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Lack of *WRN* Results in Extensive Deletion at Nonhomologous Joining Ends[1]

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ABSTRACT

Loss of *WRN* causes the genomic instability progeroid syndrome, Werner syndrome. *WRN* encodes a multifunctional nuclear protein with 3′→5′ exonuclease and 3′→5′helicase activities. Linear plasmids with noncompatible ends introduced to Werner syndrome cells underwent extensive deletions at nonhomologous joining ends, particularly at the 3′ protruding single-stranded end. This extensive deletion phenotype was complemented by wild-type *WRN*. These results suggest that *WRN* can out-compete other exonucleases that participate in double-strand break repair or stabilize the broken DNA end.

INTRODUCTION

WS[4] (1–3) is a progeroid syndrome characterized by genomic instability and the early onset of age-related pathologies. WS patients exhibit an external appearance of accelerated aging, such as premature graying and thinning of hair, skin atrophy, and regional atrophy of s.c. fat. They develop, during middle age, many disorders commonly associated with senescence, including bilateral ocular cataracts, type 2 diabetes mellitus, gonadal atrophy, osteoporosis, several forms of arteriosclerosis, and cancers. Two major causes of death are cancer and myocardial infarction in the late 40s. Cells isolated from WS patients show genomic instability and a shorter replicative life span. The genomic instability has been characterized by variegated translocation mosaicism at the chromosomal level (4) and extensive deletions at the single locus level (5).

WS patients commonly develop a variety of benign and malignant neoplasms (1, 2) Cancer is the most common cause of death among Japanese WS patients, whose overall cancer risk is increased 30- to 50-fold over that of the general population (6). The mean age of cancer detection is 44 years (range, 25–64 years), with tumor-specific risks increased up to 1000-fold (in the case of acral lentigenous melanoma). Neoplasms in WS patients are disproportionately derived from mesenchymal cells. The ratio of carcinoma:sarcoma is ∼1:1, as opposed to 10:1 in the general population. Thyroid carcinoma is the most common epithelial neoplasm. The most common malignancies in WS are soft tissue sarcomas, osteosarcomas, acral lentigenous melanomas, and meningiomas (6).

WS is caused by mutations at the *WRN* locus (7). The *WRN* gene encodes a M_r 180,000 nuclear protein that contains an NH_2-terminal exonuclease domain (8) and a central RecQ-type helicase domain (9). RecQ is an *Escherichia coli* ATP-dependent 3′→5′ helicase. It consists of seven helicase motifs: I (ATPase), Ia, II, III, IV, V, and VI, and belongs to the DEAH (Asp-Glu-Ala-His) subfamily of DNA and RNA helicases. *E. coli* and yeast have one RecQ helicase, whereas human have at least five: *RecQL*, *WRN*, *BLM*, *RecQ4* or *RTS*, and *RecQ5*. Mutations in *BLM* and *RecQ4/RTS* cause Bloom and Rothmund-Thomson syndromes, respectively, both of which are cancer-prone syndromes in humans (3, 10). Human diseases caused by the mutations in *RecQL* or *RecQ5* have not been identified.

Several unusual substrates have been tested as potential physiological targets for WRN helicase activity. The WRNp efficiently unwound a G4 quartet made by two hairpin loops (G′2 biomolecular tetraplex) of d(CGG)n (11). Although its presence has not been demonstrated *in vivo*, a G4 quartet can potentially form from two GC rich regions of unwound single-stranded DNA (or RNA) during replication, repair, recombination, or transcription. Another interesting structure is a recombination intermediate, or α structure. WRNp promoted branch migration of a Holliday junction (12). The WRN helicase appeared to dissociate the α structure better than a simple DNA duplex, as assessed by the length of migration. dsDNA with mismatched tails is also a preferred substrate for WRN helicase (13). dsDNA substrates with multiple bp mismatches within the duplex (termed “bubbles”) and structures resembling Holliday junction are more susceptible to WRN exonuclease digestion than are simple DNA duplexes (14, 15) These structures can be formed during both recombination and replication, such as break-induced DNA replication or repair of stalled replication forks.

Thus far, biochemical and cell biological studies suggest a potential role for WRNp in DNA replication, repair, recombination, and/or transcription (10). Among these DNA transactions, those that might lead to the phenotypes of WS patients (*e.g.,* repair, recombination) are likely to be most significant for the pathogenesis of WS, whereas those demonstrated only *in vitro* (*e.g.,* transcription) may be less significant. Evidence supporting a role for WRNp in DSB repair includes the 3′→5′ directionality of the helicase and exonuclease (8, 9), hypersensitivity of WS cells to certain genotoxic agents (16–18) and to interaction with Ku70/80 (19–21), and DNA-PKcs (22), and DNA polymerase δ (23–25). The interaction with Ku70/80, DNA-PKcs, and DNA polymerase δ strongly suggests a role for WRNp in NHEJ.

Despite physical and functional interactions with proteins that are important for NHEJ, it is not known how WRNp is involved in NHEJ or how the lack of WRNp alters the efficacy or accuracy of NHEJ. In this report, we examined how WS cells join the noncompatible ends of linearized plasmids. We speculated that cells must process noncompatible ends prior to joining and that without WRNp, this process might be compromised. Here we present evidence that, in the absence of WRNp, dsDNA ends lose an excessive number of nucleotides prior to nonhomologous joining. This suggests that a major role of WRNp is to minimize the removal of nucleotides during DSB repair, preventing the creation of relatively large and potentially oncogenic deletions.

MATERIALS AND METHODS

Cell Lines. Primary fibroblasts from the WS patients 73-26 and AMIE1010 were obtained from the International Registry of Werner Syndrome (Seattle, WA). Patient 73-26 is a Japanese female and carries a homozygous mutation, IVS25–1G→C, which causes skipping of exon 26 followed by a frameshift

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[4] The abbreviations used are: WS, Werner syndrome; WRNp, WRN protein; dsDNA, double-stranded DNA; DSB, dsDNA break; NHEJ, nonhomologous end joining; PSS, protruding single strand; hTERT, catalytic subunit of human telomerase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit.

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(7). AMIE1010 is a French male patient with the homozygous mutation, c.2425C→T, which alters amino acid 732 from CGA(Arg) to TGA(Stop). Lines 88-1 and 82-6 were from normal individuals (26). The cells were immortalized with retrovirus carrying the hTERT (provided by Dr. Robert A. Weinberg, Massachusetts Institute of Technology, Boston, MA) in the pBabe-Puro vector (27). The WRN-complemented line, 73-26hTERT-WRN was generated by introducing the full-length human WRN cDNA in the LXSN vector (28) into the immortalized 73-26 line. Western analysis confirmed that the parent 73-26 line was devoid of WRNp, whereas the complemented cells expressed near-normal levels of WRNp (22).

Plasmids and Transfection. pCMS-end is a pUC19-based 6305-bp plasmid (Fig. 2). It was linearized by digesting with two restriction enzymes overnight to generate incompatible ends. The digestion used *Xho*I to create a 5′ PSS, *Apa*I to create a 3′ PSS, or *Eco*47III to create a blunt end. pBlueScript-KS(−) (pBS-KS; Stratagene, La Jolla, CA) was digested with *Xho*I, *Sac*I, or *Eco*RV to generate a 5′ PSS, 3′ PSS, or blunt end, respectively.

For transfection, 2×10^5 cells were plated on 35-mm dishes, and 24 h later, 5 μg of linearized DNA were transfected using Transfast (Promega, Madison, WI), according to the manufacturer's directions. Thirty-six h later, cells were trypsinized and pelleted for analysis.

Isolation of the Plasmid and Sequencing. Plasmids were isolated from the cell pellets by an alkaline lysis method using a Qiagen Miniprep kit, and used to transform XL1-Blue competent cells. Plasmids were isolated from individual colonies, digested with restriction enzymes to estimate the extent of the deletion, and subsequently sequenced across the junction of recircularization.

Statistical Analysis. For the comparison of two sample sets, a *t* test assuming unequal variances was used. *P*s for the 95% confidence limits were used for Figs. 3 and 4.

RESULTS

To investigate the role of WRNp in DSB repair, we examined how normal and WS cells joined the noncompatible ends of the linearized pCMS-end plasmid DNA. Fig. 1 illustrates this NHEJ assay. pCMS-end was linearized with two restriction enzymes to create three different noncompatible ends: (*a*) combinations of a 5′ protruding single-stranded (PSS) end and a 3′ PSS end; (*b*) a blunt end and a 5′ PSS end; or (*c*) a blunt end and a 3′ PSS end. The linearized pCMS-end DNA was transfected to normal and WS fibroblasts that were immortalized by expressing the hTERT (Table 1). pCMS-end plasmids that were repaired by the cells to circular forms were recovered from the transfected cells and were transformed into *E. coli* to obtain single colonies containing a single circular pCMS-end plasmid. The regions across the joined ends were sequenced to determine how the noncompatible ends were removed during repair in the cell. The number of nucleotides missing from plasmids that contained protruding or blunt ends was quantified to determine the extent of the deletions.

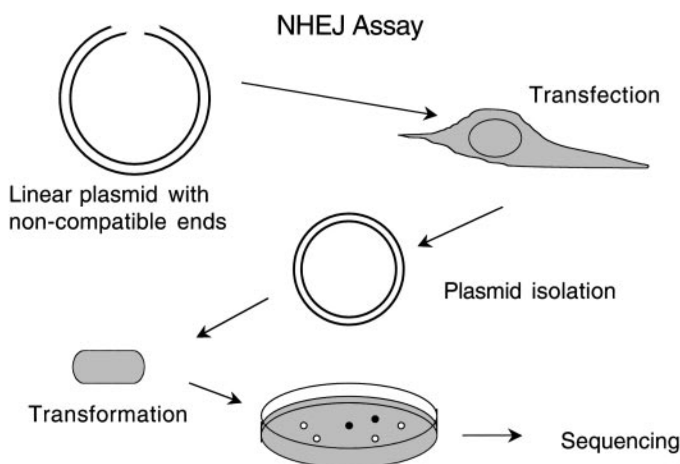

Fig. 1. Schematic diagram of the NHEJ assay.

Table 1 *Genotypes of hTERT cell lines*

hTERT line	Origin	*WRN* mutation	WRN genotype
88-1 hTERT	Caucasian	None	*WRN+/+*
82-6 hTERT	Caucasian	None	*WRN+/+*
73-26 hTERT	Japanese	IVS25-1G→C	*WRN−/−*
AMIE hTERT	French	c.2425C→T	*WRN−/−*
73-26 hTERT + WRN	Japanese	IVS25-1G→C	*WRN−/− + WRN*

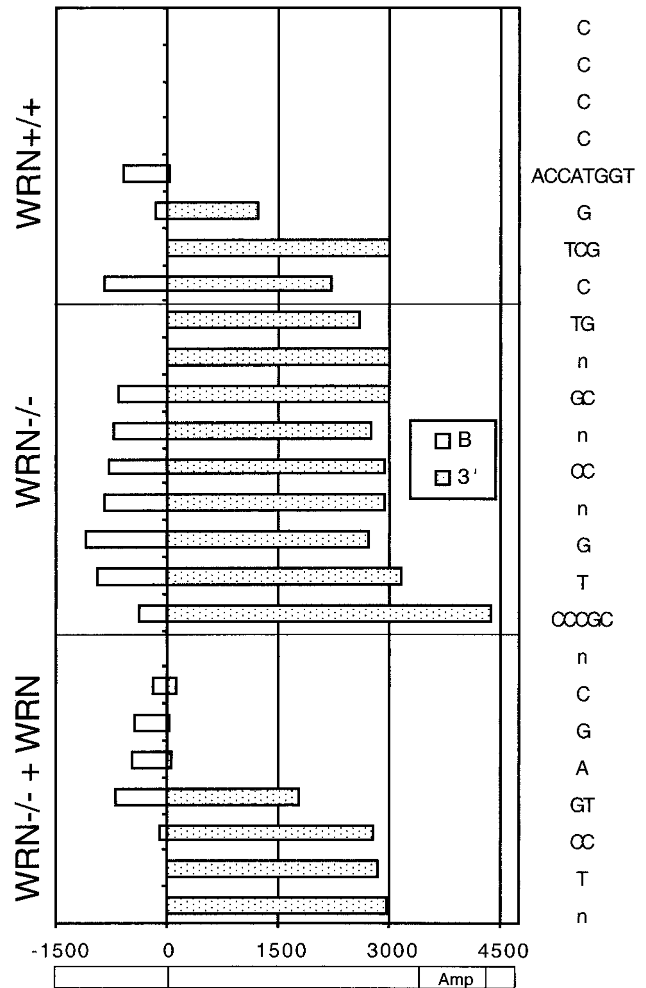

Fig. 2. Extent of the deletions from the 3′ PSS (▦) and blunt ends (□) in pCMS-end plasmids. pCMS-end plasmids were rescued from the hTERT-immortalized fibroblasts derived from control individuals (WRN+/+), WS patients (WRN−/−), and WS hTERT cells complemented with WRN (WRN−/− + WRN). *Bottom diagram*, the site of linearization (at nt 1) and the location of ampicillin-resistant gene of pCMS-end. *Nucleotides on the right side* (*outside of the graph*), the microhomology at the NHEJ junction.

Fig. 2 shows the extent of the deletions of recovered pCMS-end plasmids that had 3′ PSS and blunt ends prior to circularization in the normal (WRN+/+), WS (WRN−/−), and complemented (WRN−/− + WRN) hTERT-immortalized cell lines. When linear pCMS-end plasmid with a blunt end and 3′ PSS end was introduced to the WS line, the 3′ PSS ends were extensively degraded before circularization, compared with the degradation that occurred in the normal 82-6 and 88-1 cells (Fig. 3, ▦). In general, blunt-ends were subject to less degradation than 3′ PSS ends, but even then, more deletions occurred in the WS cells compared with the normal cells (Fig. 3, □). These findings suggest that WRNp is required to limit the extent of deletions during NHEJ. Consistent with this idea, the exten-

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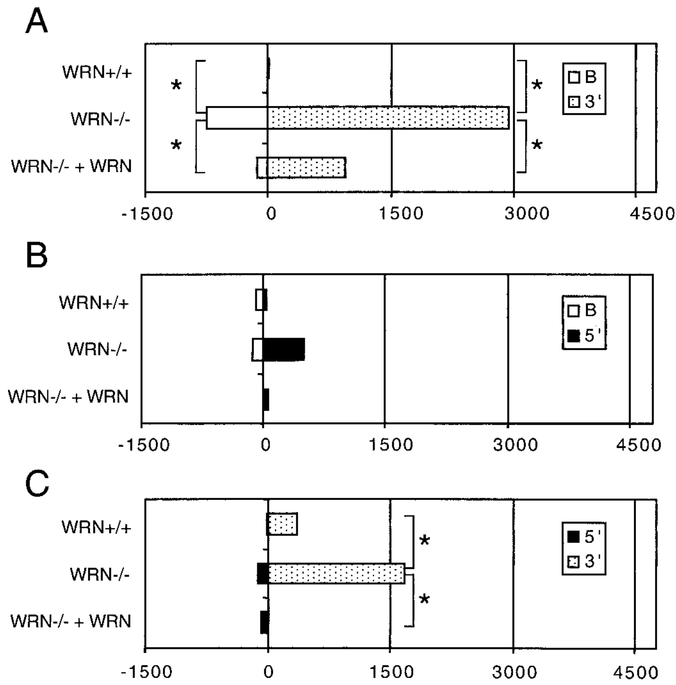

Fig. 3. Median values of the extent of nucleotide loss during NHEJ in pCMS-end at 3′ PSS (▨), 5′ PSS (■) and blunt ends (□). *A*, pCMS-end with 3′ PSS (▨) and blunt ends (□). *B*, pCMS end with 5′ PSS (■) and blunt ends (□). *C*, pCMS-end with 5′ PSS (■) and 3′ PSS ends (▨). The site of linearization is indicated at nt 1. WRN+/+ are the combined data of 82-hTERT and 88-1hTERT cells. WRN−/− are the combined data of 73-26hTERT and AMIEhTERT cells. WRN−/− + WRN are from 73-26hTERT+WRN. *, statistically significant differences.

sive deletions of the 3′ PSS and blunt ends in plasmids recovered from cells were substantially reduced in the WS cells into which the full-length human WRN cDNA had been introduced (WRN−/− + WRN). Because these cells are a nonclonal population, there is likely to be variability in the level of WRNp expression among individual cells (Fig. 2).

NHEJ in mammalian cells is believed to be mediated by microhomology. We did not, however, observe any significant difference in the extent of microhomology at the joined ends among the cell lines tested (Fig. 2).

In many plasmids rescued from the WS cell line, 3′ PSS deletions extended up to the ampicillin-resistant gene (nt 3465–4325 from the end of the 3′ PSS; Fig. 2. diagram at the bottom) and blunt end deletions extended to the origin of replication (nt −1103 from the site of linearization; Fig. 2). Thus, it is likely that some plasmids lost nucleotides beyond the sequences required for their rescue and, hence, were not scored in these experiments.

Because of the large variation among the recovered plasmids, overall loss was assessed by determining the median values of the nucleotide loss (Fig. 3). Median values of the deletions shown in Fig. 2 are shown in Fig. 3*A*. The median deletion at 3′ PSS ends placed opposite to blunt ends (Fig. 3*A*) was 6 bp for control cells, 2936 bp for WS cells, and 952 bp for the complemented WS cells. The median deletion at blunt ends placed opposite to 3′ PSS ends was 0 bp for control cells, 758 bp for WS cells, and 136 bp for the complemented WS cells. The differences between WS and control cells were highly significant, using a *t* test that assumed unequal variances for both the 3′ PSS ends ($P < 0.01$) and blunt ends ($P < 0.01$). The differences between WS and complemented WS cells were also significant ($P = 0.01$ for blunt ends; $P < 0.01$ for 3′ PSS ends), whereas differences between control and complemented cells were not significant ($P = 0.16$ and 0.15 for blunt and 3′ PSS ends, respectively).

When pCMS-end plasmids with 5′ PSS and blunt ends were introduced to the same control, WS, and complemented cell lines, the 5′ PSS ends showed a similar, but less dramatic, trend in the extent of deletion that was seen with 3′ PSS ends: a median loss of 30 bp in normal cells, 484 bp in WS cells, and 58 bp in the WS cells complemented with *WRN* (Fig. 3*B*, ■). The blunt ends were also less deleted in the blunt-5′ PSS combination than in blunt-3′ PSS combination: 96 bp in normal cells, 124 bp in WS cells, and 0 bp in the complemented WS cells (Fig. 3*B*, □). Unlike the differences in extent of plasmids with 3′ PSS and blunt ends (Fig. 3*A*), the differences in extent of deletion in plasmids with 5′ PSS and blunt ends were not statistically significant among the normal, the WS, and the complemented cells (Fig. 3*B*).

pCMS-end plasmids containing 5′ PSS and 3′ PSS ends but no blunt ends (Fig. 3*C*) also showed more extensive deletion in cells devoid of WRNp: median 3′ PSS end deletions were 360 bp, 1678 bp, and 21 bp for normal, WS, and complemented cells, respectively, and median 5′ PSS end deletions were 6 bp, 128 bp, and 70 bp for these cells, respectively. The difference in the extent of deletions on the 3′ end between WS and wild-type control was significant ($P < 0.01$) as was the difference between WS and WRN complemented mutant ($P < 0.01$). Interestingly, the deletions at 3′ PSS ends were more extensive when the other side of the plasmid had a blunt end compared to when the other side had a 5′ PSS end, in both the normal and WS cells (compare ▨ in Figs. 3*A* and 3*C*). Likewise, the deletions at 5′ PSS ends were more extensive when the other side of the plasmid had blunt ends instead of 3′ PSS ends [compare black bars (■) in Figs. 3*B* and 3*C*]. Thus, by this assay, 3′ PSS ends are more susceptible to the removal or loss of nucleotides during NHEJ in both normal and WS cells. The susceptibility to deletion during NHEJ was 3′ PSS > 5′ PSS > blunt ends. The lack of WRNp resulted in more extensive deletion at all three types of ends. We speculate that deletions at the blunt ends were less dramatic than those at PSS end because blunt ends are not as accessible as overhanging ends to exonucleases (or helicases).

We also analyzed deletions during NHEJ using the pBS-KS plasmid. For this assay, pBS-KS was used in place of pCMS-end in the assay shown in Fig. 1. The orientation of the 3′ PSS and blunt ends in pBS-KS are opposite to those in pCMS-end, with respect to the replication origin and ampicillin-resistant gene. pBS-KS plasmids recovered from normal, WS, and complemented cells showed a deletion spectrum similar to that of pCMS-end plasmids: lack of WRNp caused more extensive loss of nucleotides at a 3′ PSS end placed opposite to a blunt end (Fig. 4*A*). Several other trends seen in the NHEJ assay using pCMS-end plasmids were also observed using pBS-KS plasmids. First, 5′ PSS end deletions in WS cells were smaller than 3′ PSS end. Second, both 5′ PSS and 3′ PSS ends lost more nucleotides when the PSS ends were combined with blunt ends than when they were combined with other PSS ends. Third, introduction of functional WRNp partially or almost completely restored the extensive deletions. Overall, deletions in pBS were smaller than those in pCMS-end, probably because of the differences in total size of the original plasmids. Statistically significant differences were seen only in limited comparisons (* in Fig. 4, *A*, *B*, and *C*).

DISCUSSION

DSBs are induced by external insults (*e.g.,* ionizing radiation) as well as internal causes (*e.g.,* endogenous oxygen radicals). DSBs can lead to an arrest of cell division or cell death if unrepaired. Repair can be accomplished by either homologous recombination or illegitimate recombination, also known as NHEJ. Whereas homologous recombination is generally free of errors, NHEJ tends to result in a loss of

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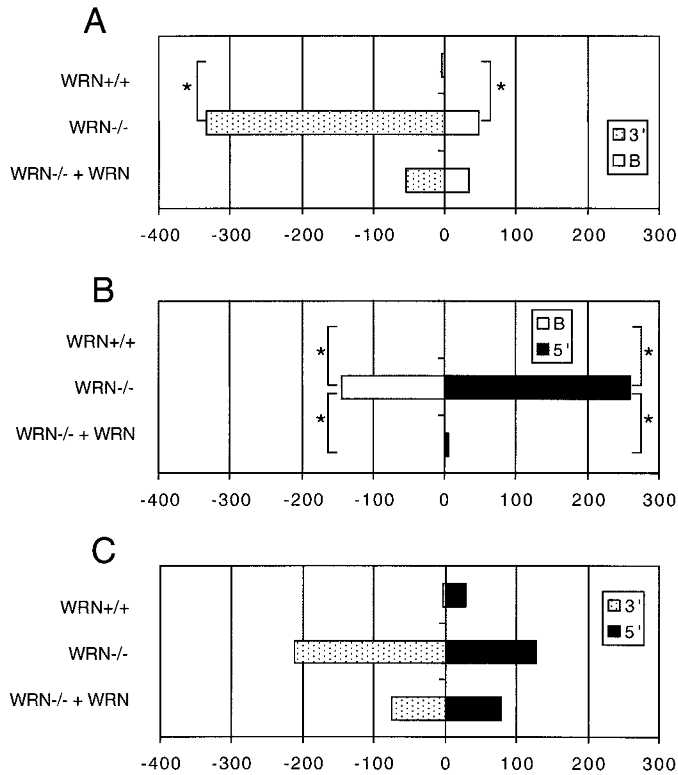

Fig. 4. Median values of the extent of nucleotide loss in pBS-KS in normal (WRN+/+), WS (WRN−/−), and complemented WS (WRN−/− + WRN) cells. *A*, pBS-KS with 3′ PSS (▨) and blunt ends (□). *B*, pBS-KS with 5′ PSS (■) and blunt ends (□). *C*, pBS-KS with 5′ PSS (■) and 3′ PSS ends (▨). The site of linearization is indicated at nt 1. *, statistically significant differences.

nucleotides. Mammalian cells are thought to use NHEJ as a primary means to repair DSBs (29).

It has been proposed that an exonuclease participates in NHEJ processes mediated by the DNA-PK complex (DNA-PKcs plus Ku70/80), and is needed to trim the dsDNA broken ends and provide regions containing microhomology (30). One study showed an interaction between the Ku complex and Mre11, suggesting that Mre11 might provide this exonuclease activity (31). However, the finding that WRNp also interacts with Ku (19–21) and DNA-PKcs (22) raises the possibility that WRNp could also provide this activity. Here, we tested the possibility that WRNp is functionally involved in NHEJ. WRNp can potentially open up DSBs for microhomology scanning and/or trim the DSB ends in preparation for the completion of repair by XRCC4 and ligase IV. Alternatively, WRNp might stabilize the Ku-DNA end-joining complex. Our data show that, in the absence of WRNp, there is extensive deletion at ends joined by NHEJ. WRNp, although itself a helicase and exonuclease, appears to suppress other more promiscuous or efficient helicase or exonuclease activities *in vivo*. Thus, WRNp minimizes nucleotide loss during NHEJ. The mechanism by which WRNp suppresses extensive nucleotide loss might be direct competition with other helicases or exonucleases for dsDNA binding. WRNp might function as a competitive suppressor, because it is a relatively weak exonuclease and/or helicase. The putative competitor(s) of WRNp is likely to have a 3′→5′ helicase, 3′→5′ single-strand exonuclease, or 5′→3′ exonuclease activity, which can use dsDNA with 3′ PPS ends as preferable substrates. The cellular phenotypes in WS, such as the variegated translocation mosaicism (4) and extensive deletions of endogenous loci (5), may not be caused by a lack of WRNp enzymatic activities *per se*. Rather, they may reflect the functions of more efficient or less discriminate enzymes, which are suppressed by the presence of WRNp. It is also possible that, because WRNp prevents nucleotide loss from all three combinations of dsDNA ends (3′ PSS, 5′ PSS and blunt ends), WRNp may modulate the stability of DNA-PK-DNA complex at DSB ends.

In prokaryotes and yeasts, the rate and mechanism of recombination is controlled, in part, by RecQ helicases, which optimize the levels of homologous and illegitimate recombination by regulating the of recombination intermediates. Unlike mammalian species, prokaryotes and yeasts have only one gene that encodes a *RecQ* type helicase. In *E. coli*, RecQ mutants have increased illegitimate recombination (32), and *E. coli* RecQ has been shown to initiate or disrupt recombinational intermediates (33). In *Saccharomyces cervisiae*, Sgs1 mutants exhibit increased homologous and illegitimate recombination rates, which can be suppressed by a *WRN* as well as by a *BLM* cDNA (34). Biochemical studies demonstrated that Sgs1p and BLMp can unwind unusual DNA structures that are potentially generated during recombination (35). Whereas the cytogenetic abnormalities of WS cells are mainly increased translocation mosaicism (4), the hallmark of BS cells is increased sister chromatid exchange, which reflects increased mitotic recombination rate (36). BLMp has been shown to interact with hRad51 that is involved in homologous recombination (37). Taken together, a primary cellular function of *WRN* may be to primarily suppress illegitimate recombination (and, perhaps to a lesser extent, homologous recombination; Ref. 38), whereas *BLM* may primarily suppress homologous recombination (and, to a lesser extent, illegitimate recombination). The ability to unwind recombination intermediates can also be used to resolve unusual DNA structures that are accidentally generated during DNA replication, repair, and transcription. *WRN* may have evolved to "fine tune" illegitimate (and, perhaps secondarily, homologous recombination), as higher eukaryotes came to use illegitimate recombination more than homologous recombination to reduce the accidental use of repetitive sequences in the genome. The idea that WRNp evolved to fine tune DNA processing, rather than to provide an essential repair function, is consistent with the late appearance of somatic phenotypes in WS patients.

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