

# Werner syndrome helicase contains a 5'→3' exonuclease activity that digests DNA and RNA strands in DNA/DNA and RNA/DNA duplexes dependent on unwinding

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## ABSTRACT

We show that WRN helicase contains a unique 5'→3' exonuclease activity in the N-terminal region. A deletion mutant lacking 231 N-terminal amino acid residues, made in a baculovirus system, did not have this activity, while it showed ATPase and DNA helicase activities. This exonuclease activity was co-precipitated with the helicase activity using monoclonal antibodies specific to WRN helicase, indicating that it is an integral component with WRN helicase. The exonuclease in WRN helicase does not digest free single-stranded DNA or RNA, but it digests a strand in the duplex DNA or an RNA strand in a RNA/DNA heteroduplex in a 5'→3' direction dependent on duplex unwinding. The digestion products were identified as 5'-mononucleotides. Our data show that WRN helicase needs a single-stranded 3' overhang region for efficient binding and unwinding of duplex molecules, while blunt-ended or 5' overhang duplex molecules were hardly unwound. These findings suggest that the WRN helicase and integral 5'→3' exonuclease activities are involved in preventing a hyper-recombination by resolving entangled structures of DNA and RNA/DNA heteroduplexes that may be generated during replication, repair and/or transcription.

## INTRODUCTION

Werner syndrome (WS) is a human autosomal recessive genetic disorder causing symptoms of premature aging accompanied by an increased risk of cancer and shows a genomic instability in patient cells (1–7). The gene for WS (WRN) has been identified as a homolog of the *Escherichia coli* RecQ gene that encodes a DNA helicase (8,9). To date, five members have been identified in the human RecQ DNA helicase family, including the Q1 (10), BLM (Bloom syndrome) (11), WRN, Q4 and Q5 helicases (12), although the biological role(s) of these helicases remains unknown. The Q4 gene has recently been identified by us as a causative gene of Rothmund–Thomson syndrome, a genetic

disease associated with a genomic instability (13). Two other eukaryotic homologs of the *E.coli* recQ DNA helicase thus far identified are *Saccharomyces cerevisiae* SGS1(14,15) and *Schizosaccharomyces pombe* rqh1+ (16).

DNA helicases have important roles in cellular processes, such as genome replication, recombination, repair and transcription, by unwinding the duplex DNA (17). *Escherichia coli* DNA helicase RecQ is involved in double-strand break repair (18) and acts as a suppressor of illegitimate recombination (19). Mutation of yeast SGS1 suppresses the slow growth phenotype of a mutant cell that has a mutation in the topoisomerase-3 gene and also causes missegregation of chromosomes during meiosis and mitosis (14,15). The rqh1+ gene of *S.pombe* has been isolated from mutant cells that are sensitive to hydroxyurea (HU) and rqh1– mutant cells were found unrecovered from HU arrest at S phase because of a high level of recombination (16). In this context, we showed that the human BLM and WRN helicases can suppress increased homologous and illegitimate recombinations in a *S.cerevisiae* sgs1 mutant, implying a role of BLM and WRN helicases in controlling genomic stability in human cells (20).

We and others have demonstrated that the WRN helicase generated in insect cells has DNA-dependent ATPase and DNA unwinding activities (21,22). We also showed that intact WRN helicase migrates to the nucleus due to a nuclear transport signal (NLS) in the C-terminus proximal region, while truncated WRN proteins in most WS patients that lack the NLS are unable to do so. Importantly, this finding clearly explains why WS patients show a set of similar clinical phenotypes no matter what type of mutation they carry (23,24). Recently, we showed that gene expression of WRN helicase is highly up-regulated in virus-transformed cells, immortalized cells and various tumor cell lines and that WRN helicases exist mainly in the nucleoplasm (25). Apart from these defined biochemical properties, the following important problems are unsolved. Does WRN helicase unwind DNA of any special structure or regular duplex DNA? Does WRN helicase contain another enzymatic activity besides the helicase? In this context, we have shown that WRN helicase can unwind RNA/DNA heteroduplexes in addition to regular duplex DNA and that the amounts of the released RNA and DNA oligonucleotides are significantly reduced from those of the starting material, suggesting that part of

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the oligonucleotides are digested upon duplex unwinding (21). Regarding this potential presence of a nuclease activity in WRN helicase, Mushegian *et al.* (26) and others (27–29) predicted that the N-terminal globular domain of WRN helicase may contain a nuclease activity, after their computer homology searches; they suggested that the nuclease-like sequence is contained only in WRN helicase and not in other members of the RecQ family of helicases and that the combined nuclease and helicase domains may be involved in DNA repair or RNA processing.

To address the problems raised above and also to understand the reasons behind the unexpected digestion of the released products, we generated a mutant WRN helicase that lacked the predicted N-terminal exonuclease domain, purified it from contamination with cellular nuclease and characterized its enzymatic activities in comparison with the intact enzyme. As a result, we found that the N-terminal region of WRN helicase contains a 5'→3' exonuclease activity as described in this paper. This exonuclease does not digest free single-stranded DNA or RNA, but can digest the DNA or the RNA strand that is unwound from DNA/DNA or DNA/RNA duplexes, respectively, in a helicase-dependent manner.

During this work, Huang *et al.* (30), Shen *et al.* (31) and Kamath-Loeb *et al.* (32) reported that a 3'→5' DNA exonuclease resides in the N-terminal region of WRN helicase and that this nuclease activity is physically and functionally separable from the helicase activity. These findings are similar to our findings regarding the presence of an exonuclease in the N-terminal region of WRN helicase, but differ in the direction of nuclease reaction, as well as the independent nature of the exonuclease activity in the duplex unwinding by helicase.

## MATERIALS AND METHODS

### Production of wild-type and mutant WRN helicases in insect cells

The recombinant baculovirus that expresses a full-length WRN cDNA was prepared as described by Suzuki *et al.* (21). The recombinant baculovirus producing a deletion mutant lacking the N-terminal 231 amino acid residues was made by the following procedure. A cDNA fragment coding for a deletion mutant protein lacking the N-terminal 231 amino acid residues was made by PCR using *Pfu* polymerase and primers with additional *SpeI* and *XhoI* restriction cleavage sites and was inserted into the transfer vector pFastBacHTc (Gibco BRL). After recombining with Bacmid DNA in *E. coli* DH10BAC™, the purified recombinant Bacmid DNA containing deletion mutant cDNA and a sequence encoding an N-terminal hexahistidine tag was transfected into insect cells by lipofection. The resulting recombinant viruses that express the mutant WRN helicase protein were confirmed by nucleotide sequencing and western blot analysis.

### Purification of wild-type and mutant WRN helicases

Both the wild-type WRN (wtWRN) helicase and the deletion mutant WRN ( $\Delta$ N231) helicases were produced in Sf9 cells ( $2 \times 10^8$  and  $1 \times 10^9$  cells, respectively) by infecting with a recombinant virus at a multiplicity of infection of 5 and the cells were harvested from monolayer flasks after 48 h culture at 27°C. The cells were washed once with cold phosphate-buffered saline (PBS), pelleted by centrifugation (1000 *g* for 15 min at 4°C) and stored at –80°C. The early steps in the purification of both proteins by Ni–nitrilotriacetic acid–agarose column chromatography were as described by us

before (21). The partially purified wtWRN and  $\Delta$ N231 proteins were subjected to a further purification after dilution to a salt concentration of 75 mM NaCl and were applied first to a heparin–cellulose column (HiTrap; Pharmacia). After washing the columns with 10 column vol of K buffer (50 mM HEPES–KOH buffer, pH 7.9, containing 100 mM KCl and 10% glycerol), the bound proteins were eluted by increasing concentrations of KCl. The pooled peak fractions eluted at 350–650 mM KCl from the heparin–cellulose column were diluted to a concentration of 100 mM KCl and the mixture was loaded onto a Q-Sepharose HP column (HiTrap; Pharmacia). After washing the column with K buffer, the proteins were eluted in steps using an increasing concentration of 100–800 mM KCl in a total volume of 25 ml. All fractions were characterized using 10% PAGE in the presence of 0.1% SDS (SDS–PAGE) by staining with Coomassie brilliant blue R-250 and by western blot analysis using a monoclonal antibody specific to the C-terminal region of WRN helicase.

### Construction of the substrate DNAs for helicase and exonuclease assays

The standard substrate DNA was generated by the following procedure. The 3'-<sup>32</sup>P-labeling of an *EcoRI*+*SphI*-digested M13mp18 RF phage M13 DNA fragment (41mer) was performed with a fill-in reaction by the Klenow fragment of DNA polymerase I with dTTP and [ $\alpha$ -<sup>32</sup>P]dATP. The labeled DNA fragment was denatured, annealed to an excess amount of the single-stranded M13 DNA and purified by spin filtration. A *HindIII*+*EcoRI*-digested fragment (43mer) of M13 duplex DNA was 5'-labeled with <sup>32</sup>P by T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP, after phosphatase treatment and digestion with *KpnI*. The oligodeoxynucleotides used for helicase and exonuclease assays were: 5'-GTCGACTCTA-GAGGATCCCCGGGTACCGAGCTCGAATTCCG-3' (40mer), 5'-CGAATTTCGAGCTCGGTACCCGGGGATCCTCTAGAGTC-GACGATCATCCTA-3' (50mer) and 5'-ATCCTACTAGCGAAT-TCCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGAC-3' (50mer). They were labeled with <sup>32</sup>P at the 5'-end using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP. The 3'-<sup>32</sup>P-labeling of oligoribonucleotide was by RNA ligase (Nippon Gene) and [ $\alpha$ -<sup>32</sup>P]pCp (33). The labeled oligonucleotides were purified by electrophoresis in 10% polyacrylamide gels; the band of labeled oligonucleotides was detected by autoradiography, was excised from the gel and the oligonucleotide was extracted from the gel slice by soaking in 0.3 M sodium acetate containing 1 mM EDTA and 0.1% SDS overnight at room temperature. The labeled oligonucleotides were annealed with 2.5  $\mu$ g M13mp18 DNA and non-hybridized oligonucleotides were removed by spin filtration. To prepare short duplex oligodeoxynucleotides with various terminal structures, the oligodeoxynucleotides were annealed and the resulting duplex molecules were purified by 20% PAGE under non-denaturing conditions and were recovered from the gel.

### Helicase and ATPase assays

The labeled substrate DNA (~2.5 pg) was incubated at 37°C with purified WRN protein (5–10 pg) in a reaction mixture (20  $\mu$ l) consisting of 50 mM Tris–HCl buffer (pH 7.5), 1 mM MgCl<sub>2</sub>, 2 mM ATP, 2 mM 2-mercaptoethanol (2-ME) and 0.5 mg/ml BSA. After 60 min incubation, the reactions were stopped by adding 5  $\mu$ l Tris–HCl (pH 8.0) buffer containing 0.5 mg/ml proteinase K, 200 mM EDTA and 2.5% SDS and by continuous incubation at 37°C for 10 min. The reaction products were

analyzed by 3% agarose gel electrophoresis. The radioactivity of the released oligonucleotides was quantified by excising the bands from the dried gel and by measuring the radioactivity using a Beckmann scintillation counter.

#### Measurement of exonuclease activity by PAGE and identification of the reaction product

Exonuclease activity was measured using gel electrophoresis using a 3'-<sup>32</sup>P-labeled substrate DNA by the method of Mummenerbrauer *et al.* (34). The reaction mixture (20  $\mu$ l) was the same as that for the helicase assay, but the reaction was run for a much shorter time to detect the intermediate reaction products. The reaction was started by adding 5–10  $\mu$ g protein, was continued for 2 min at 37°C and was then stopped by adding 5  $\mu$ l of a formamide–dye solution (95% deionized formamide, 40 mM EDTA). After incubation at 95°C for 4 min, the products were analyzed by 10% PAGE under denaturing conditions. The gel was dried on Whatman 3MM paper and was analyzed using a FUJI Bas (Fuji) image analyser.

#### Identification of the exonuclease reaction product

After incubating the 5'-<sup>32</sup>P-labeled oligonucleotide 5'-AGCTTG-CATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTAC-3' annealed to the single-stranded circular M13 DNA with wtWRN helicase in the helicase reaction mixture, the products were analyzed using high voltage paper electrophoresis (35).

#### Co-immunoprecipitation of exonuclease with WRN helicase by specific monoclonal antibodies

Purified mouse monoclonal antibodies 4F8 and 8H3 (2.5  $\mu$ g each) specific for WRN helicase (25) were mixed with the purified wtWRN protein in 20  $\mu$ l of 50 mM HEPES–KOH (pH 7.9) containing 120 mM NaCl. The mixture was kept overnight at 4°C and then the WRN protein that reacted with the antibodies was removed by adding 30  $\mu$ l of protein G–Sepharose 4FF (Pharmacia) and the resulting precipitate centrifuged. The helicase and exonuclease activities in the supernatant and pellets were measured as described in the legend to Figure 2. As a negative control, mouse IgG (Sigma) was substituted for the monoclonal antibody.

## RESULTS

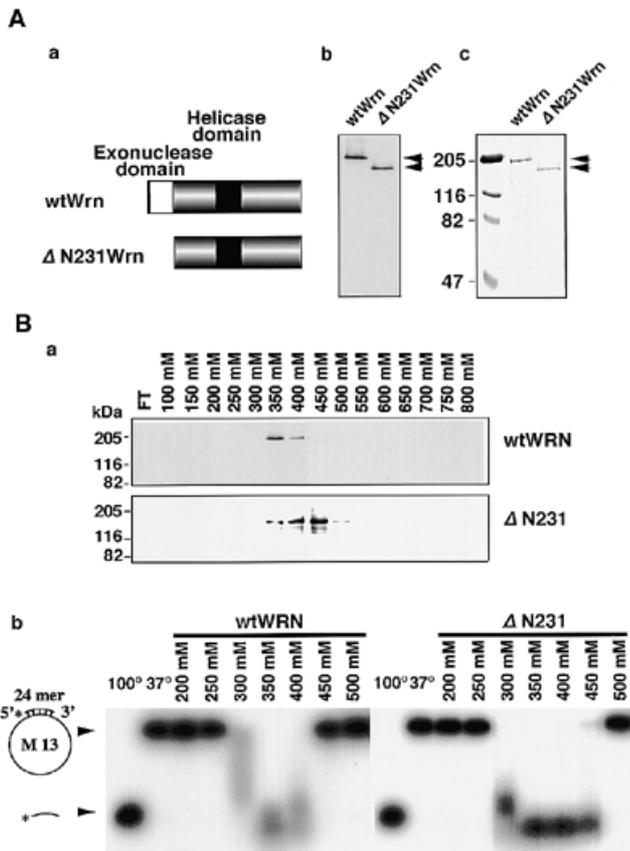
#### DNA helicase activity in a mutant WRN helicase lacking 231 N-terminal amino acid residues

Protein alignment studies have shown that the N-terminus proximal region (amino acid residues 60–231) of WRN helicase is homologous to the functional domain of bacterial RNase D and to a 3'→5' proofreading exonuclease domain of bacterial DNA polymerase I (Pol A) (26–29). To examine if this region codes for an exonuclease, we generated a recombinant baculovirus that expresses a deletion mutant of WRN helicase ( $\Delta$ N231) lacking the predicted exonuclease domain but containing an N-terminal hexahistidine tag (Fig. 1Aa). After infecting Sf9 cells with these viruses, the nuclear extracts were prepared and analyzed by immunoblotting using a monoclonal antibody specific for the C-terminal region of WRN helicase (Fig. 1Ab). The nuclear lysate containing the wtWRN helicase tagged N-terminally with hexahistidine was similarly analyzed as a reference. The lysates

of cells expressing  $\Delta$ N231 WRN helicase proteins showed a distinct band with a relative mass of ~140 kDa, less than the 180 kDa wtWRN helicase and consistent with the N-terminal deletion of 231 amino acid residues. This  $\Delta$ N231 protein was purified extensively by a series of column chromatographies using nickel-chelate NTA, heparin–Sepharose and anion exchange Q-Sepharose HP resins, as described in Materials and Methods. Figure 1Ac shows the SDS–PAGE analysis stained with Coomassie brilliant blue for both the intact and mutant WRN helicases. Although they were not purified to a homogenous state, they were free from contamination with cellular nuclease(s) (Fig. 4A, lanes 1–3). To compare the biochemical features strictly, the wtWRN helicase was purified at the same time as the  $\Delta$ N231 protein under the same conditions. Figure 1Ba shows the elution profiles of wtWRN helicase and its derivative  $\Delta$ N231 protein in the last column chromatography with a negative ion exchange Q-Sepharose HP resin. Major parts of the  $\Delta$ N231 protein were eluted at slightly higher concentrations of the KCl gradient than that for wtWRN helicase, consistent with an increased net negative charge of –3 for the  $\Delta$ N231 protein due to the N-terminal deletion. When DNA helicase assays were carried out for the fractions containing these WRN proteins using a 5'-<sup>32</sup>P-labeled oligodeoxynucleotide (24mer) annealed to single-stranded M13 DNA as a substrate, the fractions containing the wtWRN helicase (300–400 mM KCl fractions) clearly showed a DNA unwinding activity as established by us previously (Fig. 1Bb, left; 21). Fractions containing  $\Delta$ N231 (300–450 mM KCl fractions) also showed DNA helicase activity (Fig. 1Bb, right), suggesting that the  $\Delta$ N231 protein contains a DNA helicase activity that unwinds an oligonucleotide–M13 DNA complex. Similar to the wtWRN helicase (21), this helicase activity of the  $\Delta$ N231 protein was dependent on the presence of ATP and the fraction containing  $\Delta$ N231 protein showed a  $\gamma$ ATPase activity similar to the wtWRN helicase (data not shown). These findings suggest that the N-terminal region, particularly the N-terminal 231 amino acid residue, is not needed for the ATP-dependent DNA helicase activity.

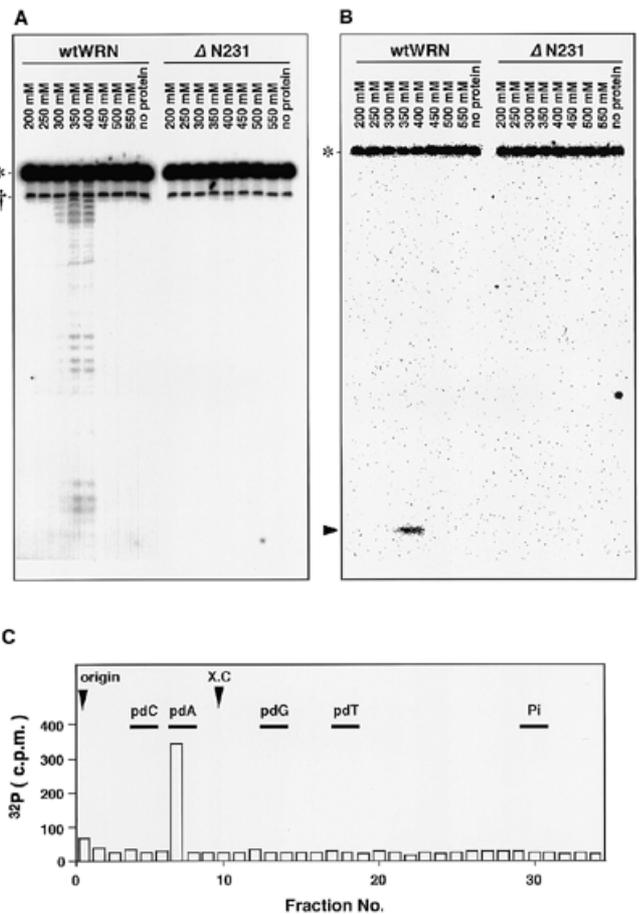
#### A 5'→3' exonuclease activity in wtWRN helicase, but not in mutant $\Delta$ N231 helicase

When the purified wtWRN and mutant  $\Delta$ N231 helicases were analyzed for exonuclease activity using substrate DNA consisting of 3'-<sup>32</sup>P-labeled oligodeoxynucleotide annealed to single-stranded circular M13mp18 DNA, we found that the exonuclease activity was associated with wtWRN helicase, but not with the mutant  $\Delta$ N231 helicase (Fig. 2). The wtWRN helicase, eluted at 300–400 mM KCl on Q-Sepharose HP column chromatography, released labeled shortened oligonucleotides from a large substrate complex that were resolved as a ladder by 10% PAGE (Fig. 2A, wtWRN). The ladder-like resolution profile of the reaction products was characteristic of an exonuclease reaction after short-term incubation. In contrast, the mutant  $\Delta$ N231 helicase, eluted at 300–450 mM KCl, released no or only a trace amount of labeled oligonucleotides under the same conditions (Fig. 2A,  $\Delta$ N231), suggesting that the  $\Delta$ N231 helicase contains no or a very low level of exonuclease activity, even though it contains a comparable level of helicase activity. When the same reaction was performed using a different substrate DNA consisting of a 5'-<sup>32</sup>P-labeled oligonucleotide annealed to single-stranded circular M13mp18 DNA, all the reaction products released by wtWRN



**Figure 1.** Identification of a helicase activity in the purified intact and mutant WRN proteins expressed in insect cells. (A) (a) Structures of intact WRN helicase and ΔN231 mutant WRN protein. (b) Proteins in cells expressing wtWRN and ΔN231 mutant helicases were resolved by SDS-PAGE and analyzed by immunoblotting using a monoclonal antibody (8H3) (25). (c) Analysis of purified wtWRN and ΔN231 WRN proteins by SDS-PAGE followed by staining with Coomassie brilliant blue dye. (B) (a) Purification of wtWRN and ΔN231 WRN proteins by Q-Sepharose column chromatography and detection by western blotting using a WRN-specific monoclonal antibody: wtWRN and mutant ΔN231 WRN proteins are shown in the upper and lower panels, respectively. FT, flow-through. Numerals represent KCl concentrations (mM) used for eluting proteins. (b) Presence of helicase activities in both intact wtWRN protein (left) and mutant ΔN231 WRN protein (right). Aliquots of wtWRN and ΔN231 WRN proteins were analyzed for helicase activity by a displacement assay using 5'-<sup>32</sup>P-labeled oligodeoxynucleotide (24mer) annealed to M13 DNA. The products in 10 μl of reaction mixture were analyzed by 3% agarose gel electrophoresis. The results from a reaction mixture heat-denatured at 100°C for 4 min (lane 100°) or from a mixture containing no WRN protein but processed similarly to other samples (lane 37°) are shown as controls.

helicase migrated at high speed in the gel (Fig. 2B, wtWRN). With ΔN231 helicase, however, no apparent reaction occurred and only a very faint sign of radioactivity was observed at the position of the mononucleotide (Fig. 2B, ΔN231). The small molecular weight product made by incubation with wtWRN helicase was identified as 5'-<sup>32</sup>P-labeled dAMP, as it co-migrated with authentic 5'-dAMP in high voltage paper electrophoresis (Fig. 2C). These results collectively indicate that the exonuclease activity associated with wtWRN helicase digests the oligonucleotide exonucleolytically in a 5'→3' direction. Gray *et al.* (22) showed that WRN helicase binds to one strand of duplex DNA and migrates in a 3'→5' direction. Thus, the 5'→3' polarity of the exonuclease reaction that digests one strand of duplex DNA is

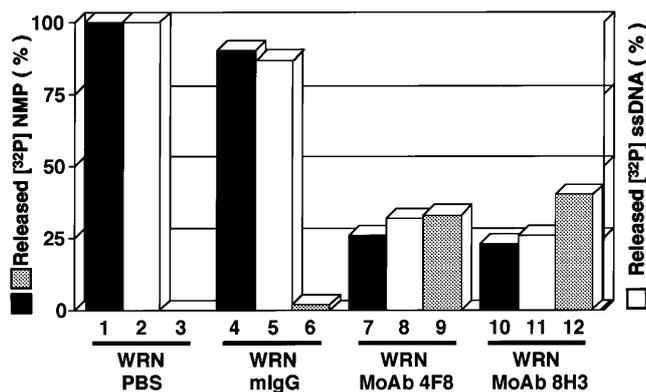


**Figure 2.** Identification of the exonuclease activity in the intact WRN helicase but not in the mutant ΔN231 helicase. Both the purified wtWRN and ΔN231 WRN proteins after Q-Sepharose column chromatography were analyzed for exonuclease activity using two different substrates. (A) 3'-<sup>32</sup>P-labeled oligodeoxynucleotide (41mer) annealed to M13 DNA was used as a substrate. (B) 5'-<sup>32</sup>P-labeled oligodeoxynucleotide (43mer) annealed to M13 DNA was used as a substrate. The reaction conditions were similar to that for the helicase assay, except that the incubation time was shorter (2 min). The products were resolved by 10% PAGE, after denaturing the substrate DNA complex by heating (100°C, 2 min) and subsequent chilling in ice. Asterisks represent the <sup>32</sup>P-labeled oligonucleotide substrate. A dagger in (A) indicates a short <sup>32</sup>P-labeled contaminant oligonucleotide in the 41mer oligonucleotide. (C) High voltage paper electrophoresis to identify the reaction product obtained with 5'-<sup>32</sup>P-labeled substrate. X.C indicates xylene cyanol dye used as a standard migration marker. pdC, pdA, pdG and pdT represent the 5'-deoxyribo-nucleotides of cytosine, adenine, guanine and thymine, respectively. Pi represents inorganic phosphate.

compatible with the 3'→5' migration on the complementary strand for DNA unwinding. In agreement with this, when the oligonucleotides labeled at either the 3'- or 5'-end were incubated in their free single-stranded forms with the wtWRN helicase, no exonucleolytic digestion occurred, as shown below, suggesting that the exonuclease acts on the oligonucleotide in a way that depends on helicase action.

#### Co-immunoprecipitation of exonuclease activity with WRN helicase by monoclonal antibodies specific to WRN helicase

We recently developed several monoclonal antibodies (mAbs), among which mAbs 4F8 and 8H3 were defined as having



**Figure 3.** Immunodepletion of the WRN helicase and exonuclease activities by WRN-specific monoclonal antibodies. Mouse mAbs 4F8 and 8H3, specific for WRN helicase protein, were mixed with the purified intact WRN helicase and incubated at 4°C for 12 h. As controls, PBS alone or mouse IgG were also used. The resulting immunocomplexes formed between WRN protein and antibodies were captured by protein G-Sepharose 4FF gel matrix and were removed by centrifugal precipitation. Both helicase and exonuclease activities remaining in the supernatant fractions were measured by analyzing 2 µl aliquots of the supernatant. Also, the unwinding-dependent exonuclease activity was monitored in the immunoprecipitated fractions. The helicase assay was done using 5'-<sup>32</sup>P-labeled oligonucleotide (24mer) annealed to M13 DNA as substrate. The exonuclease assay was carried out using 5'-<sup>32</sup>P-labeled oligonucleotide (43mer) as shown in Figure 2B. The activities of helicase and exonuclease were quantitated by measuring the intensities of released products, oligonucleotide and mononucleotide, respectively, in the autoradiograms. The remaining activities in the supernatant of one of the control samples (incubated with PBS) were assumed to contain 100% activity. The activities (shown as percentages) remaining in the supernatant fractions are represented for exonuclease by a black column and for helicase by a white column. The unwinding-dependent exonuclease activities recovered from pellets are shown by a hatched column.

epitopes in the N-terminal region (amino acid residues 232–368) of WRN helicase adjacent to the deduced coding region for WRN exonuclease (amino acid residues 1–231) (25). To verify if an exonuclease activity exists in the WRN helicase molecule, we carried out an immunoprecipitation experiment using these monoclonal antibodies. The purified WRN helicase (after the Q-Sepharose column chromatography step) was incubated with mAbs 4F8 and 8H3 and the immunocomplex formed between the mAbs and WRN helicase was precipitated by binding to a protein G-agarose gel. As a reference, non-immune mouse IgG was used instead of specific antibodies. When each of the supernatant fractions was analyzed for DNA helicase and exonuclease, we found that the exonuclease activity was immunodepleted together with the WRN helicase activity by mAbs specific for WRN helicase (Fig. 3, columns 7, 8, 10 and 11). In contrast, the non-immune IgG, used as a negative control, did not precipitate either helicase activity or exonuclease activity (Fig. 3, columns 4 and 5). In addition, ~30–40% of the total exonuclease activity dependent on DNA unwinding by helicase (see below) was recovered from the immunoprecipitates (hatched columns 9 and 12). These results, together with ΔN231 WRN lacking an exonuclease activity, indicate that the exonuclease is an integrative component of wtWRN DNA helicase.

#### Characterization of WRN-associated exonuclease

The enzymatic properties of the 5'→3' exonuclease (for short 5'-exonuclease) associated with WRN helicase were studied

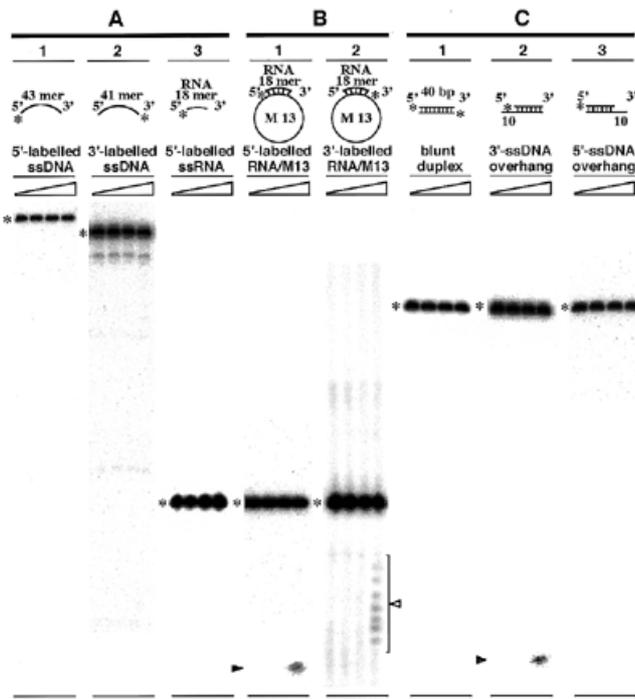
using the purified preparation obtained by Q-Sepharose column chromatography (Table 1). The exonuclease required ATP to degrade an oligonucleotide annealed to single-stranded M13 DNA. A non-hydrolyzable ATP analog, ATPγS, failed to substitute for ATP, but it inhibited the exonuclease activity by 90% at 2 mM concentration, which also inhibited helicase activity by 90% (data not shown). The pH range for the exonucleolytic reaction was broad, 6.5–8.5, with the optimum around 7.5. The exonuclease required the divalent cation Mg<sup>2+</sup> or Mn<sup>2+</sup> for the reaction, while Zn<sup>2+</sup> and Ca<sup>2+</sup> did not substitute and adding EDTA abolished the activity. It was sensitive to a high concentration of salts: sodium chloride and potassium chloride showed half-maximal inhibition at 125 mM. All these enzymatic profiles resemble those of WRN helicase, consistent with the hypothesis that the 5'-exonuclease activity is dependent on the duplex unwinding reaction of WRN helicase.

**Table 1.** Reaction conditions required for exonuclease associated with WRN helicase

Assay conditions	Degraded DNA
Complete standard assay	1.0
pH 6.5	~1.0
pH 8.5	~1.0
2 mM Mn <sup>++</sup>	0.8
2 mM Zn <sup>++</sup>	0.0
2 mM Ca <sup>++</sup>	0.0
20 mM EDTA	0.0
2 mM ATPγS	0.1
125 mM potassium chloride	0.5
125 mM sodium chloride	0.5

The complete reaction mixture (20 µl) contained purified WRN helicase (~10 pg), 5'-<sup>32</sup>P-labeled oligonucleotide (43mer) annealed to M13 DNA and other ingredients described in Materials and Methods. The reaction mixture was incubated for 15 min at 37°C and the reaction product, a mononucleotide, resulting from unwinding and digestion was analyzed by 10% PAGE. An autoradiogram was made from the dried gel and exonuclease activities under various conditions were estimated by measuring the intensities of the released mononucleotide radioactivity in the autoradiogram. Reaction conditions deviating from that of the complete reaction mixture are indicated in the left column. The right column shows the relative activities of exonuclease, assuming the activity in the complete mixture as 1.0.

To investigate the potential substrate for the 5'-exonuclease in WRN helicase, we tested a total of 10 different possible substrates (Fig. 4). Among them, single-stranded 43mer and 41mer DNA labeled with <sup>32</sup>P at the 5'- and 3'-ends, respectively, and a 5'-<sup>32</sup>P-labeled 18mer oligoribonucleotide were resistant to digestion by the exonuclease associated with WRN helicase (Fig. 4A1–A3). Although resistant in their free forms, both the oligodeoxyribonucleotides and the oligoribonucleotide were digested by WRN exonuclease after they were hybridized to the complementary DNA, as shown in Figure 2A and B and in Figure 4B1 and B2. The 5'-<sup>32</sup>P-labeled RNA 18mer in an RNA/M13 DNA heteroduplex yielded mononucleotides consistent with 5'→3' exonucleolytic digestion by an increased amount of WRN helicase (Fig. 4B1) and the 3'-<sup>32</sup>P-labeled RNA 18mer in the same



**Figure 4.** Structure of nucleic acids required for the exonuclease reaction. Oligodeoxyribonucleotides or oligoribonucleotides labeled either at the 5'- or 3'-end were tested for susceptibility to being digested by the exonuclease associated with WRN helicase. They were tested either in the single-stranded form (A) or in the duplex form annealed to M13 DNA (B) or to complementary oligonucleotides (C). The structures of substrate DNA or RNA are shown at the top of the panel. The reactions were done in the complete mixture with a short incubation time (2 min). The reaction products were analyzed by 10% PAGE. An arrowhead shows the position where mononucleotides migrate.

heteroduplex gave rise to a ladder-like profile of digested intermediate products and mononucleotides (Fig. 4B2). Short DNA molecules (with 5'-labeled 40mers) containing different terminal structures showed that a blunt-ended 5'-<sup>32</sup>P-labeled duplex is hardly digested by the exonuclease (Fig. 4C1), while a 5'-<sup>32</sup>P-labeled 40mer in a partial duplex containing a 3' overhang sequence can be digested and yields a 5'-<sup>32</sup>P-labeled mononucleotide (Fig. 4C2). In contrast, the 5'-<sup>32</sup>P-labeled blunt-ended 40mer in the same partial duplex containing a 5' overhang on the other strand was not digested (Fig. 4C3). These results support the view that WRN helicase unwinds the duplex DNA or RNA/DNA heteroduplexes in the 3'→5' direction by binding to a single-stranded region and simultaneously (but occasionally) digesting the counterstrand exonucleolytically in a 5'→3' direction.

#### Structure of substrates required for efficient unwinding and exonucleolytic digestion by WRN helicase

To understand in more detail the molecular structure(s) optimal for DNA unwinding and exonucleolytic digestion by WRN helicase, we prepared several DNA molecules that included a blunt-ended DNA duplex 40mer, duplex DNAs (40mers) containing a 10 base 3' overhang or a 10 base 5' overhang (Fig. 5A), and they were tested for their susceptibility to WRN helicase and exonuclease (Fig. 5A1–A3). The data show that WRN helicase unwinds the DNA with a 3' overhang effectively with simulta-

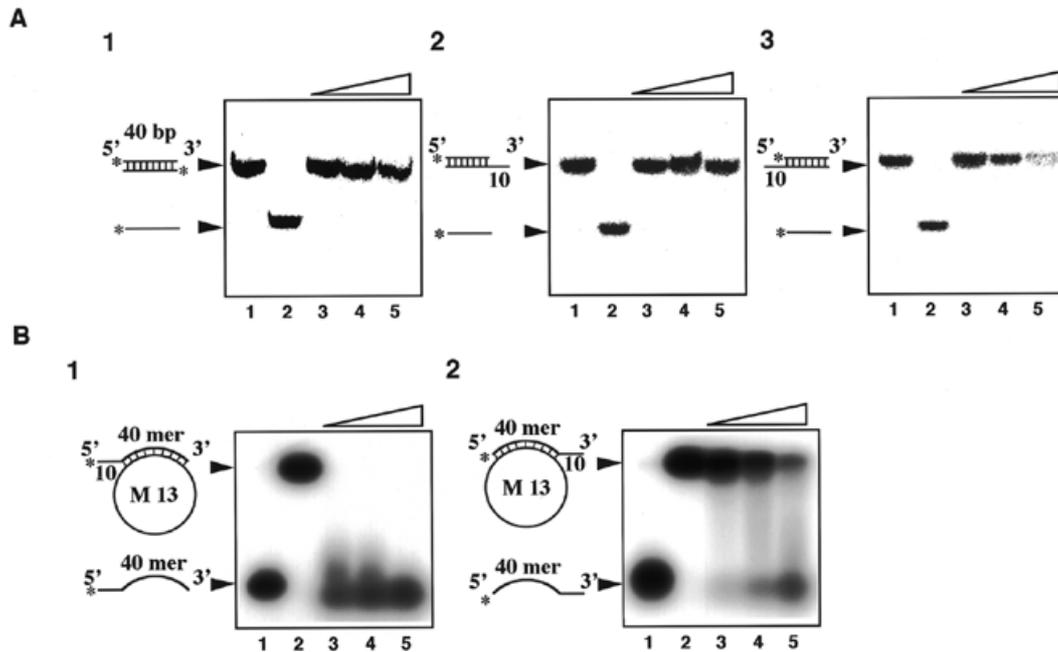
neous digestion of the 5'-end of the indented strand (Fig. 5A3). It is noteworthy that the blunt-ended DNA structure and DNA with a 5' overhang appears to be resistant to unwinding and to being digested exonucleolytically (Fig. 5A1 and A2). We showed previously that an oligonucleotide (40mer) annealed to single-stranded M13 DNA is released from M13 DNA by WRN helicase and that the same 40mer is released much faster when unpaired fringe 10mers are added at both the 3'- and 5'-ends (21).

To extend the search for the most favorable DNA structure to be unwound by WRN helicase, two DNA complexes, consisting of single-stranded M13 DNA and the 40mer oligodeoxyribonucleotides containing a 5' or 3' unpaired fringe sequence of 10mers, were prepared as a model for illegitimate base pairing of DNA strands (Fig. 5B). When incubated with WRN helicase, the oligonucleotide with a 5' unpaired fringe sequence (Fig. 5B1) was released much faster than that with a 3' fringe sequence (Fig. 5B2). The data indicate that WRN helicase binds most favorably to a single-stranded fork region of DNA and unwinds the base paired region by migrating from the 3'- to the 5'-end. The susceptibility of the DNA terminus to unwinding and to digestion by WRN helicase and 5'-exonuclease prompts us to speculate that WRN helicase binds to the single-stranded region of DNA, migrates in the 3'→5' direction and exonucleolytically digests the other strand as the strand is unwound (Fig. 6).

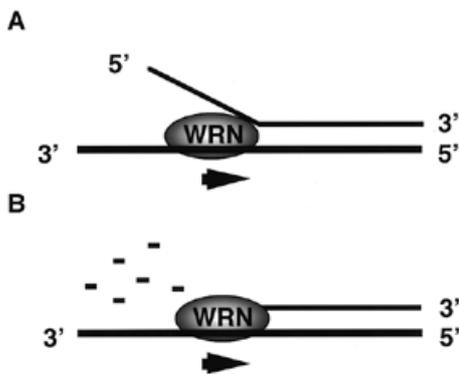
## DISCUSSION

We showed previously that the WRN gene product is a DNA helicase that unwinds DNA and RNA/DNA heteroduplexes (21). Gray *et al.* (22) also showed that WRN helicase unwinds DNA in a 3'→5' direction, migrating along the DNA to which it binds. Despite these defined biochemical characteristics of the WRN gene product, how the defective helicase causes WS remains obscure and more information about the nature of WRN helicase is needed to decipher the biological functions and/or cellular events in which WRN helicase is involved.

In this study, we showed that an exonuclease activity is associated with the WRN helicase. Our comparative studies with purified wtWRN helicase protein and its N-terminally truncated ΔN231 protein showed that wtWRN helicase, but not the ΔN231 protein, contains 5'-exonuclease activity, whereas both proteins contain helicase activity (Figs 1 and 2). The helicase activity associated with ΔN231 protein has been confirmed through multiple column chromatography steps during purification (data not shown). In Figure 1Bb, the profile of helicase activity in ΔN231 protein appears to deviate slightly from that of proteins eluted from the columns by high concentrations of KCl (350–500 mM). However, this is primarily due to an inhibitory effect of high KCl concentrations, as shown in Table 1. The helicase and 5'-exonuclease activities in wtWRN helicase were co-purified by multiple column chromatography and they were co-precipitated by monoclonal antibodies specific to WRN helicase (Fig. 3). These results provide us with several important insights into the structure and biochemical function of WRN helicase. First, the WRN helicase contains three enzymatic activities, helicase, ATPase and 5'-exonuclease. Second, the 5'-exonuclease activity is unnecessary for the activity of wtWRN helicase because the ΔN231 protein lacking the 231 N-terminal amino acids and exonuclease activity retains an equivalent ATP-dependent helicase activity. Third, the exonuclease activity is most probably encoded in the 231 N-terminal amino acid region of the wtWRN helicase; this view is supported by our experimental data



**Figure 5.** Substrate DNA structures required for optimal unwinding and exonucleolytic digestion. Reactions were made by incubating DNA substrates for 90 min at 37°C in the complete reaction mixture with purified wtWRN helicase (pooled 350–400 mM KCl fractions of Fig. 1Ba) and the products were analyzed using electrophoresis with 20% polyacrylamide and 3% agarose gels. (A) 5'-<sup>32</sup>P-labeled oligodeoxyribonucleotide (40mer) was annealed to the complementary oligodeoxyribonucleotides of various sizes to generate duplex oligomers containing different terminal structures. These structures are at the side of each panel. In each panel, lanes 1 and 2 show the unreacted substrate DNA and the heat-denatured substrate DNA, respectively. Lanes 3–5 show reactions for increasing concentrations of wtWRN helicase. (B) Two types of 5'-<sup>32</sup>P-labeled oligodeoxyribonucleotides (50mer) were annealed to M13 DNA and were tested for susceptibility to be unwound and digested by wtWRN helicase and the associated exonuclease. The oligonucleotides were designed to form a partial duplex (with M13 DNA) with extra unpaired 5' (1) or 3' (2) fringes. Lanes 1 and 2 show the heat-denatured substrate DNA and the substrate DNA before incubation with wtWRN helicase.



**Figure 6.** Schematic representation of a possible cooperative duplex unwinding and exonucleolytic digestion by the 3'→5' WRN helicase and the 5'→3' exonuclease. (A) Unwinding the duplex without exonucleolytic digestion. (B) Unwinding the duplex with exonucleolytic digestion.

that the purified ΔN231 protein lacks exonuclease activity, as well as an amino acid homology search by Mushegian *et al.* (26).

The 5'-exonuclease activity associated with WRN helicase does not digest single-stranded DNA or RNA molecules in their free form (Fig. 4). However, it digests DNA or RNA strands in DNA/DNA homoduplexes or RNA/DNA heteroduplexes as the helicase unwinds the duplex. The reaction conditions required for exonuclease activity are identical to those required for helicase (Table 1), suggesting that the 5'-exonuclease activity is dependent

on the unwinding of duplexes catalyzed by helicase. Perhaps, the 5'-exonuclease in the N-terminal region works together with the helicase in the helicase domain in the middle of the molecule (Fig. 1Aa, darkened area). The data obtained from experiments to examine the terminal structures of DNA or RNA/DNA duplexes required for unwinding and 5'-exonucleolytic digestion (Figs 4 and 5) are consistent with this hypothesis, i.e. no exonucleolytic digestion of substrate DNA or RNA occurs unless the substrate duplexes are unwound. For example, blunt-ended duplexes are not good substrates and, accordingly, no digestion occurs (Figs 4C1 and 5A1). In contrast, duplex molecules with a 3' overhang structure are good substrates for both helicase and exonuclease (Figs 4C2 and 5A3 and B2).

All our results with various DNA and RNA substrates having different terminal structures are consistent with the view that WRN helicase needs an open single-stranded DNA region to bind before its migration in the 3'→5' direction and duplex unwinding. In this study, we found that the direction of the exonuclease reaction is 5'→3', indicating that exonuclease digests the unwound strand as helicase proceeds to unwind the duplex DNA and RNA/DNA heteroduplex (Fig. 6), the products of the digestion being 5'-monophosphorylated nucleotides. In an *E. coli* protein complex recBCD, necessary for DNA recombination, the component protein complexes recB and recC have a DNA unwinding activity and recD has an exonuclease activity (36). A similar assembly of helicase and exonuclease might have occurred in a structurally fused form in WRN helicase.

Our finding that WRN helicase has an ability to unwind RNA/DNA heteroduplexes in addition to duplex DNA is intriguing, because this ability extends the possible biological role(s) of WRN helicase to RNA synthesis-mediated cellular events, such as transcription. Recently, Bennett *et al.* (37) reported that Sgs1 DNA helicase, a yeast homolog of WRN helicase, is also capable of unwinding RNA annealed to DNA. If the unwinding of RNA/DNA heteroduplexes is an intrinsic physiological function of WRN helicase (or Sgs1 helicase), defining the RNA species (transcripts of RNA polymerase I or II or both) that are involved in the potential heteroduplex substrate will be of great importance, because WRN helicase resides mainly in the nucleoplasm (25), but it can be found also in the nucleolus (38,39; our unpublished data).

Genomic instability resulting from illegitimate recombination and deletion has been noted in the cells of WS patients (7,40) where no intact WRN helicase exists in the nucleus (23,24). To search for the true biological function of WRN helicase, we tested a DNA structure as a model of illegitimate DNA interaction, using a 50mer oligonucleotide in which a 40mer region is base paired with M13 DNA and the remaining 10mer is unpaired at the 5'- or 3'-end (Fig. 5B). With these DNA substrates, the release of oligonucleotides by WRN helicase is faster with the 5' unpaired fringe 10mer than the 3' unpaired fringe (Fig. 5B). The intensities of the substrates decreased from the original level, which we expected from the action of unwinding-dependent 5'-exonuclease. However, why only part of the released oligonucleotides was digested by exonuclease remains to be clarified. We speculate that the 5'-exonuclease may need free 5'-ends for the exonucleolytic reaction to proceed, which occurs efficiently when the free 5'-end is in close proximity to the duplex region to be unwound (Fig. 6A), but less efficiently when the free 5'-ends are distal from the site of the unwinding reaction (Fig. 6B). Further study is needed to resolve the discrepancy between the 5'→3' exonuclease described in this paper and the 3'→5' exonuclease activity previously reported by Huang *et al.* (30), Shen *et al.* (31) and Kamath-Loeb *et al.* (32) and to determine if WRN helicase is involved in disrupting DNA (or RNA) structures formed by the illegitimate interaction between DNA strands (or between DNA and RNA strands) during DNA repair, replication and transcription.

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