminal fragment (sequence 940−1432) of WRN that physically interacts with FEN-1 is capable of stimulating FEN-1 cleavage of a 1 nt 5′ flap substrate (*47*). To determine if this FEN-1 interaction domain of WRN, devoid of the helicase and exonuclease domains, is sufficient to stimulate FEN-1 cleavage of a longer 5′ flap substrate, we tested the effect of the WRN protein fragment on FEN-1 cleavage of the 26 nt 5′ flap substrate. Using 10 fmol of FEN-1 and increasing amounts of the protein fragment His−$WRN_{940-1432}$, the cleavage reaction was stimulated in a dose-dependent manner (Figure 2C,D). FEN-1 cleavage was stimulated 2-fold at a His−$WRN_{940-1432}$ amount of 10 fmol (Figure 2C, lane 3). At 20 fmol of His−$WRN_{940-1432}$, product formation began to plateau at 16%, a 4-fold increase compared to the reactions containing only FEN-1 (Figure 2C, lane 4, and Figure 2D). The level of stimulation by the C-terminal WRN fragment was approximately 3-fold less than the full length WRN protein at the 80 fmol level (Figure 2A−C). We also observed that the full-length WRN protein was a more effective stimulator of FEN-1 than the WRN C-terminal protein fragment on a 1 nt 5′ flap substrate (*47*). The 26 and 27 nt products detected in reactions containing His−$WRN_{940-1432}$ and FEN-1 were also detected in reactions containing only FEN-1 at higher concentrations of FEN-1 (data not shown), indicating that the cleavage site specificity of FEN-1 is not altered by the WRN C-terminal protein fragment. These results indicate that the protein domain of WRN that physically binds to FEN-1 is capable of stimulating FEN-1 cleavage of the 26 nt flap substrate.

WRN Helicase Activity Alters the Ability of WRN to Stimulate FEN-1 Cleavage. Recently, our laboratory demonstrated that WRN helicase efficiently unwinds a 26 nt 5′ flap substrate (shown in Table 2), releasing the 5′ flap oligonucleotide, while leaving the duplex species still containing the upstream oligonucleotide (*24*). Analyses of the labeled upstream oligonucleotides of 5′ flap substrates on denaturing polyacrylamide gels revealed that the upstream primer was >95% intact, indicating that it was not degraded to any significant amount by the 3′ to 5′ exonuclease activity of WRN under reaction conditions identical to those used in this study (*24*). Since a FEN-1 cleavage substrate that is dependent on the presence of an upstream primer (such as the 26 nt 5′ flap substrate used in this study) is rendered a less efficient substrate for FEN-1 cleavage when a gap as short as one nucleotide is introduced between the two duplexes (*48*), we reasoned that WRN stimulation of FEN-1 cleavage may be altered under conditions in which WRN is able to unwind the 5′ flap substrate. The abilities of WRN to unwind the 19 bp downstream duplex and to bind ssDNA (*49*), which may prevent reannealing of the released single strand to its partner before FEN-1 binds, suggested that WRN helicase activity may alter the efficiency of FEN-1 cleavage. To determine the outcome of 5′ flap processing by FEN-1 on a substrate that is also unwound by WRN, we examined both DNA unwinding (release of the 5′ flap oligonucleotide) and FEN-1 cleavage of the 5′ flap substrate in the presence or absence of the appropriate energy source (nucleoside triphosphate) that fuels the WRN helicase reaction. The results of these studies are shown in Figure 3. The products of the reactions were analyzed by electrophoresis on a denaturing (Figure 3A) or native (Figure 3B) polyacrylamide gel and quantitated. WRN (20 fmol) stimulated cleavage of the flap substrate by FEN-1 (5 fmol) approximately 4-fold in the absence of nucleotide (Figure 3A, lanes 10 and 11) or the presence of ADP (Figure 3A, lanes 8 and 9) or GTP (Figure 3A, lanes 12 and 13). Under these conditions, no detectable release of the 5′ flap oligonucleotide was detected for reactions containing WRN in the absence (Figure 3B, lane 2, and data not shown) or presence (Figure 3B, lanes 9,

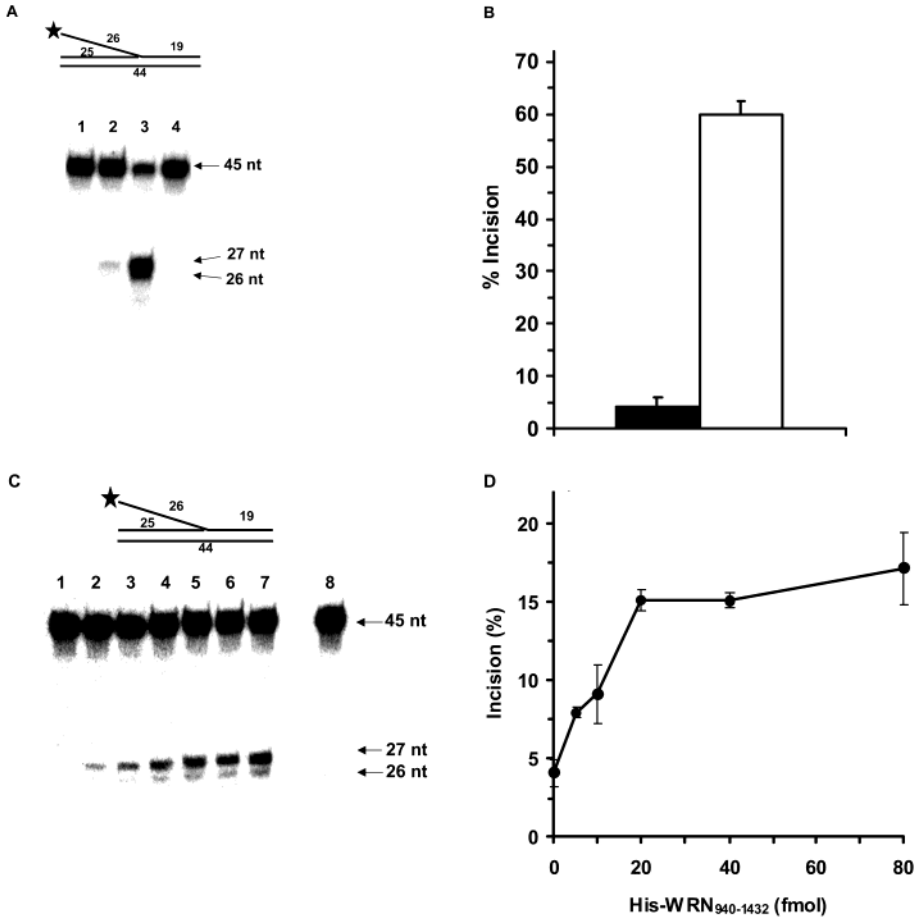


FIGURE 2: WRN stimulates FEN-1 cleavage activity on a 26 nt 5′ flap substrate. Panel A: Reaction mixtures (20 μL) containing 10 fmol of a 26 nt 5′ flap DNA substrate, 10 fmol of FEN-1, and 80 fmol of WRN were incubated at 37 °C for 15 min under standard conditions. A phosphoimage of a typical gel is shown. Substrate and cleavage products are as indicated. Lane 1, no enzyme; lane 2, FEN-1; lane 3, FEN-1 + WRN; lane 4, WRN. Panel B: % incision from panel A (mean value of three experiments) with SD indicated by error bars. Panel C: Reactions mixtures (20 μL) containing 10 fmol of 26 nt 5′ flap substrate, 10 fmol of FEN-1, and the indicated amounts of His−$WRN_{940-1432}$ were incubated at 37 °C for 15 min. A phophoimage of a typical gel is shown. Lane 1, no enzyme; lane 2, FEN-1; lane 3, FEN-1 + 5 fmol His−$WRN_{940-1432}$; lane 4, FEN-1 + 10 fmol His−$WRN_{940-1432}$; lane 5, FEN-1 + 20 fmol His−$WRN_{940-1432}$; lane 6, FEN-1 + 40 fmol His−$WRN_{940-1432}$; lane 7, FEN-1 + 80 fmol His−$WRN_{940-1432}$; lane 8, 80 fmol His−$WRN_{940-1432}$. Panel D: % incision from panel C (mean value of three experiments) with SD.

11, and 13) of FEN-1. In contrast, WRN was able to utilize ATP to catalyze release of the 5′ flap oligonucleotide in the absence (Figure 3B, lane 1) or presence (Figure 3B, lane 7) of FEN-1. Similarly, WRN was able to catalyze release of the 5′ flap oligonucleotide using dATP as the energy source in the absence (data not shown) or presence (Figure 3B, lane 5) of FEN-1. Analysis of the incision products from the reactions containing WRN, FEN-1, and either ATP or dATP demonstrated an approximately 2-fold stimulation of the FEN-1 cleavage reaction by WRN (Figure 3A, lanes 4−7), although the difference was only statistically significant for the reactions containing dATP (data not shown). The size of the cleavage products (26, 27 nt) were the same for the reactions in which WRN unwound the flap structure (ATP, dATP) as for the reactions in which WRN did not unwind the flap substrate (ADP, GTP, H_2O) (Figure 3A). The results indicate that a significantly diminished, but still detectable, stimulation of FEN-1 cleavage was evident when WRN catalyzed release of greater than 80% of 5′ flap DNA substrate molecules. Thus, the ability of WRN to stimulate FEN-1 cleavage is optimal under conditions in which WRN does not unwind the 5′ flap structure.

The results from these experiments suggested that the level of WRN-catalyzed unwinding of the 5′ flap substrate would incrementally alter the stimulation of FEN-1 cleavage by WRN. To address this hypothesis, we examined FEN-1 cleavage and WRN-catalyzed DNA unwinding as a function of ATP concentration (Figure 3C). WRN helicase activity increased proportionately with ATP concentration up to 100 μM ATP, resulting in 45% 5′ flap displacement at 100 μM ATP. At 200 μM ATP, the level of DNA unwinding began to achieve a plateau of 55% substrate unwound. A maximum level of unwinding of 60% was achieved at 1000 μM ATP. The midpoint of the ATP titration for WRN helicase activity on the 5′ flap DNA substrate was in the range of 20−100 μM, a result consistent with the published K_m value for ATP in the WRN helicase reaction on a 20 bp oligonucleotide duplex substrate with flanking 3′ and 5′ ssDNA tails on opposite ends of the same strand (*22*). These reaction products were also examined on denaturing gels to quantitate FEN-1 cleavage products. FEN-1 (5 fmol) alone cleaved 5% of the substrate in the presence of ATP (Figure 3A,B) or the absence of ATP (data not shown). When WRN (20 fmol) was present in the reaction, FEN-1 cleavage was stimulated

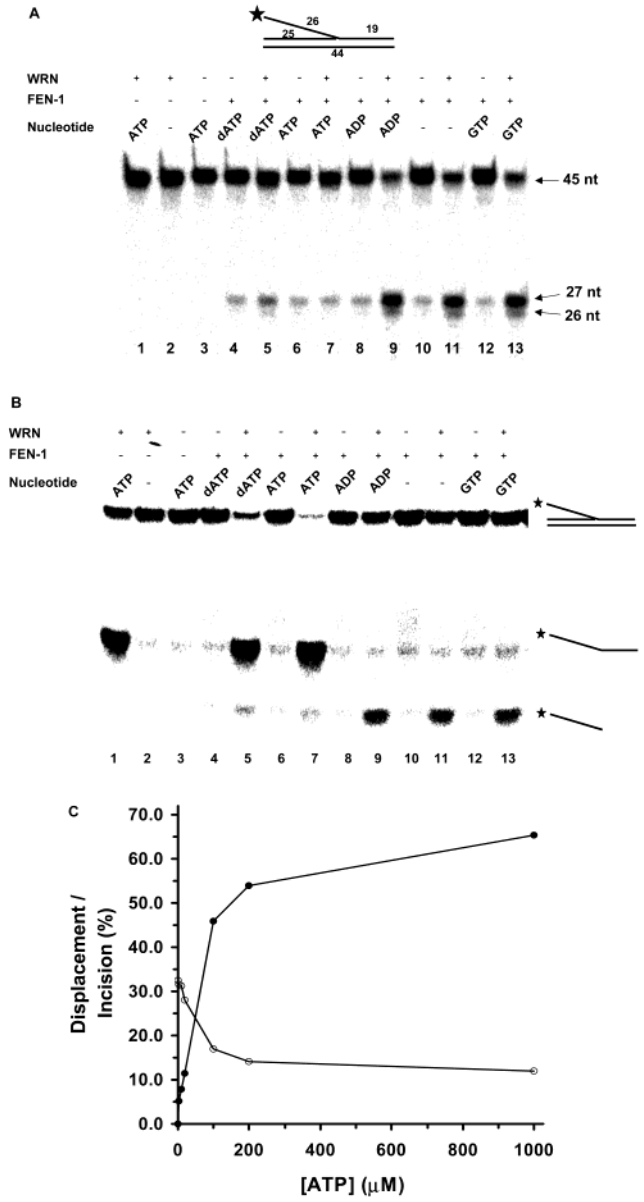


FIGURE 3: Nucleoside triphosphate dependence of WRN helicase activity and stimulation of FEN-1 cleavage. Reaction mixtures (20 μL) containing 10 fmol of a 26 nt 5′ flap DNA substrate, 5 fmol of FEN-1, and 40 fmol of WRN were incubated in the presence of different nucleotides as indicated at 37 °C for 15 min under standard conditions. Nine-microliter aliquots of reaction volumes were quenched with 5 μL Formamide Stop Solution or Helicase Stop Solution and electrophoresed on a 20% polyacrylamide 7 M urea denaturing gel (panel A) or 12% polyacrylamide nondenaturing gel (panel B), respectively, and analyzed by PhosphorImager analysis. Panel C: Dependence of WRN helicase activity and stimulation of FEN-1 cleavage on ATP concentration. Reactions were conducted as described above in the presence of the indicated concentrations of ATP. Percent incision and strand displacement are shown by open circles and filled circles, respectively.

6.5-fold (34% incision) in the absence of ATP and 2-fold (12% incision) in the presence of 2 mM ATP. As ATP concentration was increased, FEN-1 cleavage of the 5′ flap substrate decreased. At 100 μM ATP, FEN-1 incision decreased to 17%, a 2-fold reduction in FEN-1 cleavage compared to the reactions containing FEN-1 and WRN conducted in the absence of ATP. Slightly greater reduction of FEN-1 cleavage was observed at higher concentrations of ATP. The ATPase/helicase-deficient WRN mutant protein,

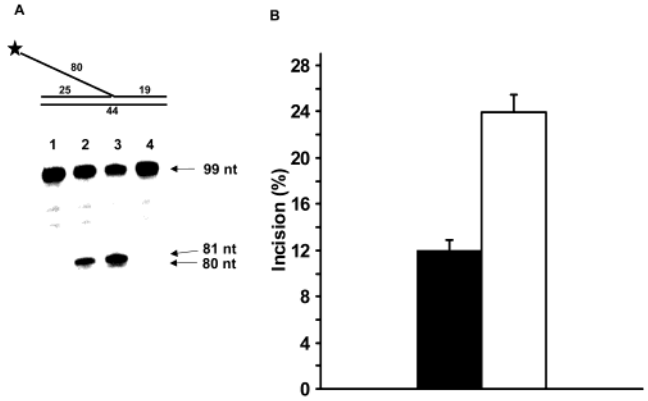


FIGURE 4: WRN is able to stimulate FEN-1 cleavage of an 80 nt 5′ flap substrate. Panel A: Reaction mixtures (20 mL) containing 10 fmol of an 80 nt 5′ flap DNA substrate, 5 fmol of FEN-1, and 10 fmol of WRN were incubated at 37 °C for 15 min under standard conditions as described in Materials and Methods. A phosphorimage of a typical gel is shown. Lane 1, no enzyme; lane 2, FEN-1; lane 3, FEN-1 + WRN; lane 4, WRN. Panel B: % incision from panel A (mean value of three experiments), with standard deviation (SD) indicated by error bars.

WRN−K577M, stimulated FEN-1 cleavage similarly under all ATP concentrations (0−2 mM) (data not shown), providing further evidence that WRN stimulation of FEN-1 cleavage is downregulated by the action of the WRN unwinding function rather than some other effect of ATP on the WRN−FEN-1 functional interaction. The ability of the WRN−K577M WRN mutant protein to stimulate FEN-1 cleavage of the 26 nt 5′ flap substrate equally well in the presence or absence of ATP indicates that the stimulation of FEN-1 cleavage by WRN is independent of WRN ATPase/helicase activity. This result is consistent with earlier work on a 1 nt 5′ flap substrate (*47*) and also with the results presented in Figure 2C,D that demonstrate the C-terminal WRN protein fragment that is devoid of catalytic domains (His−$WRN_{940-1432}$) can mediate the functional interaction with FEN-1. We conclude that WRN activation of FEN-1 cleavage activity on the 5′ flap substrate can be altered by the 5′ flap strand displacement catalyzed by WRN helicase.

WRN Stimulates FEN-1 Cleavage of a Very Long 5′ ssDNA Flap Substrate. The processing of Okazaki fragments may require that very long 5′ flaps arising from strand displacement during efficient DNA synthesis by polymerase δ/PCNA be removed. Although the initiator RNA and DNA fragment synthesized by polymerase δ is 30−40 nucleotides long, it is conceivable that efficient strand displacement by the polymerase δ/PCNA complex coupled with helicase-catalyzed unwinding may give rise to longer 5′ flap structures. Therefore, we were interested in what effect WRN might have on FEN-1 cleavage of a long 80 nt 5′ flap substrate. In the presence of 5 fmol of FEN-1, 12% of the substrate was incised (Figure 4A, lane 2, and Figure 4B). In the presence of equimolar WRN (10 fmol), FEN-1 incised 24% of the 80 nt 5′ flap molecules (Figure 4A, lane 3, and Figure 4B). Importantly, WRN alone did not cleave the long 5′ flap substrate to yield the 80 nt incision product (Figure 4A, lane 4). These results demonstrate that WRN is able to stimulate FEN-1 cleavage of substantially long 5′ flaps that might arise from strand displacement during lagging strand synthesis.

Biotin-Streptavidin Complexes Positioned in the Upstream Duplex Do Not Block WRN Stimulation of FEN-1 Flap Cleavage. Presently, the site on the DNA substrate where WRN acts to stimulate FEN-1 is not known. The mechanism for stimulation of FEN-1 cleavage by WRN may require that WRN track along the upstream DNA sequence that resides below the flap, as reported for PCNA (*56*). PCNA, a homotrimeric toroidal protein, is required to encircle the double-stranded upstream region to be functional. Although we do not know the active assembly state of WRN as a helicase on DNA, it is relevant that recombinant WRN protein has been reported to exist in a trimer-hexamer equilibrium (*57*). In addition, a recombinant WRN protein fragment spanning residues 70−240 was found to exist in a trimer−hexamer equilibrium in solution, and the hexamer form is stabilized upon binding DNA (*58*). Interaction of WRN_{70-240} with PCNA also induces hexamer formation (*58*). These studies suggest a structural similarity of WRN with PCNA and raise the possibility that WRN may function in certain contexts such as PCNA. Alternatively, the ability of WRN to recognize the flap junction to initiate DNA unwinding (*24*) may preclude a requirement for WRN to load onto the upstream duplex to exert its stimulatory effect on FEN-1 cleavage. To address these possibilities, we have tested WRN for stimulation of FEN-1 cleavage of a flap substrate with specifically positioned biotin-streptavidin complexes. The streptavidin−biotin complex is an extremely strong interaction ($K_D \sim 10^{-15}$ M) (*59*), and the diameter of a streptavidin tetramer, ~45 Å (*60*), has been previously shown to block PCNA from sliding on to the linear duplex DNA when it is positioned at the DNA end below the flap thereby preventing stimulation of FEN-1 cleavage. Initially, we tested a DNA substrate that has a biotin moiety conjugated to the terminal 3′ nt of the strand complementary to the upstream primer (Figure 5A). Gel shift analysis of the biotin-conjugated DNA flap substrates that were preincubated in the presence of streptavidin indicated nearly 100% of the substrate was bound by streptavidin (data not shown). The results in Figure 5A,B provide clear evidence that WRN is able to efficiently stimulate FEN-1 when the biotin conjugate at the DNA end below the flap is bound to streptavidin. Quantitation of the incision products demonstrated that FEN-1 (5 fmol) incision was stimulated 7-fold by WRN (40 fmol) when streptavidin is bound to the DNA substrate (Figure 5A, lanes 6 and 7, and Figure 5B), a similar fold increase observed for the substrate not bound to streptavidin (Figure 5A, lanes 2 and 3, and Figure 5B). Importantly, WRN alone did not catalyze cleavage of the 5′ flap substrate (Figure 5A, lanes 4 and 8). These results suggest that WRN is not required to load onto the end of the upstream duplex of the flap substrate to stimulate FEN-1 cleavage. Although the DNA loading steps of WRN to 5′ flap substrates has not been determined, we have observed that WRN can effectively unwind 5′ flap substrates with a biotin−streptavidin complex bound to the upstream duplex end (*24*). These results suggest that WRN does not load from a free end to catalyze unwinding or stimulate FEN-1 cleavage of the flap substrate.

The 5′ flap DNA substrate with streptavidin bound to biotin at the terminus of the upstream sequence provided 25 bp for WRN to load in the upstream duplex adjacent to the 5′ flap. To address the possibility that WRN required an unobstructed upstream duplex adjacent to the junction of the

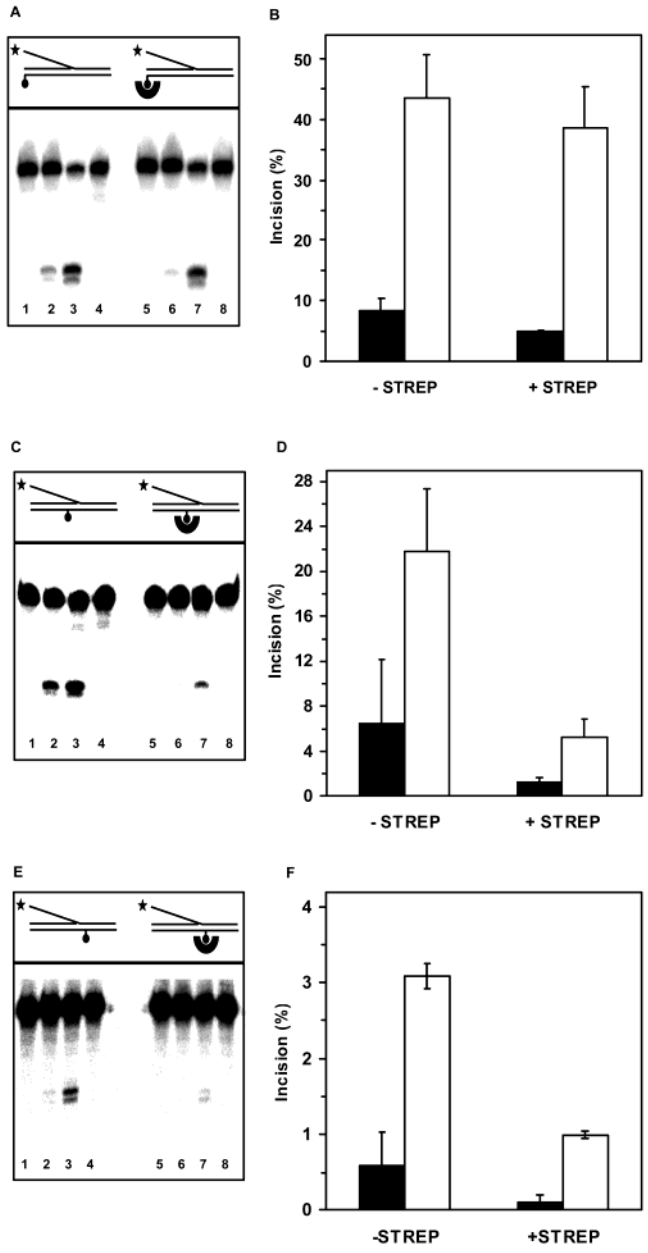


FIGURE 5: WRN is able to stimulate FEN-1 cleavage of the 5′ flap substrate despite the presence of a streptavidin−biotin complex in the upstream or downstream duplex regions. A 26 nt 5′ flap substrate with biotin conjugated to the upstream duplex terminal 3′ nt (panel A), a nucleotide positioned 6 nt upstream of the flap junction (panel C), or a nucleotide positioned 3 nt downstream of the flap junction (panel E) preincubated in the absence (lanes 1−4) or presence (lanes 5−8) of streptavidin (15 nM) for 10 min at 37 °C was subsequently incubated with FEN-1 (5 fmol, panel A, or 20 fmol, panels C and E) and WRN (40 fmol) at 37 °C for 15 min as described in Materials and Methods. A phosphorimage of a typical gel for each substrate is shown. Lanes 1 and 5, no enzyme; lanes 2 and 6, FEN-1; lane 3 and 7, WRN + FEN-1; lanes 4 and 8, WRN. Panels B, D, and F: % incision from panels A, C, and E, respectively. Data (mean value of three experiments with SD) for reactions containing FEN-1 or FEN-1 + WRN are shown by black and white bars, respectively.

5′ ssDNA flap rather than a free DNA end at the upstream sequence to activate FEN-1 cleavage, we tested the same 5′ flap substrate; only the biotin was positioned 6 nt upstream of the junction where the "elbow" of the 5′ flap resides (Figure 5C). Streptavidin was effectively bound to the DNA

substrate throughout the time course of the experiment as detected by a gel-shifted species when streptavidin is present (data not shown). For this substrate, the presence of the biotin conjugate 6 nt upstream of the cleavage site partially inhibited FEN-1 incision (Figure 5C, lane 2, and Figure 5D) such that a 4-fold greater amount of FEN-1 (20 fmol) was required to achieve the same level of incision as observed when 5 fmol of FEN-1 was used in the cleavage reaction with the flap substrate that had biotin attached to the end of the upstream sequence (Figure 5A,B). For the internal biotin-conjugated flap substrate, WRN (40 fmol) stimulated FEN-1 to cleave 22% of the flap substrate, a 3-fold increase compared to the reaction of FEN-1 alone (Figure 5C, lanes 2 and 3, and Figure 5D). When streptavidin was bound to this substrate, FEN-1 cleavage was reduced to a reproducibly measurable 1.5% incision (Figure 5C, lane 6, and Figure 5D). In the presence of WRN, FEN-1 cleavage was reproducibly stimulated ~3-fold, yielding 5% incision (Figure 5C, lane 7, and Figure 5D). The effect of a biotin−streptavidin complex positioned downstream of the junction on WRN stimulation of FEN-1 cleavage was also examined (Figure 5D,E). For this substrate, the presence of the biotin conjugate positioned 3 nt downstream of the junction also partially inhibited FEN-1 incision, so 20 fmol of FEN-1 was used to achieve 0.6% incision (Figure 5E, lane 2, and Figure 5F). In the presence of WRN (40 fmol), FEN-1 cleavage was stimulated ~5-fold for the streptavidin-free 5′ flap substrate (Figure 5E, lane 3, and Figure 5F). The presence of streptavidin bound to the biotin moiety positioned just downstream of the 5′ flap junction reduced FEN-1 incision to a barely detectable level of 0.1% incision (Figure 5E, lane 6, and Figure 5F). However, WRN reproducibly stimulated FEN-1 cleavage of the streptavidin-bound 5′ flap substrate to a level of 1% substrate incised (Figure 5E, lane 7, and Figure 5F). These results indicate that WRN is able to stimulate FEN-1 cleavage of the 5′ flap substrate, even when FEN-1 cleavage is partially inhibited by a steric block in the upstream or downstream duplex that is in close proximity to the junction.

WRN Activates FEN-1 Cleavage on Pseudo-Y Substrates. For certain DNA sequences, pseudo-Y substrates that lack the upstream primer are cleaved less efficiently by FEN-1 (*48*). The pseudo-Y version of the 5′ flap substrate used in Figure 2 is closely related in sequence to a substrate that belongs to this class of upstream primer-dependent FEN-1 cleavage substrates (*48*). We tested the pseudo-Y substrate for cleavage by FEN-1 (40, 80 fmol) and found that it was weakly acted upon by the endonuclease (Figure 6A, lanes 2 and 6). Four percent of the substrate was incised using 40 fmol of FEN-1 (Figure 6A, lane 6, and Figure 6B), yielding detectable products ranging from 19 to 23 nt. Cleavage of the same substrate with an upstream primer present in the structure using 40 fmol of FEN-1 yielded 15% incision and resulted exclusively in the formation of the 26 and 27 nt oligonucleotide products (Figure 2A). Thus, the upstream primer alters the efficiency and specificity of FEN-1 cleavage. In the presence of WRN (80 fmol), FEN-1 cleavage of the pseudo-Y substrate was dramatically stimulated in reactions containing either 40 fmol (Figure 6A, lane 3) or 80 fmol (Figure 6A, lane 7) of FEN-1. Quantitative analysis of the results demonstrated a 10-fold stimulation of FEN-1 cleavage when WRN was present in the reaction containing

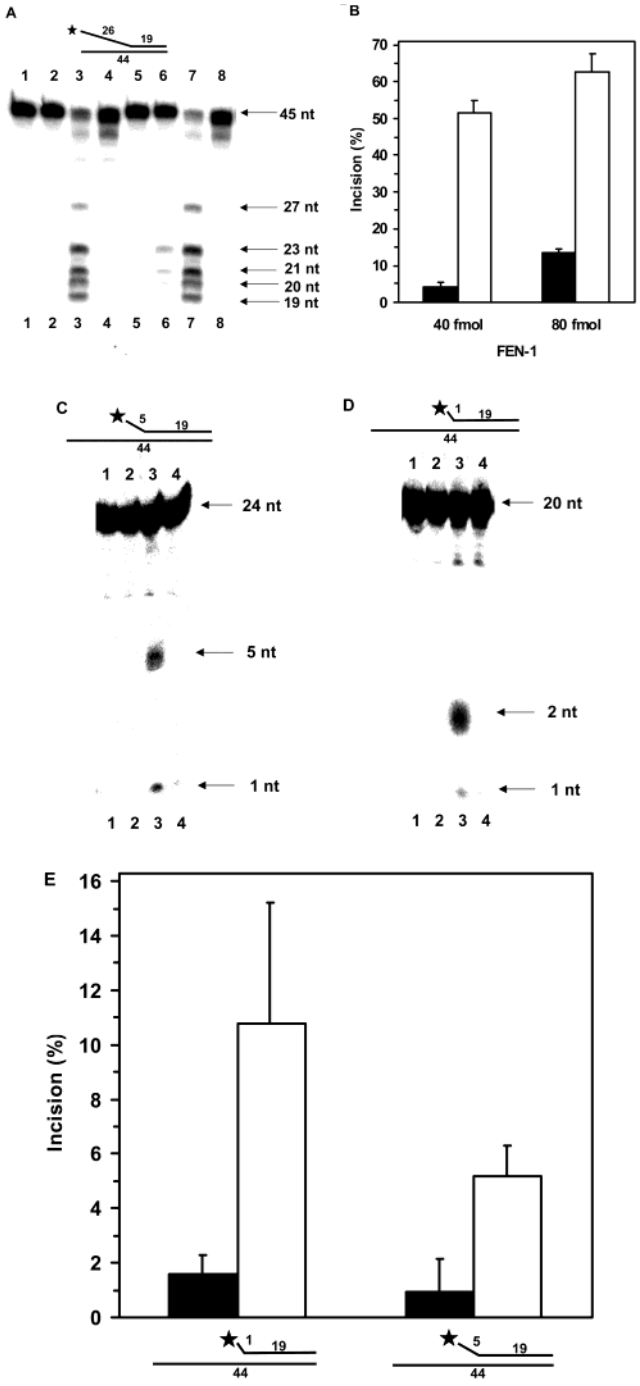


FIGURE 6: WRN stimulates FEN-1 cleavage on pseudo-Y substrates that lack the upstream primer. Panel A: Reaction mixtures (20 μL) containing 10 fmol of a 26 nt 5′ tail pseudo-Y substrate, 40 or 80 fmol of FEN-1 as indicated, and 80 fmol of WRN were incubated at 37 °C for 15 min under standard conditions. A phosphorimage of a typical gel is shown. Substrate and cleavage products are as indicated. Lane 1, no enzyme; lane 2, FEN-1 (40 fmol); lane 3, FEN-1 (40 fmol) + WRN; lane 4, WRN; lane 5, no enzyme; lane 6, FEN-1 (80 fmol); lane 7, FEN-1 (80 fmol) + WRN; lane 8, WRN. Panel B: % incision from Panel A (mean value of 3 experiments) with SD indicated by error bars. Panels C and D: Reaction mixtures (20 μL) containing 10 fmol of a pseudo-Y substrate with a 5 nt (panel C) or 1 nt (panel D) 5′ ssDNA tail, 80 fmol of FEN-1, and 80 fmol of WRN were incubated at 37 °C for 15 min under standard conditions. A phosphorimage of a typical gel for each substrate is shown. Substrate and cleavage products are as indicated. Lane 1, no enzyme; lane 2, FEN-1; lane 3, FEN-1 + WRN; lane 4, WRN. Panel E: % incision from panels C and D. Data (mean value of three experiments with SD) for reactions containing FEN-1 or FEN-1 + WRN are shown by black and white bars, respectively.

40 fmol of FEN-1 (Figure 6B). Using 80 fmol of FEN-1, the stimulation by WRN was less (4−fold), but still significant (Figure 6B). An analysis of the cleavage pattern demonstrated that the primary products were the same as those in the reactions containing only FEN-1 ranging in size from 19 to 23 nt, but the 27 nt product was also observed to a lesser extent (4% of the substrate cleaved). Importantly, WRN alone did not catalyze significant cleavage of the pseudo-Y DNA substrate (Figure 6A, lanes 4 and 8). The band migrating just below the intact 45-mer in lanes 4 and 8 results from WRN 3′ to 5′ exonuclease digestion of a fraction of the forked duplex substrate molecules from the blunt duplex end, as previously reported (*61*). No exonuclease digestion products were observed when the substrates were incubated with a WRN-E84A mutant protein that is devoid of exonuclease activity (data not shown). These results indicate that WRN can effectively activate FEN-1 on the cleavage-resistant pseudo-Y structure. WRN primarily activates FEN-1 to cleave within the 5′ ssDNA tail of the pseudo-Y structure at sites upstream of the single stranded-double stranded DNA junction but also to a minor extent 1 nt within the duplex junction.

To further characterize the effect of WRN on FEN-1 cleavage of the pseudo-Y substrate, we tested pseudo-Y substrates with shorter 5′ ssDNA tails of 5 or 1 nt as compared to the substrate with the longer 26 nt tail tested above. The presence of upstream primers in the 5 and 1 nt 5′ flap substrates, like the 26 nt 5′ flap substrate, display structure-specific cleavage to yield incision products of 5 and 6 nt species for the 5 nt 5′ flap and 1 and 2 nt for the 1 nt 5′ flap (*47*). The results of the FEN-1 incision assays with the pseudo-Y substrates with these shorter 5′ tails are shown in Figure 6C,D. Both pseudo-Y substrates were nearly completely resistant to cleavage by FEN-1 (80 fmol) (Figure 6C,D, lane 2, and Figure 6E). In the presence of WRN (80 fmol), FEN-1 incised 5% of the pseudo-Y 5 nt 5′ tail substrate to produce the 6 nt product (Figure 6C, lane 3, and Figure 6E). Likewise, WRN activated FEN-1 to incise 10% of the pseudo-Y 1 nt 5′ tail substrate to generate the 2 nt product (Figure 6D, lane 3, and Figure 6E). WRN alone did not catalyze significant cleavage of the pseudo-Y DNA substrate (Figure 6C,D, lane 4). These results demonstrate that the presence of WRN in the FEN-1 cleavage reaction activates the endonuclease to incise pseudo-Y structures with variable lengths of 5′ ssDNA tails.

Recently it was reported that a purified recombinant human WRN protein fragment spanning amino acid residues 70−240 (hWRN−N_{70-240}) exhibited a 5′ protruding ssDNA endonuclease activity (*58*). We have not observed this endonucleolytic activity of full-length recombinant WRN on DNA substrates with 5′ protruding ends and/or 3′ protruding ends ((*24*); this study) or 5′ flap structures with either short (*47*) or long 5′ ssDNA tracts (this study). We conclude that the purified recombinant human full-length WRN protein does not possess an endonucleolytic activity on any of the DNA substrates tested thus far and cannot readily explain the discrepancy between the reported endonucleolytic activity of the hWRN−N_{70-240} protein fragment (*58*) and our results with the full length WRN protein.

WRN Does Not Obviate the Requirement for a 5′ ssDNA Tract in the Flap for FEN-1 to Efficiently Cleave the Flap Substrate. FEN-1 has been shown to cleave the 5′ ssDNA flap by recognizing the 5′ end, tracking the length of the tail, and incising at the ssDNA-dsDNA junction. The unannealed 5′ tail must be single-stranded; flap substrates with foldback structures within the 5′ flap effectively inhibit FEN-1 cleavage (*62*). A model for genomic instability and repeat sequence expansion has been proposed based on the resistance of such secondary structures in the 5′ flap to FEN-1 cleavage (*63*). It was conceivable that WRN might alleviate the inhibition of FEN-1 cleavage imposed by secondary structure. To address this question, we tested the effect of WRN on FEN-1 cleavage of a DNA substrate containing complementary sequences in the 5′ flap that generate a stem-loop structure with 24 bp in the annealed region. This flap substrate was previously demonstrated to effectively block the FEN-1 structure-specific cleavage activity at the base of the flap (*62*). Instead, FEN-1 incised the 5′ end of the self-annealed 5′ tail, resulting in mono- and dinucleotide products (*62*). The control substrate that contains a standard 26 nt 5′ flap structure was cleaved by FEN-1 to yield the 26 and 27 nt products (*62*), a result that we also obtained (Figure 2).

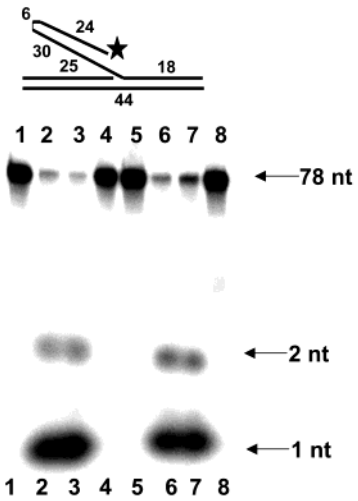
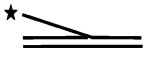
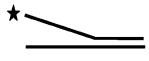


FIGURE 7: WRN does not stimulate FEN-1 cleavage of a foldback flap structure. Reaction mixtures (20 μL) containing 10 fmol of a foldback substrate, 100 fmol of FEN-1, and 80 fmol of WRN were incubated at 37 °C for 15 min under standard conditions. Reactions were conducted either in the absence (lanes 1−4) or presence (lanes 5−8) of 2 mM ATP. A phosphorimage of a typical gel is shown. Substrate and cleavage products are as indicated. Lanes 1 and 5, no enzyme; lanes 2 and 6, FEN-1; lanes 3 and 7, FEN-1 + WRN; lanes 4 and 8, WRN.

The results of a typical experiment with WRN and FEN-1 on the secondary structure flap substrate are shown in Figure 7. FEN-1 (100 fmol) cleavage of the flap substrate with the 24 bp foldback resulted in the generation of mono- and dinucleotide products (Figure 7, lane 2). The additional presence of WRN (80 fmol) with FEN-1 did not alter the FEN-1 cleavage pattern (lane 3). WRN clearly did not activate FEN-1 cleavage at the base of the flap to yield the 60 nt product (lane 3). WRN alone did not incise the substrate, as expected (lane 4). Similar results were obtained in the presence of ATP (Figure 7, lanes 5−8). In reactions containing the secondary structure flap substrate and reduced amounts of FEN-1 (1, 10 fmol), FEN-1 cleavage resulted in a reduced amount of mono- and dinucleotide products, and no effect of WRN on FEN-1 cleavage was observed (data not shown). These results indicate that WRN cannot activate FEN-1 incision of a flap substrate containing secondary

Table 3: Kinetic Parameters for FEN-1 Cleavage

Substrate	(#)	Reaction	K_m (nM)	V_{max} (nM/s)	Fold Stimulation of V_{max}
	(11)	FEN-1 only	3.9 ± 2	9.1 ± 3 x 10^{-5}	
		FEN-1 + WRN	3.9 ± 1	1.5 ± 0.2 x 10^{-3}	16
	(2)	FEN-1 only	13 ± 4	6.8 ± 2 x 10^{-4}	
		FEN-1 + WRN	14 ± 5	4.7 ± 0.1 x 10^{-3}	6.9
	(7)	FEN-1 only	11 ± 2	2.4 ± 0.2 x 10^{-3}	
		FEN-1 + WRN	11 ± 3	1.5 ± 0.3 x 10^{-2}	6.3

FEN-1 reactions for Michaelis-Menton kinetic studies contained 5 fmol FEN-1, 100 fmol WRN, and the indicated DNA substrate (0 - 40 nM). Double reciprocal plots (1/ product formation velocity (nM/s)) versus (1/ substrate concentration (nM)) were used to determine the kinetic parameters K_m and V_{max} by linear regression analysis.

structure that is resistant to structure-specific cleavage. The inability of WRN to enable FEN-1 to bypass the flap secondary structure to cleave at the base of the flap suggests that WRN does not directly bind FEN-1 to the single stranded-double stranded DNA junction from solution. The results also suggest that WRN does not circumvent the obligatory step of FEN-1 tracking on the 5′ flap substrate.

Effect of WRN on the Kinetic Parameters for FEN-1 Cleavage. To better understand the mechanism whereby WRN stimulates the FEN-1 cleavage reaction, we conducted FEN-1 incision assays with DNA substrate concentrations ranging from 0 to 40 nM using 5 fmol of FEN-1 in the presence or absence of WRN (100 fmol). WRN (100 fmol) alone did not cleave any of the DNA substrates tested. Quantitation of the incision products demonstrated that at the highest concentrations of DNA substrate tested (20, 40 nM), reaction velocities (nM/s) achieved a plateau (data not shown). The kinetic incision data obeyed Michaelis−Menton kinetics and were transformed by Lineweaver−Burke analysis to determine the kinetic parameters K_m (Michaelis−Menton constant) and V_{max} (maximal reaction velocity). The results of these analyses are shown in Table 3. For a 5′ flap substrate with a single unannealed 5′ deoxynucleotide (1 nt 5′ flap), the kinetic parameter K_m was virtually unchanged by the presence of WRN in the FEN-1 cleavage reaction. In contrast, the V_{max} of the incision reaction was elevated 16-fold by the presence of WRN. Similarly, no significant effect of WRN on the K_m value for FEN-1 cleavage of the 26 nt 5′ flap substrate was detected, whereas a 7-fold increase in V_{max} was observed. Analysis of the related 26 nt 5′ tailed substrate that lacked the upstream primer also showed a significant increase (7.5-fold) of the kinetic parameter V_{max}, but no effect on K_m, when WRN was present. These results indicate that WRN stimulates the FEN-1 cleavage reaction of all three DNA substrates by increasing the reaction velocity, suggesting that WRN improves the efficiency of the FEN-1 cleavage reaction, but not DNA substrate binding by FEN-1.

DISCUSSION

In this study, we have examined mechanistic aspects of the functional interaction between WRN protein and FEN-1. The determination of how WRN stimulates the FEN-1 cleavage reaction is important to understanding how these proteins are likely to function together within a physiological context of dynamic protein−protein and protein-DNA interactions that occur during DNA replication or repair in vivo.

The mechanism for WRN stimulation of FEN-1 cleavage was investigated by determining the steady-state kinetic parameters of the reaction. Kinetic analyses of the FEN-1 cleavage reaction for both 1 and 26 nt 5′ flap substrates, as well as a pseudo-fork structure, demonstrate that the presence of WRN increases the V_{max} of the cleavage reaction but has no significant effect on K_m. These results suggest that WRN improves the efficiency of the FEN-1 cleavage reaction but does not alter the reaction rate at subsaturating DNA concentrations. It is possible that the protein interaction between WRN and FEN-1 alters the structure of FEN-1 to improve its catalytic substrate turnover capacity or the orientation of FEN-1 on the DNA substrate. The impact of WRN on K_m and V_{max} for the FEN-1 cleavage reaction of the 26 nt flap substrate is distinct from the reported effect of PCNA on FEN-1 cleavage of a similar substrate in which the V_{max} was improved only 2-fold whereas the K_m was reduced 11.5-fold (*48*). The results of Tom et al. (2000) are consistent with a mechanism in which PCNA enhances FEN-1 binding stability resulting in enhanced cleavage. Our experimental results from kinetic analyses suggest that WRN stimulates FEN-1 cleavage by a mechanism distinct from that of PCNA.

To begin to understand the DNA structural elements important for WRN to stimulate FEN-1 cleavage of the 5′ flap substrate, we tested the effect of WRN on FEN-1 cleavage of a 5′ flap substrate with a specifically positioned steric block in the upstream duplex adjacent to the 5′ flap junction. WRN was able to effectively stimulate FEN-1 cleavage of the 5′ flap substrate when streptavidin was bound to biotin positioned at the end of the upstream duplex. These results indicate that WRN does not require a free upstream duplex end to load on to the substrate for FEN-1 activation. Similar studies have indicated that the entry of PCNA on to the 5′ flap substrate is blocked by the presence of streptavidin bound to the end of the upstream duplex, suggesting that PCNA must be on the 5′ side of the flap to stimulate FEN-1 (*56*). We further tested WRN for stimulation of FEN-1 cleavage on a flap substrate with the biotin−streptavidin complex positioned 6 nt upstream or 3 nt downstream of the flap junction. WRN still retained the ability to stimulate FEN-1 cleavage despite the fact that the position of the complex (upstream or downstream) inhibited the FEN-1 cleavage reaction. In the absence of a cocrystal structure for a Rad2 family member with a DNA flap substrate, it could be speculated that the requirement for a greater amount of FEN-1 to cleave the 5′ flap substrate with biotin positioned close to the junction suggests that important DNA contacts or structural integrity elements facilitate recognition and/or catalysis by human FEN-1. Consistent with this notion, it was recently proposed that Arg-47 and Arg-70 in human FEN-1 are involved in the interaction with the upstream duplex of a 5′ flap substrate (*64*). The crystal structure of *Pyrococcus furiosus* FEN-1 suggests that the highly conserved H3TH motif also functions in DNA binding via its interactions with the phosphodiester backbone of the dsDNA of the flap substrate (*65*). Importantly, the stimulatory effect of WRN on FEN-1 cleavage using the upstream streptavidin-bound DNA substrate is consistent with the ability of WRN to effectively unwind the same streptavidin-bound substrate in the presence of ATP (*24*), suggesting that WRN utilizes its ability to recognize the junction of the 5′

flap substrate to stimulate FEN-1 cleavage. These results, together with the kinetic analyses, provide solid evidence that the mechanism by which WRN stimulates FEN-1 cleavage is different from that of PCNA.

The ability of WRN to unwind the 26 nt 5′ ssDNA flap structure suggests that WRN may coordinate the strand displacement reaction with activation of FEN-1. We previously reported that WRN is capable of stimulating FEN-1 cleavage of the 1 nt 5′ flap substrate (also used in this study) in the presence or absence of ATP (*47*). Analysis of WRN helicase activity on the 1 nt 5′ flap substrate indicates that the substrate is inefficiently unwound by WRN (*24*); consequently, the presence of ATP does not significantly modulate the ability of WRN to stimulate FEN-1 cleavage of the 1 nt 5′ flap substrate. The 26 nt 5′ flap substrate is unwound significantly more efficiently by WRN compared to the 1 nt 5′ flap substrate (*24*), consistent with the results presented here showing a dependence of WRN stimulation of FEN-1 cleavage of the long (26 nt) 5′ flap structure on ATP concentration. These results might suggest that WRN stimulation of FEN-1 cleavage of short 5′ flaps is likely to be an important feature of the WRN−FEN-1 interaction whereas WRN helicase activity on longer 5′ flap substrates prevails in vivo. In support of a specific function of FEN-1 on short 5′ flap structures, it was recently demonstrated that a *rad27* disruption mutant displayed reduced NHEJ in a pathway that requires the removal of short 2 nt 5′ flaps (*66*). A model was presented in which FEN-1 removes the 5′ flaps generated after end alignment prior to ligation. A role for WRN in NHEJ may involve its ability to stimulate FEN-1 in the trimming of short 5′ flaps during the end-processing step. Alternatively, WRN may regulate patch size during BER by its stimulation of FEN-1 cleavage of 5′ ssDNA flaps that are intermediates of the pathway. Yet another possible role for WRN is the stimulation of FEN-1 cleavage of short 5′ flaps during Okazaki fragment processing. Further studies are necessary to determine precisely the cellular pathways WRN directly plays a role in and how its interaction with FEN-1 is biologically important.

The demonstration that WRN stimulation of FEN-1 cleavage of the 26 nt 5′ flap substrate is reciprocally related to helicase displacement of the 5′ flap ssDNA strand suggests that factors which modulate WRN helicase activity on the 5′ flap structure may affect the ability of WRN to stimulate FEN-1 cleavage. It is possible that other cellular proteins that participate in DNA replication or repair may stimulate or inhibit WRN helicase function, thereby modulating the effect of WRN on FEN-1 cleavage. For example, the single stranded DNA binding protein RPA strongly stimulates WRN helicase activity (*23*), whereas DNA-PK_{CS} reportedly inhibits WRN helicase activity (*67*). The results presented in this study suggest that WRN serves dual functions as a helicase and activator of FEN-1 cleavage on longer 5′ flap structures. The tentative identification of WRN in a replication complex (*32*) supports the notion that other proteins in the complex may affect the functional interaction between WRN and FEN-1.

The genomic instability and replication defects of WS cells are consistent with the notion that WRN has a direct role in DNA replication. Stimulation of FEN-1 cleavage of 5′ flaps may be an important role of WRN in Okazaki fragment processing during lagging strand synthesis at the replication fork. One current model for Okazaki fragment processing based on biochemical studies of SV40 viral DNA replication using mammalian cell extracts proposes that RNase H1 removes the initiator RNA of the primed DNA lagging strand, leaving a single ribonucleotide at the RNA-DNA junction that is subsequently incised by FEN-1 (*35*). Evidence presented here demonstrates that WRN is able to stimulate FEN-1 cleavage of the 1 ribonucleotide 5′ flap efficiently. Longer 5′ flaps that potentially arise during strand displacement by polymerase δ also need to be cleaved in order for proper maturation of the newly synthesized lagging strand. Recently, a model for Okazaki fragment processing was proposed in which pol PCNA displacement of the RNA/DNA fragment synthesized by pol δ/primase eliminates the replication errors introduced by pol δ since it lacks a proofreading subunit (*35*). In vitro studies indicate that the size of the displaced strand may be modulated by the concerted action of polymerase δ, PCNA, RPA, and FEN-1 (*68*). Our results suggest that the processing of longer 5′ flaps (such as the 26 or 80 nt 5′ flap in this study) may involve WRN stimulation of FEN-1 cleavage to facilitate its removal. It was proposed that the yeast endonucleases Dna2 and Rad27 (FEN-1) act sequentially to remove long RNA-primed 5′ flaps during Okazaki fragment maturation in *S. cerevisiae* (*51*). In addition, the yeast helicase/endonuclease Dna2 is able to remove secondary structure in the 5′ flap in a reaction aided by RPA, thereby assisting the Dna2 endonuclease activity (*69*). WRN, on the other hand, did not stimulate FEN-1 cleavage of the fold-back flap structure used in this study suggesting that WRN may not resolve certain secondary structures that may arise in the flap during a process such as Okazaki fragment maturation.

WRN activation of FEN-1 cleavage of 5′ ssDNA tracts of pseudo-Y forked structures may be relevant in certain circumstances of lagging strand synthesis. If polymerase δ pauses during lagging strand synthesis, gaps between Okazaki fragments may arise. This might occur by the action of a DNA helicase (such as WRN) that continues to unwind the flap substrate by releasing the strand with the 5′ ssDNA tail, even after pol δ has stalled during the strand displacement reaction. A pseudo-Y structure with an elongating 5′ ssDNA tail would be produced that may become resistant to FEN-1 cleavage if it forms a secondary structure (*62*). The ability of WRN to stimulate FEN-1 cleavage of 5′ ssDNA tracts in pseudo-Y structures, thereby keeping them short, may prevent the formation of secondary structures that are resistant to FEN-1 cleavage even in the presence of PCNA, RPA (*62*), or WRN (this study).

The activation of FEN-1 cleavage by WRN is likely to have biological importance. A prediction from our observation is that WS cells might display some of the defects observed in *rad27* null mutants. Like WS cells, *rad27* mutants have increased spontaneous mutagenesis, genomic instability, and impaired S phase progression (*39*, *40*−*46*). The notion that human and yeast FEN-1 function similarly is supported by the demonstration that human FEN-1 protein complements a number of phenotypes of *rad27* null mutants, including mutagen sensitivity and genome stability (*70*). The observation that *rad27* temperature-sensitive mutants accumulate short DNA fragments of the expected size for unprocessed Okazaki fragments at the restrictive temperature suggests that FEN-1 participates in lagging strand synthesis

in vivo (*71*). In human cells, delayed action of FEN-1 cleavage may be deleterious because of the large number (millions) of Okazaki fragments that are produced each time the genome is replicated. The *rad27* null phenotypes of hyper-recombination (41;43) and elevated DNA rearrangements induced by inverted repeats such as Alu sequences (*72*) (that are also abundant in the human genome) suggest that similar aberrant processes may prevail in WS cells. Double strand breaks that arise from defective Okazaki fragment processing or a stalled replication fork may be lead to genomic instability in WS.

WS cells are noted for their mutator phenotype characterized by an increased frequency of deletions and chromosomal rearrangements. A nucleotide sequence analysis of the human HPRT gene mutations in WS cells revealed an elevated frequency of deletions between short repeated sequences (*73*). Sequence alignments of the deletion junction and donor sequences suggest a pathway of nonhomologous recombination was used for the end-joining. More recently, it was observed that linearized plasmids with noncompatible ends transfected into hTERT-immortalized WS fibroblasts undergo extensive deletions at nonhomologous joining ends (*74*). Importantly, the deletion phenotype was complemented by wild-type WRN (*74*), suggesting a direct role of the protein in facilitating joining of broken DNA ends with fidelity. The ability of WRN to facilitate efficient FEN-1 cleavage of 5′ flap structures may be important for the trimming process after sequence alignment during nonhomologous end-joining. A subphysiological rate of FEN-1 cleavage may result in the failure to process single DNA strands or aberrant structures that are susceptible to helicases/nucleases, ultimately giving rise to extensive degradation at broken DNA ends. Characterization of WS mutant cell lines that have been stably transfected with the WRN domain responsible for physical and functional interaction with FEN-1 should help to elucidate the importance of the WRN−FEN-1 interaction in vivo.

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