Non-B DNA-forming Sequences and WRN Deficiency Independently Increase the Frequency of Base Substitution in Human Cells*

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Although alternative DNA secondary structures (non-B DNA) can induce genomic rearrangements, their associated mutational spectra remain largely unknown. The helicase activity of WRN, which is absent in the human progeroid Werner syndrome, is thought to counteract this genomic instability. We determined non-B DNA-induced mutation frequencies and spectra in human U2OS osteosarcoma cells and assessed the role of WRN in isogenic knockdown (WRN-KD) cells using a supF gene mutation reporter system flanked by triplex- or Z-DNA-forming sequences. Although both non-B DNA and WRN-KD served to increase the mutation frequency, the increase afforded by WRN-KD was independent of DNA structure despite the fact that purified WRN helicase was found to increase genomic hybridization techniques has revealed the frequentoccurrence of DNA sequence motifs capable of forming alternative (non-B) DNA conformations (e.g. triplex, quadruplex, Z-DNA, cruciforms, slipped structures) at the breakpoint junctions of chromosomal alterations (gross deletions and duplications) associated with human genetic disease, including cystic fibrosis, mental retardation, and multiple congenital anomalies (1). These observations have served to extend the generality of previous work that aimed to elucidate the molecular mechanisms underlying recurrent translocations (2–6) and the genetic instability observed in many model systems (7–16), both of which were suggestive of a direct mutagenic role for non-B DNA. In the same vein, analyses of DNA sequence motifs flanking human gross deletion breakpoints (9), genomic inversions that distinguish the human from the chimpanzee genome (17), and DNA sequence tracts involved in pathological gene conversion events (18) have provided evidence for a wide ranging role for DNA secondary structure in promoting genomic rearrangements. Despite these recent advances, few studies (8, 11) have attempted to address systematically the extent of the influence of non-B DNA-forming sequences in modulating mutational spectra. It therefore remains unclear whether other types of mutation, such as single base substitutions, might also be induced by the presence of non-B DNA.

Considerable work in vitro has documented the ability of helicases, such as BLM, WRN, DHX9, and FANCJ, to unwind non-B DNA conformations (19–23), consistent with their postulated role in maintaining genome integrity (reviewed in Ref. 24) (25, 26). The heritable deficiency of WRN, Werner syndrome (WS),2 is a rare recessive disorder characterized by the early onset of an aged appearance and age-related disorders including bilateral cataracts, skin changes, short stature, graying and loss of hair, osteoporosis, diabetes, and premature death (21, 27, 28). WS patients invariably carry inactivating mutations in the WRN gene encoding WRN (27, 29, 30), an evolutionarily conserved member of the RecQ helicase family

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§ The abbreviations used are: WS, Werner syndrome; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside; 8-oxodG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; iz, 2-amino-5′-[(2-deoxy-b-D-erythro-pentofuranosyl)amino]-4H-imidazol-4-one.

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that possesses both 3’ → 5’ helicase and exonuclease activities (31). Fibroblast cultures from WS patients display a variety of chromosomal abnormalities, including reciprocal translocations, deletions, and inversions (reviewed in Ref. 31), elevated single base pair mutation frequencies (32–34), and modified bases (reviewed in Ref. 35). Fibroblasts from both WS patients and siRNA-mediated WRN knockdown exhibit a marked reduction in proliferation in culture, which is exacerbated by DNA damaging agents (36). An increase in DNA damage response nuclear foci and other defects thought to be associated with the resolution of either arrested DNA replication forks (36, 37) or mitotic recombination intermediates (38) are also evident in the absence of WRN.

WRN deficiency has also been shown to be associated with a profound reprogramming of genome-wide gene expression profiles (39–42) and increased intracellular levels of reactive oxygen species, suggesting that cellular senescence may result from the pleiotropic effect of several genes in association with redox imbalance (43–46).

To explore the complex interrelationship between non-B DNA structures, WRN deficiency, and mutagenesis, we used a plasmid system containing triplex- and Z-DNA-forming sequences flanking a reporter gene in transfection experiments performed in WT and WRN knockdown (WRN-KD) U2OS human osteosarcoma cell lines. Our results revealed that both non-B DNA and WRN deficiency serve to induce mutations. Further, mutational spectra were characterized by single base changes, mostly at G-C pairs. These mutations were found to be strongly dependent upon base stacking with neighboring bases, suggesting a role for oxidative damage through electron loss to the local environment leading to redistribution of the outer electron cloud between adjacent DNA bases (hole migration). Suggesting a role for oxidative damage through electron loss to such lesions on sequence context as would be predicted by a hole migration model. Hence, electron loss and redistribution within the DNA molecule appear to underlie mutations arising from such diverse sources as alternative DNA conformations, WRN deficiency, and the process of tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Plasmids pCEX, pMEY, pMEYr, pGG32y, and pSCG14 are derivatives of plasmid pSP189 in which inserts (Fig. 1A) were cloned between the EcoRI and XhoI restriction sites. Triplex-forming plasmids (Fig. 1B) formed concatenated multimer species during bacterial cell growth. Because plasmid dimerization limits the detection of single base substitutions and could alter deletion/rearrangement frequencies by providing a substrate for homologous recombination, we first determined the most suitable *Escherichia coli* genetic background to obtain monomer plasmids. Of the DH5α, HB101, DL795, DL733, and DL1649 strains tested, the highest yields of monomer DNA were obtained in CaCl₂-competent *E. coli* strain HB101. The fraction of monomer DNA was additionally increased by transforming closed circular, relaxed, monomer plasmid prepared by linearization with PvuI followed by ligation with T4 DNA ligase. The ratios of monomer to concatenated multimer species ranged from >95% for pCEX to ~70% for pGG32y as assessed by agarose gel electrophoresis (data not shown). Structural transitions from B-DNA to non-B DNA require negative supercoiling. Therefore, to separate closed circular DNA from functionally ineffective open circular DNA, large scale plasmid DNA preparations were purified by equilibrium centrifugation in CsCl-ethidium bromide continuous gradients (47) on a Beckman Optima LE-80K ultracentrifuge and resuspended.

**Cell Types and Relevant Genotypes**— *E. coli* strains DH5α (supE44 ΔlacY169 (860 lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1) and HB101 (F supE44 hsdS20 (r59 m56) recA13 ara-14 leuB6 Δ(gpt-proA)62 Δ(mcr-C-mrr) thi-1 lacY1 galK2 rpsL20 xylI-5 mtl-1 λ−) were obtained from the ATCC (Manassas, VA). The *E. coli* K12 SH28-derivative strains DL795 (deoA Δ(mrr hsdR51 mcrBC2 mcrA e14) sbcC201 supE44 recA4:CamB3), DL733 (IM83 sbcC202 phoR:Trn10), and DL1649 (MGL1565 ΔlacZ (ΔsbcDC/PsbcDC-lacZaph) were a kind gift from David R. Leach (University of Edinburgh, Edinburgh, UK). Control and WRN knockdown gene expression were obtained by stable transfection of the human U2OS osteosarcoma cell line with pSilencer 3.1-H1 hygro (AM5766) in which either a scrambled shRNA sequence (WT) with no homology with known genes or the WRN-targeting shRNA (WRN-KD) insert GGATCC CCGTGAAGAGGACGTTACTCTGGCCTCAAGAGAGGAGGT-AACCTGCTCTTCAATTTTGGAGAAAGCTT was cloned between the BamHI and HindIII restriction sites (48) to yield pshSCR and pshWRN, respectively.

**Mutation-Reporter Plasmid Transfection in WT and WRN-KD Cells**—WT and WRN-KD U2OS human osteosarcoma cells were maintained in hDMEM (DMEM containing glucose/glutamine/sodium pyruvate supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 1× streptomycin/penicillin (Invitrogen) solution, and 200 µg/ml hygromycin B (Calbiochem, La Jolla CA). For transfections, ~80% confluent cells were treated with 1× trypsin (Invitrogen), and ~1.2 million cells were mixed with 110 µl of premixed cell line nucleofector kit V (Lonza), 1.8 µl of plasmid DNA at 2 µg/µl and electroporated. Transfected cells were allowed to recover for 24 h in McCoy’s 5A medium (Invitrogen) supplemented with 1× l-glutamine and 10% fetal bovine serum and then grown for an additional 48-h period in hDMEM. Plasmid DNA was isolated using QIAprep spin miniprep kits (Qiagen); cleaved with DpnI, AatII, and Tth111I (to remove MBM7070 to read through amber stop codons in the chromosomal lacZ gene (Fig. 1D)); hence, *supF* mutants are found as...
white colonies among blue wild-type colonies on X-gal/IPTG/ampicillin-containing agar plates. For screening, 50 and 100 μl (of 1 ml) of transfected E. coli strain MBM7070 cells (47) were plated on X-gal/IPTG/ampicillin-containing plates and grown for ~16 h at 37 °C. When replating was required to optimize the number of colonies on the plates, E. coli solutions, which were kept at 4 °C overnight, were prewarmed to 37 °C. This heating step was necessary to overcome extensive cell death observed with pGG32y-containing cells, a feature of E. coli harboring triplex-containing plasmids (7). All of the white colonies were picked and streaked on X-gal/IPTG/ampicillin-containing plates to confirm their phenotype. Mutation frequencies were determined by dividing the number of white (mutant) colonies by the total number of colonies (blue + white). The experiments were repeated 3–6 times.

Plasmid DNA was isolated from randomly selected white colonies and supF mutations were determined by direct DNA sequencing using primers 189 (5'-CAAAAAAGGAATAGGGCG-3') and 201 (5'-CGTTTCTGGTGAGCAAAA-3'), which hybridize within the ampicillin resistance gene. The mutations induced by non-B DNA conformations were assessed by comparing the mutation frequencies and spectra obtained with sequences cloned upstream of the supF gene (Fig. 1A), which included: 1) a control sequence (pCEX) unable to form any known non-B DNA structure; 2) two sequences from the human c-MYC promoter (pMXExy and pMExr) capable of adopting metastable triplex structures (Fig. 1B); and 3) two sequences (pGG32y and pSCG14) predicted to fold into stable triplex and Z-DNA conformations (Fig. 1B), respectively.

Helicase Assays in Vitro—For plasmid DNA, 1 μg of plasmid DNA was incubated with 60 nM purified WRN protein (50) at 35 °C for 30 min. Fifty units of muan bean nuclease were added for 5 min, and the reactions were stopped by chilling on ice. The products were separated by 0.8% agarose gel electrophoresis, and the percentage of unwinding by WRN was determined from the total amount of supercoiled plasmid remaining after the reactions. For synthetic triplex DNA, PAGE-purified 41R 5'-AAAGAGAGGTAAGCCCAAGTTTCC-3', 41Y 5'-GGAGACTTGGCCCTTCCGCCT-3', and 31R (3'-over-hang) 5'-GGAAAGGTCATGGGGGAGGGCGTACAGCAGC-3' (triplex region underlined) oligonucleotides were 5'-end-labeled and annealed, and 5 nM labeled triplex DNA was incubated with purified WRN (19). The reaction products were resolved by 12% PAGE and quantified (Molecular Dynamics, Sunnyvale, CA).

8-OxodG Analysis—The concentration of 8-oxodG (8-oxodG/dG) was determined by HPLC (51) on genomic DNA isolated from wild-type and WRN-KD U2OS cells using the QIAamp DNA mini kit (Qiagen). DNA was digested and dephosphorylated by nuclease P1 and alkaline phosphatase and analyzed by HPLC using a reverse phase analytical column coupled with a photodiode array detector (SPD-M10A; Shimadzu, Columbia, MD) followed by electrochemical detection (CoultArray; ESA, Chelmsford, MA).

Databases and Statistical Analyses—A data set of 54,422 germline missense and nonsense mutations was retrieved from the Human Gene Mutation Database (52). These mutations, as well as those mutations identified in the WT and WRN-KD cells, were recategorized according to their occurrence in the 10 possible alternative dinucleotide pairs. To allow the spectrum of mutations found in the WT and WRN-KD cells to be compared directly with that of the inherited disease mutations recorded in the Human Gene Mutation Database, each type of mutation at a particular position was counted only once. A Chi square test was then performed to assess the significance of the disproportionate occurrence of certain types of mutation found in the WT and WRN-KD cells within a specific dinucleotide pair as compared with those mutations recorded in the Human Gene Mutation Database. All of the results were corrected for multiple testing.

Approximately 50,000 somatic single nucleotide substitutions identified in a non-small cell lung cancer genome (53) were used to determine the number of mutations that occurred at G-C base-pairs (39,615 of 50,675 or 78%) in the context of 64 possible NGNN and their complementary NNCCN sequences (using in-house Perl scripts) during the process of tumorigenesis. Mutations were mapped onto the reference human genome assembly hg19; the numbers of each tetrancleotide sequence (i.e. AGAA, TGAC, etc.) mutated in the lung cancer genome were divided by the number of tetrancleotide sequences present either within 1 kb of each mutation site (F_{kb}) or genome-wide (F_{gw}).

A one-way analysis of variance Tukey test was used to determine the statistical significance of the mutation frequencies. Differences in the numbers of transversions and transitions generated were assessed by means of McNemar’s test between two correlated proportions.

RESULTS
The ability of repetitive DNA sequences to adopt non-B DNA conformations and to induce genomic instability is well documented (2–6, 8–11, 17, 18). However, few studies have reported on the mutational spectra induced by such elements (8, 10, 11). In addition, the potential role of those proteins whose function is to preserve genome stability, such as WRN, in counteracting non-B DNA-induced mutagenesis has not yet been explored in vivo. To this end, we measured mutation frequencies and spectra in a plasmid reporter system (Fig. 1A) in which the supF gene was preceded by sequences capable of forming either triplex structures of increasing thermal stability or a Z-DNA conformation (Fig. 1B). These plasmids were transfected into human osteosarcoma U2OS cells containing an integrated plasmid encoding either a control shRNA (WT) or a targeting shRNA designed to stably knockdown WRN (WRN-KD) (48). Under standard cell culture conditions, the average level of WRN protein in WRN-KD cells relative to its WT counterpart was found to be 0.26 ± 0.06 (mean ± S.D.), as determined by immunoblot analysis (Fig. 1E and supplemental text).

Non-B DNA-forming Sequences and WRN-KD Both Increase Mutation Frequencies—We postulated that if one function of the WRN helicase were to resolve triplex and Z-DNA structures, as observed in vitro (8, 11, 23), then mutation frequencies might increase in WRN-KD cells as compared with WT cells because both the number and stability of such structures would
be significantly greater in the former cell type. We verified by native PAGE that purified WRN protein (50) was able to unwind the third, purine-rich, strand of a synthetic triplex in vitro and to partially protect from mung bean nuclease cleavage the Z-DNA-forming repeat in supercoiled pSCG14 (data not shown).

To determine the spontaneous and non-B DNA-induced mutation frequencies in WT and WRN-KD cells, 3–6 separate experiments were performed in both cell lines in parallel using plasmids pCEX, pGG32y, and pSCG14 (Fig. 1). In WT cells, from a total of 232,938 colonies, 129 were confirmed to be mutant (white), with relative mutation frequencies of 2.1 $\pm$ 1.0 $\times$ 10$^{-5}$ (mean, S.E.) for pCEX, 2.4 $\pm$ 1.0 $\times$ 10$^{-5}$ for pGG32y, and 3.4 $\pm$ 1.0 $\times$ 10$^{-5}$ for pSCG14 (Fig. 2A). The WRN-KD cells, a total of 144,832 colonies were counted, and of these, 190 were mutants, yielding mutation frequencies of 4.6 $\pm$ 2.0 $\times$ 10$^{-5}$ for pCEX, 4.4 $\pm$ 2.0 $\times$ 10$^{-5}$ for pGG32y, and 2.0 $\pm$ 1.0 $\times$ 10$^{-5}$ for pSCG14 (Fig. 2A). Thus, the mutation frequencies generated in the WRN-KD cells were invariably ~2-fold (1.9–2.2) greater than in WT cells, irrespective of the relative ability of the various plasmids to adopt non-B DNA conformations.

Pair-wise statistical (z-test) comparisons within the data set indicated that in both WT and WRN-KD cells, the non-B DNA-forming plasmid inserts increased the mutation frequencies over background levels ($p < 0.001$) (Fig. 2B). The increases in the number of mutant colonies observed in WRN-KD cells relative to WT cells for each of the plasmids were also significant ($p = 9.8 \times 10^{-5}$ to 3.0 $\times$ 10$^{-5}$) (Fig. 2B). Additional experiments performed in WT cells, which also included the less stable triplex-forming plasmids pMEXr and pMEXp (Fig. 2C), further indicated that the mutation frequency was directly proportional to the stability of the non-B DNA structure (Fig. 1B) predicted to form in the respective plasmids.

**Mutational Spectra in Human Cells**—To characterize the mutational spectra, we sequenced the supF mutation-reporter gene in 12 clones, randomly chosen, for each of the plasmids studied. For the control pCEX plasmid in WT cells, nine clones (75%) revealed the presence of one or more base changes within the supF gene, whereas in the remaining three clones, the entire supF gene was deleted (Fig. 2D). The prevalence of single base mutations remained unchanged when the non-B DNA-forming plasmids were used. Indeed, the ratio of single base changes to deletions did not appear to be affected by the type of plasmid employed. In WRN-KD cells, single base substitutions were present in 6, 8, and 11 cases in pCEX, pGG32y, and pSCG14.

**FIGURE 2. Mutation frequencies and spectra in WT and WRN-KD cells.** A, fractions of white (mutant) colonies to the total number of colonies for the indicated plasmids (x axis) isolated from WT cells (black) and WRN-KD cells (gray). B, statistical z-test pair-wise comparisons of the data from A. Solid lines, $p < 0.001$; dashed line, 0.01 < $p < 0.05$. C, as in A for WT cells; 374 mutant (white) colonies were obtained from a total of 915,386 colonies. The mutation frequencies were 1.4 $\pm$ 0.5 $\times$ 10$^{-5}$ for pCEX, 2.2 $\pm$ 0.5 $\times$ 10$^{-5}$ for pMEXp, 3.0 $\pm$ 0.5 $\times$ 10$^{-5}$ for pMEXr, 3.7 $\pm$ 0.5 $\times$ 10$^{-5}$ for pGG32y, and 4.2 $\pm$ 0.5 $\times$ 10$^{-5}$ for pSCG14; ***, $p < 0.001$; *, $p = 0.001$–0.023; pair-wise comparisons between pGG32y (or pSCG14) and pCEX (top line), pMEXp (middle line), and pMEXr (bottom line) are shown. D, mutation spectra of selected mutant colonies. Black, single nucleotide changes in the supF gene; white, deletions disrupting the supF gene.
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respective (Fig. 2D), and hence were more abundant than deletions, as found with WT cells. This conclusion was further supported by results obtained from 24 additional clones with pCEX, pMEXr, pMEXy, pSCG14, and pGG32y, 22 of which contained single base changes in the supF gene (not shown).

To determine the relative contribution of mutations arising in bacterial cells, pCEX, pGG32y, and pSCG14 were transformed directly into E. coli MBM7070 cells, which were plated on X-gal/IPTG/ampicillin-containing plates. From a total of 287,398 colonies in two separate experiments, 13 mutant colonies (4.5 × 10⁻⁵) were confirmed, without significant differences (z-test) in frequency between plasmids (3.8 × 10⁻⁵ for pCEX, 6.6 × 10⁻⁵ for pGG32y, and 3.3 × 10⁻⁵ for pSCG14). Thus, mutations in bacteria did not contribute significantly to the mutation frequencies observed in the human cells.

Plasmid DNA was also extracted from 26 mutant colonies (of 154,541 screened) derived from WT human cells transfected with pCEX and retransformed in fresh competent MBM7070 cells. Of these, six yielded blue (i.e. wild-type) colonies, indicating that mutations were present in the lac operon of the recipient bacterial cells, rather than in the plasmid supF gene, at a frequency of ~3.9 × 10⁻⁵ (6/154,541).

In summary, the following conclusions could be drawn: 1) the mutational spectra of the supF gene in human cells comprised predominantly single base changes; 2) the presence of non-B DNA conformations or WRN-KD increased mutation frequencies but did not alter the underlying mutational spectral; 3) the presence of non-B DNA structures increased mutation frequencies but did not alter the underlying mutational spectra; 4) most mutations (from 68% in pCEX to 96% in pSCG14) in the supF gene arose in the human cells. Thus, additivity (rather than synergy) with respect to mutation frequencies was evident between the presence of non-B DNA conformations and WRN helicase deficiency, allowing one to conclude that WRN helicase activity does not provide protection against non-B DNA-directed mutation.

**Figure 3. SupF-inactivating single base changes.** Sequence composition of the supF gene (below) and base changes (above) found in all sequenced mutant plasmids isolated from WT and WRN-KD U2OS cells. Δ, nucleotide deleted; **, double-base substitution; ** double underlining, GA-TC dinucleotides.

**GA-TC Dinucleotides Represent Mutation Hot Spots**—Of the 120 clones sequenced (Fig. 3), 94 (78%) were found to harbor base changes within the supF gene (47 for WT cells and 47 for WRN-KD cells), whereas only 26 clones (22%) carried deletions that abolished supF gene function (supplemental text and supplemental Fig. S1). Of the 94 clones with base changes, 92 contained single base substitutions, and 1 clone lacked a nucleotide, whereas the other displayed a double base substitution (Fig. 3). Hence, no insertions were detected. Overall, 123 base changes occurred (63 in WT cells and 60 in WRN-KD cells), or 1.3 base changes per clone on average, with up to 5 base changes per clone. A survey of the nucleotides most commonly substituted revealed three hot spots at nucleotide positions 41, 67, and 76 (Fig. 3), which together accounted for more than a third (37%) of all the base substitutions. The second and third hot spots shared a DNA sequence motif (TTTCGAA) that possesses dyadic symmetry, in which TTC is complementary to GAA. Similarly, the first hot spot occurred within the anticodon loop of the su^t^tRNA^Tyr^ molecule (Fig. 1C), which also corresponds to a region of inverted repeat symmetry. Inspection of the nucleotides that underwent sequence changes suggested a bias toward G-C base pairs when followed by A-T pairs. Indeed, in 103 of 121 (85%) cases, G-C base pairs were changed within a GA-TC dinucleotide context with a significantly higher prevalence of the G → C (49%) type of transversion over the alternative G → A (28%, p = 5.2 × 10⁻¹²), G → T (23%, p = 9.2 × 10⁻¹⁴), or combined G → A + G → T (p = 3.7 × 10⁻⁸) types of mutation. On the other hand, no difference was observed in the frequency of transversions versus transitions when either the GA-TC (p = 0.257) dinucleotide or all sequences (p = 0.252) were analyzed. In summary, 1) G → C transversions within GA-TC dinucleotides were the preferred type of mutation, and 2) tripod or Z-DNA-forming sequences and WRN-KD did not alter the propensity for G → C transversions to occur within a GA-TC context.
The central conclusion emerging from this study was the unexpected role of charge transfer (hole migration) in targeting guanine residues for oxidation, leading to mutation, in contexts as diverse as the formation of non-B conformations by specific DNA sequences, partial WRN deficiency, and lung tumorigenesis. Although these three contexts are structurally and functionally very different, they all ultimately appear to converge on the abstraction of an electron from a nucleobase and the subsequent reorganization of electron orbitals by base-base interactions along the DNA chain. Thus, a purely physical property of DNA (hole migration) appears to play a critical role in mutagenesis in a wide variety of contexts.

In addition, we observed that in the human osteosarcoma U2OS cell line, DNA sequences with the capacity to adopt either triplex or Z-DNA structures served to increase sponta-
FIGURE 4. Oxidative damage and single base changes. A, concentration of 8-oxoG divided by the concentration of dG in genomic DNA of WT and WRN-KD cells (means ± S.D. from three DNA purifications). B, schematic diagram for hole migration showing the abstraction of an electron (e⁻) (step 1) by a radical species in the solvent (not shown) from a base downstream of the target G in an NGNN sequence, forming a radical cation (hole). The hole migrates to the upstream guanine (step 2) through the reorganization of the outer electron cloud involving base stacking between the interacting bases. A drop in the ionization potential traps the hole at the oxidized guanine, which through multiple steps (step 3) (including DNA replication and repair) eventually gives rise to a mutation. C, correlation between single base changes in the supF gene and base stacking at NGNN sequences. The y axis shows the number of mutations (mean ± S.E.) at G residues (from Fig. 3; C residues were also counted with 3' 5' flanking sequences) in NGNN/NNCN sequences. The x axis shows the average free energy contribution [−ΔG(ν)] to nearest neighbor base stacking in single-stranded DNA for the NG, GN, and NN dinucleotides within NGNN (from Table 4 of Ref. 61) with εi = 2; Y, T or C; H, T, C or A; G, a G residue at either the first, third, or fourth position within NGNN. Inset, site energy (eV) for the nucleobase G in 5'NGN-3' (from Table 2 of Ref. 55). HGAH = YGAY + YGAA + AGAY. D, the fractions of single nucleotide variants from a lung cancer genome (53) at each of the 64 possible NGNN/NNCN tetranucleotide sequences (compared with the reference human genome assembly hg19) to the number of NGNN/NNCN sequences found within 1 kb (F kb) of each mutation site were calculated and averaged for the 19 CpG-containing sequences (4.66 ± 1.63 × 10⁻⁵) and the 45 non-CpG-containing sequence combinations (2.84 ± 1.02 × 10⁻⁵) (p = 0.000051). For the data normalized to the genome-wide number of NGNN/NNCN sequences (Fgw), the respective fractions were 5.23 ± 2.03 × 10⁻⁵ (CpG) and 3.36 ± 1.25 × 10⁻⁵ (non-CpG; p = 0.00032). E, the Fgw data (D) for the 45 non-CpG-containing sequences (y axis) were plotted against the average ionization potential values of the upstream G (Ref. 54). r = −0.94; p = 0.018.
neous mutation frequencies in cis in a manner that was directly proportional to their predicted thermal stability. These non-B DNA-forming sequences increased mutation frequencies without altering the basic composition of the mutational spectra. Thus, sequences capable of forming non-B DNA increase the rate of mutagenesis such that all types of mutation are increased proportionately, microlesions as well as large deletions. To our knowledge, no other type of DNA sequence has so far been reported to be capable of inducing different types of mutational events, both small and large.

We have also shown that chronic deficiency of the WRN protein led to a ∼2-fold increase in mutation frequency (both single base substitutions and deletions) irrespective of the presence or absence of non-B DNA-forming sequences. Given the ability of WRN to resolve non-B DNA (Refs. 21, 23, and 63 and this study) conformations in vitro, a synergistic effect on mutation frequency might reasonably have been expected between WRN-KD and non-B DNA-forming sequences. Because no synergy was observed, a role for WRN in reducing mutation frequencies via a mechanism(s) that is dependent upon its cellular helicase activity appears unlikely. Moreover, the higher level of 8-oxodG in WRN-KD as compared with wild-type U2OS cells suggests that the observed 2-fold increase in mutations was probably caused by an overall hyperoxidative state in the WRN-deficient cells (43).

This conclusion concurs with the view that the senescent phenotype induced by WRN deficiency is associated with oxidative stress and consequent DNA damage (43, 44). Indeed, the increase in life-span of WS patients treated with pioglitazone, a potent PPARγ agonist that mediates the up-regulation of antioxidant enzymes, such as catalase and copper/zinc superoxide dismutase, has been interpreted in terms of a reduction in oxidative stress (28). In a different type of study (46), cigarette smoke, which contains numerous reactive oxygen species and cytotoxic by-products of oxidation reactions, has been found to induce DNA damage, WRN down-regulation, and cellular senescence in lung fibroblasts. Either WRN overexpression or treatment with the antioxidant N-acetylcysteine served to attenuate WRN down-regulation, senescence-associated-β-galactosidase activity, and the senescent phenotype in human pulmonary fibroblasts in culture exposed to H₂O₂ or cigarette smoke extract (46). Hence, redox imbalance appears to play a significant role in WRN deficiency-associated senescence.

The G^+ radical cation is known to be converted to several species, including 8-oxo-dG, 2-amino-5-[(2-deoxy-β-D-erythro-pentofuranosyl)amino]-4H-imidazol-4-one (Iz), its hydrolysis product 2,2-diamino-4-[(2-deoxy-β-D-erythro-pentofuranosyl)amino]-5(2H)-oxazolone, and others (54). 8-oxo-dG and Iz have been shown to base pair with A and G, respectively, giving rise to T-A and C-G transversions (64–66). In our study, mutations at GA-TG dinucleotides yielded mostly transversions with the ranking: G-C → C-G > T-A. These base changes are consistent with Iz-G and 8-oxo-dG-A mispairing intermediates, respectively (54, 64–66). Iz has also been shown to arise from both direct depñoction of G^+ in single-stranded DNA and from 8-oxo-dG through long range hole migration (65). Thus, the high G-C → C-G transition rates observed herein are consistent with hole migration activity.

8-oxo-purines have been noted to accumulate with age in normal individuals, as well as in cells from prematurely aged WS patients and WRN-KD cells in culture (reviewed in Ref. 35). Base adducts, such as 8-oxo-purines and Fapy lesions, associate stably with the WRN protein, and the subsequent complexes are believed to trigger/initiate base excision repair (24, 35, 39, 67). In the absence (or reduction) of WRN, base lesions may accumulate, thereby increasing the mutation frequency.

The increase in mutations in the supF gene by the upstream triplex and Z-DNA forming repeats is unambiguous. However, the underlying mutational mechanism remains to be determined. If pausing at the non-B DNA-forming motifs occurred in vivo, either during DNA replication or transcription (68–70), this might have increased exposure of the downstream supF sequences to the solvent and hence to oxidative damage. Consistent with this interpretation, the genome-wide increase in mutation rates observed in late-replicating regions are believed to arise from the accumulation of single-stranded DNA regions and their exposure to endogenous oxidative DNA damage (71).

Oxidative DNA damage has been proposed as a major source of mutation both in somatic and germline cells (72). Our data support this view and point to a critical role for the early steps of the reaction, including hole migration. Those genomic regions with the propensity to fold transiently into non-B DNA secondary structures may become hot spots for mutagenesis, particularly under conditions, such as WS and cancer, that are characterized by an elevated cellular basal oxidative state.

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