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Biochemical Characterization of an Exonuclease from *Arabidopsis thaliana* Reveals Similarities to the DNA Exonuclease of the Human Werner Syndrome Protein*

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The human Werner syndrome protein (hWRN-p) possessing DNA helicase and exonuclease activities is essential for genome stability. Plants have no homologue of this bifunctional protein, but surprisingly the *Arabidopsis* genome contains a small open reading frame (ORF) (AtWRNexo) with homology to the exonuclease domain of hWRN-p. Expression of this ORF in *Escherichia coli* revealed an exonuclease activity for AtWRNexo-p with similarities but also some significant differences to hWRN-p. The protein digests recessed strands of DNA duplexes in the 3′ → 5′ direction but hardly single-stranded DNA or blunt-ended duplexes. In contrast to the Werner exonuclease, AtWRNexo-p is also able to digest 3′-protruding strands. DNA with recessed 3′-PO_4 and 3′-OH termini is degraded to a similar extent. AtWRNexo-p hydrolyzes the 3′-recessed strand termini of duplexes containing mismatched bases. AtWRNexo-p needs the divalent cation Mg^{2+} for activity, which can be replaced by Mn^{2+}. Apurinic sites, cholesterol adducts, and oxidative DNA damage (such as 8-oxoadenine and 8-oxoguanine) inhibit or block the enzyme. Other DNA modifications, including uracil, hypoxanthine and ethenoadenine, did not inhibit AtWRNexo-p. A mutation of a conserved residue within the exonuclease domain (E135A) completely abolished the exonucleolytic activity. Our results indicate that a type of WRN-like exonuclease activity seems to be a common feature of the DNA metabolism of animals and plants.

RecQ helicases play a major role in the maintenance of genome stability (1, 2). Unique members of the RecQ family of DNA helicases have been found in *Escherichia coli*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe.* Higher eukaryotes like *Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis*, *Mus musculus*, and *Homo sapiens* harbor up to five family members in their genome. In humans several inherited diseases were correlated with mutations in RecQ-like helicases (3–5). The most prominent disease is the Werner syndrome (WS),[1] an autosomal recessive genetic disorder characterized by early onset of premature aging (4) and genomic abnormalities, such as chromosome breaks, reciprocal chromosomal translocations (6), and extensive genomic deletions (7). The Werner protein (hWRN-p) responsible for WS, possesses 3′ → 5′ exonuclease and 3′ → 5′ helicase activities (8, 9). The 3′ → 5′ helicase of hWRN-p unwinds blunt-ended duplexes containing a single-stranded bubble and G4 DNA (10), mediates migration of Holliday junctions (10, 11), and mediates unwinding of DNA triple helixes with a free 3′-tail of the third strand (12) as well as of a $d(CGG)_7$ hairpin and tetraplex structures, which block DNA synthesis, to facilitate polymerase δ to traverse these unusual DNA structures and synthesize full-length DNA products (13). Recently it has been shown that hWRN-p helicase is able to unwind intermediates that occur during DNA replication and repair processes such as 5′-flap substrates and synthetic replication forks (14). The hWRN-p DNA 3′ → 5′ exonuclease is stimulated by ATP and digests preferentially the recessed strand of DNA duplexes (9), three- and four-way junctions, and substrates containing an open helical structure, such as a loop, a stem-loop, or a bubble (15). It can also initiate DNA degradation from a nick or a gap and efficiently remove a mismatched nucleotide at a 3′-recessed terminus. Single-stranded DNA, blunt-end double-stranded DNA, and DNA with a 3′-protruding strand are not preferred substrates (9, 16). However, it was shown that last two substrates are exonucleolytically degraded by hWRN-p in the presence of Ku70/80, a heterodimeric factor involved in the repair of double-strand breaks by non-homologous DNA end joining (17). Ku70/80 also allows hWRN-p to digest regions of DNA containing 8-oxoadenine and 8-oxoguanine modifications (18), which normally block the hWRN-p exonuclease activity (19).

WRN-like proteins have also been detected in mouse (20) and *Xenopus laevis* (21). We were recently able to identify seven RecQ homologues in *Arabidopsis thaliana* (22). However, no gene encoding the complete homologue of WRN protein was found within the *Arabidopsis* genome. Instead, a small ORF (AtWRNexo) encoding 285 amino acids, lacking any RecQ-like domain but showing a striking similarity to the exonuclease domain of the human Werner syndrome protein was detected (22). To characterize the biochemical properties of the WRN exonuclease homologue AtWRNexo-p, we expressed the protein in *Escherichia coli*. We could demonstrate that the protein is indeed a 3′ to 5′ exonuclease with the preference for double-stranded DNA with a 3′-recessed or protruding strand. However, also significant differences in the processing of DNA

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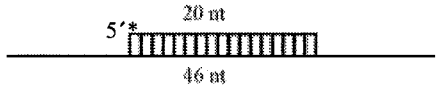
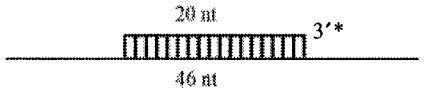
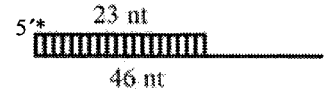
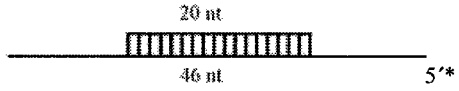
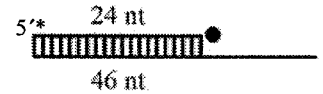
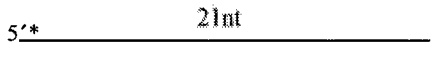
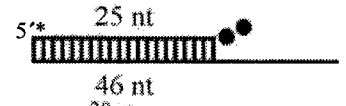
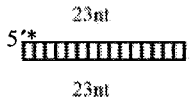
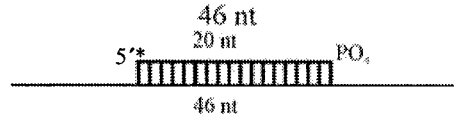
[1] The abbreviations used are: WS, Werner syndrome; hWRN-p, human Werner syndrome protein; AtWRNexo-p, *A. thaliana* Werner exonuclease; CBP, calmodulin-binding peptide; ORF, open reading frame; LIC, ligation-independent cloning; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; nt, nucleotide(s); DNA-PKcs, DNA-dependent protein kinase subunit; TRF2, TTAGGG repeat binding factor 2; FFA-1, focus-forming activity 1; Sgs1, slow growth suppressor 1; 8-oxoA, 8-oxoadenine; 8-oxoG, 8-oxoguanine; CaM, calmodulin.

TABLE I
Oligonucleotide sequences used in DNA substrate preparation

Oligonucleotide no.	Name	Nucleotide sequence
1	20-merT	5′-CGCTAGCAATATTCTGCAGC-3′
2	19-merNP	5′-Pho-CGCTAGCAATATTCTGCAGC-3′
3	46-merT	5′-TTTTTTTTTTTTTTGCTGCAGAATATTGCTAGCGTTTTTTTTTTTTTT-3′
4	T21	5′-TTTTTTTTTTTTTTTTTTTTT-3′
5	BLU1	5′-CGCTAGCAATATTTTTTTTTTTT-3′
6	BLU2	5′-AAAAAAAAAAAATATTGCTAGCG-3′
7	HPO1	5′-TGACGTGACGACGATCAGGGTACGTTCAGCAG-3′
8	OL11LATER	5′-TGACGTGACGAC(8-oxoG)ATCAGGGTACGTTCAGCAG-3′
9	OL12LATER	5′-TGACGTGACGACG(8-oxoA)TCAGGGTACGTTCAGCAG-3′
10	OL13	5′-TGACGTGACGACGATCAGGGTACGT(dU)CAGCAG-3′
11	OL14	5′-AGTGCAGACTGCTGCTGAACGTACCCTGATCGTCGTCACGTCA-3′
12	ANOK	5′-CGCGCCGAATTCCCGCTAGCAAT-3′
13	AN-1	5′-CGCGCCGAATTCCCGCTAGCAATG-3′
14	AN-2	5′-CGCGCCGAATTCCCGCTAGCAATGC-3′
15	D1	5′-GCGCGGAAGCTTGGCTGCAGAATATTGCTAGCGGGAATTCGGCGCG-3′
16	20-merP	5′-CGCTAGCAATATTCTGCAGC-Pho-3′
17	ABAS	5′-TGACGTGACGACGATCAGGGTAC(ABAS)TTCAGCAG-3′
18	CHOLE	5′-TGACGTGACGACGATCAGGGTAC(CHOLE)TTCAGCAG-3′
19	ETAD	5′-TGACGTGACGACGATCAGGGT(ETAD)CGTTCAGCAG-3′
20	HYPO	5′-TGACGTGACGACGATCAGGGT(HYPO)CGTTCAGCAG-3′

TABLE II
DNA substrates used in this study

Oligonucleotides	DNA substrate	Oligonucleotides	DNA substrate
*1 + 3		*(7, 8, 9, 10, 17, 18, 19, 20) + 11	
*2 + 3		*12 + 15	
*3 + 1		*13 + 15	
*4		*14 + 15	
*5 + 6		*16 + 3	

substrates were found in comparison to the exonuclease activity of hWRN-p.

EXPERIMENTAL PROCEDURES

Materials and Enzymes—[γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from Amersham Biosciences. High performance liquid chromatography-purified oligomers containing standard nucleotides were obtained from Metabion (Martinsried, Germany); 8-oxoadenine (8-oxoA)-, 8-oxoguanine (8-oxoG)-, uracil (dU)-containing oligomers were from Eurogentec (Liege, Belgium); and abasic site-, cholesterol-, hypoxanthine-, or ethenoadenine-containing oligomers from Midland (Midland, TX). The sequences of synthetic oligonucleotides and the positions of damaged bases are listed in Table I. Bacteriophage T4 polynucleotide kinase and the Klenow fragment of *E. coli* DNA polymerase I were purchased from New England Biolabs (Beverly, MA); leupeptin, benzamidine, and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride were from Sigma; and lysozyme was from Roche Applied Science (Mannheim, Germany).

DNA Labeling and Annealing—Single-stranded DNA oligomers were 5′-end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer. To produce DNA substrates (see Table II), labeled oligomers were annealed to the complementary strand in a 1:2 molar ratio in 70 mM Tris-HCl buffer, pH 7.6, 10 mM $MgCl_2$, incubated at 95 °C for 5 min, and then slowly cooled to room temperature. The 3′-end-labeled substrate was produced by the annealing of 19-mer DNA to the 46-mer oligonucleotide (1:2) and labeling the 3′-end of 19-mer using [α-^{32}P]dCTP and Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs) according to the manufacturer. Unincorporated labeled nucleotides were removed from DNA-labeling reactions using MicroSpin™ G-50 columns (Amersham Biosciences). DNA substrates were stored at 4 °C.

Expression and Purification of AtWRNexo Proteins—The AtWRNexo ORF was cloned by ligation-independent cloning using an Affinity LIC Cloning and Protein Purification kit (pCAL-n-FLAG, Stratagene) to yield full-length AtWRNexo protein containing an N-terminal 27-aa calmodulin-binding peptide (CBP) tag. CBP-AtWRNexo-p was overexpressed in the *E. coli* strain BL21(DE3) by 1 mM isopropyl-β-D-thiogalactopyranoside induction for 6 h at 25 °C. The purification procedure followed the instructions of the manufacturer with some modifications. The frozen cells were thawed and resuspended in lysis buffer (5 ml/g) containing 750 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, 0.1% Triton X-100, 10 mM β-mercaptoethanol, and a mixture of protease inhibitors. The lysozyme (200 μg/ml) was added, and the suspension was incubated with gently shaking on ice for 20 min. The cells were further disrupted by sonication and centrifuged at 16,000 × *g* for 20 min at 4 °C. The lysate was then applied on a 2-ml CaM resin column equilibrated in lysis buffer. The column was extensively washed with ten volumes of lysis buffer, and two volumes of buffers were supplemented with proteases inhibitors and containing either 750 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol or 500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol. Proteins were eluted in elution buffer (500 mM NaCl, 50 mM

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WRNhuman MSEKKLETTAQQRKCPEWMNVQNKRCAVEE-RKACVRKSVFEDDLPFLEFTGSIVYSYDASD 61
AtWRNexo TSVHGHEEDPNQIPNNIRRQLPRSITSSTSYKRFPLSR-CRARNFPAMRFGGRILYSKTATE 106

WRNhuman CSFLSEDISMSL------SDGDVVGFDMEWPPLYNRGKL-GKVALIQLCVSESKCYLFHVSS 116
AtWRNexo VDKRAMQLIKVLDTKRDESGIAFVGLDIAWRPSFRKGVLPGKVATVQICVDSNYCDVMHIFH 168
                                  *
WRNhuman MSVFPQGLKMLLENKAVKKAGVGIEGDQWKLLRDFDIKLKNFVELTDVANKKLKCTETWSLN 178
AtWRNexo SGI-PQSLQHLIEDSTLVKVGIGIDGDSVKLFHDYGVSIKDVEDLSDLANQKIGGDKKWGLA 229

WRNhuman SLVKHLLGKQLLKDKSIRCSNWSKFPLTEDQKLYAATDAYAGFIIYRNLEILDDTVQRF 237
AtWRNexo SLTETLVCKELLKPNRIRLGNWEFYPLSKQQLQYAATDAYASWHLYKVLKDLPDAVSGS 288
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FIG. 1. **Amino acid sequence alignment of AtWRNexo-p with the N terminus of the WRN protein from human.** The three extra amino acids resulting from an alternative splicing event of intron 5 of AtWRNexo gene are *underlined*. Identical and conserved amino acids are shown in *dark bold characters*, highly conserved amino acid stretches are represented as *gray shaded boxes*. The five amino acids thought to be critical for exonuclease activity are *dark shaded*. The point mutation E135A that abolishes the exonucleolytic activity of AtWRNexo-p is marked with a *star*. The total similarity between both protein sequences is 45%.

Tris-HCl, pH 8.0, 2 mM EGTA, 10 mM β-mercaptoethanol). Elution fractions of 0.5 ml were collected and analyzed by SDS-PAGE. The protein concentration was determined by the Bradford assay using bovine serum albumin as a standard. The purified AtWRNexo-p was supplemented with 100 μg/ml bovine serum albumin and stored in elution buffer containing 25% glycerol at −80 °C. The activity of AtWRNexo-p remained stable for at least 3 months. AtWRNexoE135A-p was purified in an identical fashion as used for AtWRNexo-p.

Production of pCAL-n-FLAG-AtWRNexoE135A—The point mutation E135A (Glu → Ala at amino acid 135) was introduced by the QuikChange site-directed mutagenesis kit from Stratagene according to the manufacturer's protocol using primers 5′-GTT GGC TTG GAT ATT GCG TGG AGA CCA AGT TTT AG-3′ and 5′-CTA AAA CTT GGT CTC CAC GCA ATA TCC AAG CCA AC-3′.

Exonuclease Assay—Reactions (10 μl) were performed in reaction buffer (40 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP, and 0.1 mg/ml bovine serum albumin); for investigation of cation requirements we used reaction buffer with pH 7.4 supplemented with respective cations as indicated in Fig. 5. For the amounts of labeled DNA substrates (DNA amounts are expressed in terms of molecules) and AtWRNexo proteins see the figure legends. The reactions were performed at 22 °C for 1 h, unless otherwise indicated, and terminated by addition of an equal volume of denaturing loading buffer (89 mM Tris borate, 2 mM EDTA, pH 8.0, 12% Ficoll, 0.01% bromphenol blue, 0.02% xylene cyanol FF, 7 M urea; Bio-Rad), immediately heated at 98 °C for 3 min, analyzed on 20% polyacrylamide 1× TBE/7 M urea gel, and visualized by autoradiography.

RESULTS

Expression and Purification of AtWRNexo-p and AtWRNexoE135A-p—Our previous analysis indicated that an ORF with similarity to only the exonuclease domain of hWRN is present in the *Arabidopsis* genome. We reported about the isolation of a cDNA that coded for an ORF of 285 amino acids (22). Further detailed analysis indicated that beside this cDNA a second slightly larger cDNA could also be amplified resulting from an alternative splicing event of intron 5. This cDNA contains an ORF that carries three amino acids more (Fig. 1) than the sequence reported previously. An ORF encoding this larger version of AtWRNexo was cloned into a bacterial vector and used for further analysis. Amino acid sequence alignment of AtWRNexo-p with the N terminus of the human WRN protein (Fig. 1) revealed conserved positions of all five amino acids (Asp-133, Glu-135, Asp-194, Tyr-263, and Asp-267 in the *Arabidopsis thaliana* sequence) regarded to be essential for exonuclease activity (16). The total identity between the N terminus of the human WRN protein and AtWRNexo-p is 32% with a similarity index of 45% (over 237 aa). At the far N terminus AtWRNexo-p is 45 amino acids longer than the human WRN protein (see Fig. 1). A point mutation from glutamic acid to alanine at position 135 (E135A) corresponding to the amino acid change E84A, previously shown to eliminate exonuclease activity of the human WRN protein (23), was introduced into the AtWRNexo ORF by site-directed PCR mutagenesis to generate pCAL-n-FLAG-AtWRNexoE135A. Otherwise the sequences of cloned fragments of AtWRNexo and AtWRNexoE135A were identical to the original cDNA of AtWRNexo. The ORFs were inserted in-frame into the vector to allow the production of calmodulin-binding peptide (CBP) fusion proteins. Recombinant proteins were overexpressed in *E. coli* and purified by one-step CaM affinity chromatography. Purified AtWRNexo-p and AtWRNexoE135A-p had a molecular mass of ~37kDa, as expected from the calculated one, including the additional N-terminal 27 amino acids of the CBP tag and 14 aa translated from the vector sequence (Fig. 2).

AtWRNexo-p Has 3′ → 5′ Exonuclease Activity—To determine whether AtWRNexo-p has any exonuclease activity, a duplex of an unlabeled 46-mer DNA oligonucleotide annealed to either a 5′-^{32}P-labeled 20-mer or 3′-^{32}P-labeled 20-mer oligonucleotide was used. A constant amount of AtWRNexo-p (100 fmol) was incubated at 22 °C with each DNA substrate (200 fmol) for different periods of time. In a second experiment, we incubated the DNA substrates (200 fmol) with increasing amounts of AtWRNexo-p. Digestion of 5′-end-labeled DNA substrates resulted in ladder-like patterns with labeled bands becoming progressively smaller with increasing incubation time and increasing enzyme concentration (Fig. 3, *A* and *B*). We observed an accumulation of 2- to 6-nt fragments already after 15 min of digestion. The size of the fragments became smaller (up to 2 nt) when incubation time was prolonged. When the DNA substrate was incubated with 500–2000 fmol of AtWRNexo-p for 1 h even a very weak band corresponding to mononucleotides was observed (Fig. 3*B*). Fragments of 3–6 nt could be a result of the fact that at this length the DNA duplex may dissociate under the conditions applied yielding non-digestible single strands (see below). In contrast, incubation of AtWRNexo-p with a 3′-end-labeled DNA substrate resulted, irrespective of the incubation time, besides the full-length substrate that disappeared with time, only in a single band migrating as a mononucleotide (Fig. 3, *C* and *D*). The intensity of degradation correlated with the amount of enzyme and the time of incubation. These data indicate that AtWRNexo-p possesses no DNA endonuclease activity and acts under the applied conditions as an exonuclease digesting the DNA from the 3′ to 5′ end.

Activity of AtWRNexo-p on Different DNA Substrates—We assayed AtWRNexo-p on different DNA substrates to determine its substrate requirements (Fig. 4). AtWRNexo-p hydrolyzes the recessed strand of DNA duplexes (see Fig. 3), as does hWRN-p (9). The efficiency of degradation increased when the reaction is performed at 37 °C (data not shown). Notably, we

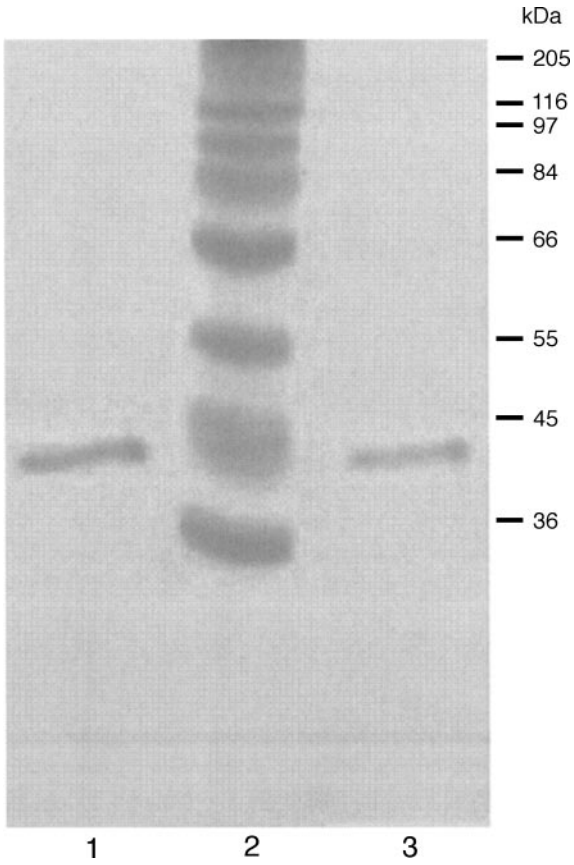


FIG. 2. **SDS-PAGE analysis of bacterially expressed and purified AtWRNexo-p and AtWRNexoE135A-p.** Both proteins, AtWRNexo-p (*lane 1*) and AtWRNexoE135A-p (*lane 3*) were purified by one-step calmodulin (CaM)-affinity chromatography, electrophoresed (0.3 μg) by 10% SDS-PAGE, and stained with Bio-Safe Coomassie Blue. *Lane 2* represents molecular markers, positions of molecular size marker proteins are indicated.

observed that AtWRNexo-p is able to degrade also the protruding strand of the same DNA substrate (Fig. 4*A*). The result of this degradation is a pattern of labeled oligonucleotides varying in length from 15 to 26 nucleotides dependent of the extent of degradation of the recessed DNA strand. This is an important difference to hWRN-p, which was reported to be unable to degrade protruding strands of DNA duplexes (9).

Similar to hWRN-p, single-stranded DNA and double-stranded DNA with blunt ends are hardly degraded by AtWRNexo-p (Fig. 4, *B* and *C*). The mutant protein AtWRNexoE135A-p was not able to process any of the substrates tested, indicating that the observed activities belong to the recombinant AtWRNexo-p and that this purified protein is free of contaminating endo- or exonuclease activities.

AtWRNexo-p Needs Mg^{2+} or Mn^{2+} for the Reaction and Is Not Stimulated By ATP—The concentration and nature of divalent cations is influencing the specificity and efficiency of various exonuclease reactions. We therefore assayed the activity of AtWRNexo-p in the presence of different divalent cations (Fig. 5, see also "Experimental Procedures"). We found that the presence of Mg^{2+} is required for optimal exonucleolytic activity of AtWRNexo-p. The activity remains stable even over the broad range of ion concentration (90–95% of DNA substrate degraded in the presence of 2–16 mM Mg^{2+}). Mg^{2+} can be partially substituted by Mn^{2+} at lower concentrations (62–66% of DNA substrate degraded in the presence of 2–6 mM Mn^{2+}). Higher Mn^{2+} concentrations have an inhibitory effect on the exonuclease activity. Zn^{2+} promotes only weak DNA degradation (only 23% of DNA was hydrolyzed at 4 mM Zn^{2+}). In the presence of Ca^{2+} no significant AtWRNexo-p activity was observed. Cations alone were not responsible for DNA degradation, and AtWRNexoE135A-p was unable to process the DNA even in presence of Mg^{2+} (data not shown).

It was reported for hWRN-p that ATP stimulates the WRN exonuclease activity on duplexes with 3′-recessed ends (9). ATP is required for structure-dependent binding of hWRN-p to DNA, whereas its hydrolysis allows the exonucleolytic degradation (15). However, another report demonstrated that ATP hydrolysis is not required for and has no significant effect on the hWRN-p exonuclease activity on DNA oligonucleotides that form a bubble-like secondary structure (24). When we incubated AtWRNexo-p with duplex DNA with a 3′-recessed end and tested the influence of ATP and its analogue with increased metabolic stability, ATPγS, on exonuclease activity, we observed no difference in DNA substrate degradation (data not shown). Quantification of the ratio between full-length oligonucleotides and degradation products revealed no significant differences. Between 6.5 and 7% of substrate remained undigested in all cases.

The Processing of DNA Modifications and Bulky Lesions by AtWRNexo-p—One can speculate that *in vivo* the exonuclease activity of AtWRNexo-p might be required during the repair of damaged DNA to remove damaged nucleotides. As described for hWRN-p previously, certain damaged or modified nucleotides incorporated into the DNA substrate inhibit or block the exonucleolytic activity (19). To investigate a possible function of AtWRNexo-p in DNA damage processing and to compare its activities with those of hWRN-p, we tested the influence of several types of DNA modifications on the exonucleolytic degradation. We used dsDNA substrates with a 5′-protruding tail, which contained modifications in the labeled strands. These modifications included helix-distorting lesions (apurinic site and cholesterol adduct), minor base modifications (8-oxoG, 8-oxoA, and ethenoadenine), and unusual bases (uracil and hypoxanthine). The sequences of oligonucleotides and the positions of respective modifications are shown in Table I. We found that AtWRNexo-p is not inhibited by uracil and hypoxanthine (Fig. 6, *A* and *C*). Only weak inhibition at the position 3′ to the modification could be observed using the substrate with ethenoadenine (Fig. 6*D*). On the contrary, the exonuclease is strongly inhibited by an apurinic site and completely blocked by a cholesterol adduct (Fig. 6, *C* and *D*). AtWRNexo-p is also inhibited by 8-oxoG and 8-oxoA at lower enzyme concentrations. Higher enzyme concentrations allow the exonuclease to pass the damaged nucleotide, in the case of 8-oxoA to a higher degree (Fig. 6*B*). Importantly, AtWRNexo-p is able to start the degradation of all DNA substrates. The unmodified substrate was fully degraded after the same time of incubation using 100 fmol of AtWRNexo-p.

AtWRNexo-p Digests Recessed DNA Strands with 3′-Terminal-mismatched Nucleotides and Degrades DNA with Either a Recessed 3′-OH or a 3′-PO_4 Terminus to a Similar Extent—For hWRN-p it was reported that its exonuclease, which is homologous to the 3′ → 5′ proofreading domain of *E. coli* DNA polymerase I (25), preferentially digests the 3′-recessed strand with a single 3′-mismatched nucleotide (9), suggesting a possible proofreading activity of hWRN-p during DNA replication. We therefore tested AtWRNexo-p on DNA substrates containing matched and one or two mismatched nucleotides on the 3′-recessed end (Fig. 7, *A–C*). We incubated 200 fmol of DNA substrate with 100 fmol of either AtWRNexo-p or AtWRNexoE135A-p for indicated periods of time at 22 °C. The efficiency of degradation of single 3′-mismatched nucleotide was within the first 10 s about a third higher than that of the complementary terminal nucleotide. Further time intervals re-

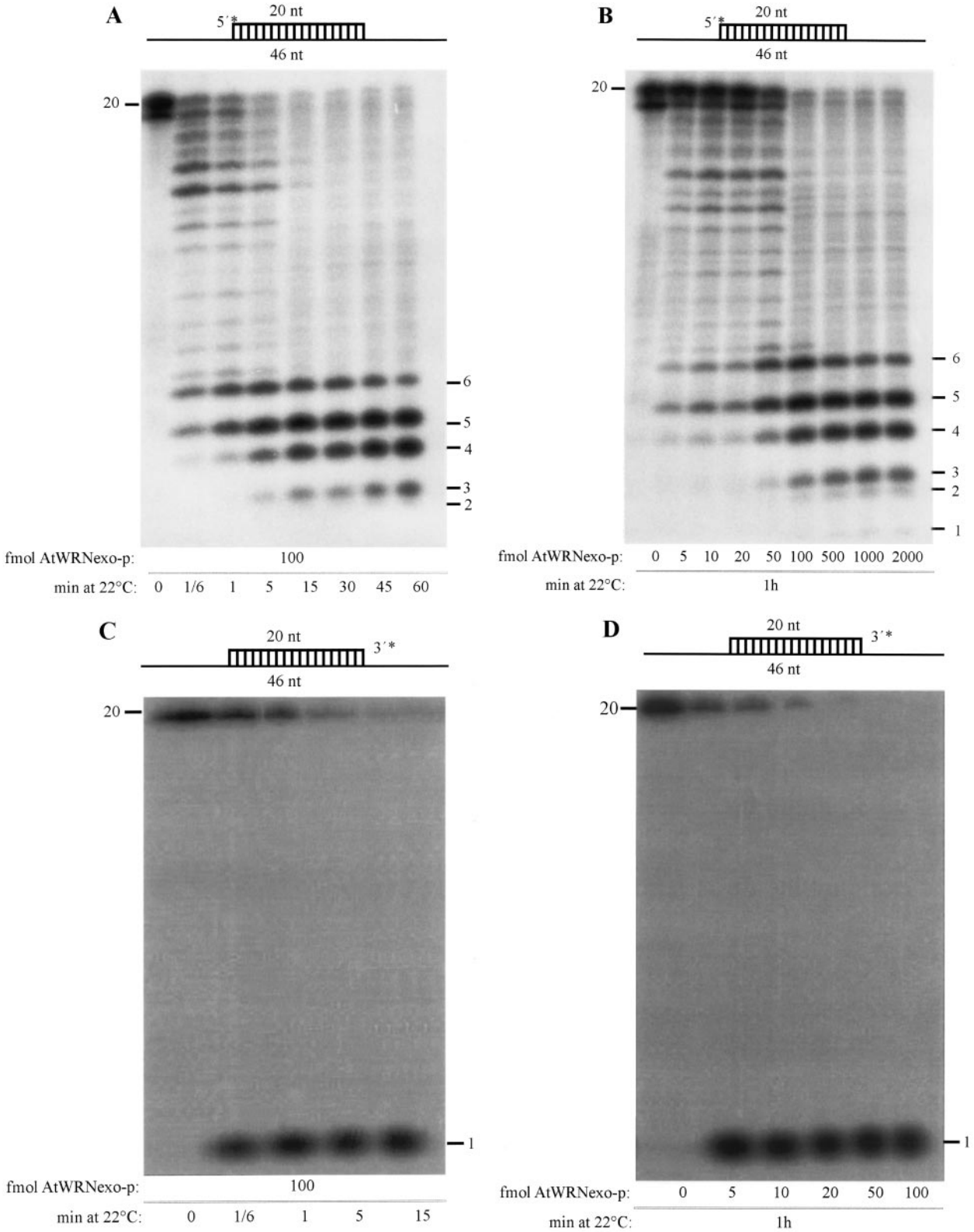


FIG. 3. **AtWRNexo-p hydrolyzes DNA in 3′ → 5′ direction.** DNA substrates (200 fmol), ^{32}P-labeled either at the 5′-end or at the 3′-end of 20-mer, were incubated with 100 fmol of AtWRNexo-p for the indicated periods of time (*A* and *C*) or with indicated amounts of AtWRNexo-p for 1h at 22 °C (*B* and *D*). The length of single-stranded DNA present on each side of the duplex region is 13 nt. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide/7 M urea gel. The length of degraded fragments is indicated. It was determined using labeled DNA oligonucleotides of known lengths.

vealed the similar extent of degradation for both matched and a single 3′-mismatched nucleotide. The hydrolysis of DNA containing two 3′-mismatched nucleotides was less efficient. After 15 min of the reaction only about 60% of the DNA substrate was hydrolyzed (Fig. 7*D*). In comparison to hWRN-p, which hardly degrades a 3′-end with two terminal mismatches, AtWRNexo-p seems to cleave such substrates, although with lower efficiency.

To determine the ability of AtWRNexo-p to start the digestion also from 3′-PO_4 termini, we incubated the DNA substrate

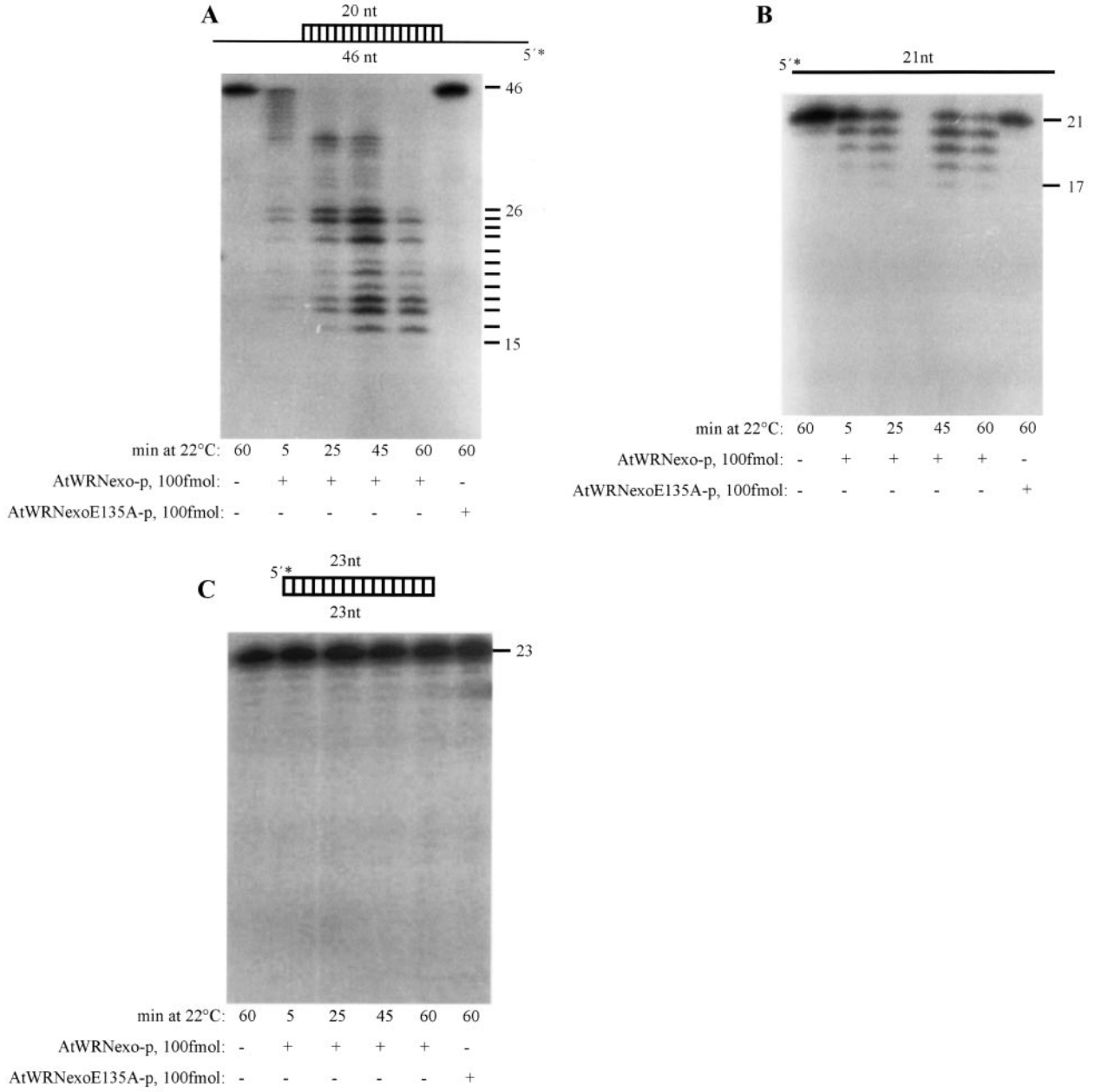


FIG. 4. **AtWRNexo-p hydrolyzes recessed and protruding strands in DNA duplexes.** DNA substrates (200 fmol) were incubated with 100 fmol of either AtWRNexo-p or AtWRNexoE135A-p for the indicated periods of time at 22 °C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide/7 M urea gel. Degradation of protruding strands (*A*), single-stranded DNA (*B*), and blunt-ended DNA (*C*). The length of degraded fragments is indicated. It was determined using labeled DNA oligonucleotides of known lengths.

containing a 3′-PO_4-recessed strand with 100 fmol of either AtWRNexo-p or AtWRNexoE135A-p for the indicated periods of time at 22 °C (Fig. 7*E*). The hydrolysis of the substrate by AtWRNexo-p is similarly efficient for both types of termini (Fig. 7*F*). The same has been reported for hWRN-p (9).

DISCUSSION

Comparison of the Exonucleolytic Activities of AtWRNexo-p and hWRN-p—A small ORF is present in the *Arabidopsis* genome (AtWRNexo) with striking similarity to only the exonuclease domain of the human Werner syndrome protein (22). In this study we were able to demonstrate that the product of the ORF indeed possesses exonuclease activity (see Fig. 1). Using a one-step affinity chromatography-purified CBP-tagged AtWRNexo protein (AtWRNexo-p) we have identified similarities but also significant differences in the processing of DNA substrates of AtWRNexo-p in comparison to the human Werner exonuclease. Both possess 3′ → 5′ exonuclease activity cleaving 3′-recessed ends of partial dsDNA molecules. AtWRNexo-p additionally digests protruding strands of duplexes. Single-stranded DNA and double-stranded DNA with blunt ends are poor substrates for both enzymes (this report and Refs. 9, 16, and 17). Because AtWRNexo-p cleaves ssDNA only poorly, digestion of 3′-protruding strands apparently needs a double-stranded region either to bind the enzyme or to stimulate the exonucleolytic activity. Degradation of such substrates by only the N-terminal part of human Werner syndrome protein (16, 26) revealed similar substrate specificities for the whole hWRN-p and its N-terminal part.

Both, hWRN-p and AtWRNexo-p do not need ATP for their exonuclease activity. However, in contrast to hWRN-p (9), no

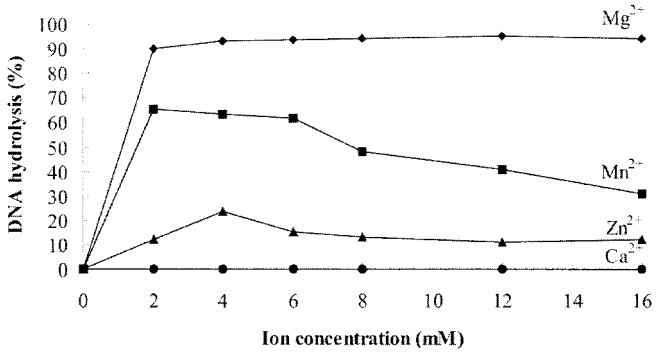


FIG. 5. **AtWRNexo-p needs $MgCl_2$ or $MnCl_2$ for exonucleolytic digestion.** The duplex DNA (labeled oligonucleotide 1 + oligonucleotide 3, 200 fmol) was incubated with 100 fmol of AtWRNexo-p in the presence of indicated concentrations of $MgCl_2$, $MnCl_2$, $CaCl_2$, or $ZnCl_2$ for 15 min at 22 °C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide/7 M urea gel, and the ratio between full-length oligonucleotide and degradation products was determined by PhosphorImager analysis.

stimulatory effect of ATP (even at protein to DNA molar ratios of 0.05 or 0.1, data not shown) or ATPγS on the AtWRNexo-p activity on partial duplex DNA containing the 3′-recessed end was observed. This is most probably due to the absence of the helicase/ATPase domain in AtWRNexo-p. The binding of ATP to the helicase part of hWRN-p might also promote a conformational change in the exonuclease domain, which could enhance the exonuclease activity.

The divalent ion Mg^{2+} is required for the exonuclease reaction. Mn^{2+} is able to replace Mg^{2+}, Zn^{2+} only partially, and Ca^{2+} not at all under the conditions used. The substitution of Mg^{2+} by Mn^{2+} in reactions catalyzed by AtWRNexo-p is in agreement with findings for hWRN-p, where Mn^{2+} was able not only to replace Mg^{2+} but even to allow a more extensive degradation of stem-loop DNA (15).

As for hWRN-p, the cholesterol adduct completely blocks AtWRNexo-p, and uracil, hypoxanthine, and ethenoadenine allow AtWRNexo-p to digest through the regions containing these modifications (19). In contrast to hWRN-p, an apurinic site strongly inhibits but not completely blocks the exonucleolytic activity of AtWRNexo-p. Oxidative DNA lesions such as 8-deoxyadenine (8-oxoA) and 8-deoxyguanine (8-oxoG) inhibit AtWRNexo-p, but such damaged nucleotides are removed at high concentrations of the exonuclease. This is not the case for hWRN-p (19).

hWRN-p degrades a DNA substrate with a single mismatched nucleotide at the 3′-recessed terminus more efficiently than a DNA with a 3′-complementary terminal base; however, DNA containing two mismatched nucleotides on the 3′-recessed strand is poorly degraded (9), whereas AtWRNexo-p hydrolyzes DNA substrates without or with a single mismatch with almost similar efficiency. In contrast to hWRN-p, AtWRNexo-p is able to digest a DNA substrate with two mismatched nucleotides at the 3′-recessed end, although at lower efficiency than substrates without or only with one mismatch. This difference might be caused by the absence of the helicase domain in AtWRNexo-p, because another report (16) observed the comparable efficiency of DNA degradation without and with a single mismatch and decreased efficiency of cleavage as the number of mismatches increases when only the N-terminal part of hWRN-p was tested.

DNA double strand breaks can result in the formation of 3′-recessed ends, terminating with either a 3′-OH or a 3′-PO_4 group. Similar to hWRN-p (9), AtWRNexo-p is able to hydrolyze both kinds of substrates efficiently.

What Could Be the Reason(s) for the Different Activities of AtWRNexo-p and hWRN-p?—In general our experiments show that certain DNA structures are better substrates for AtWRNexop than what has been reported on hWRN-p before, indicating a somehow reduced substrate specificity of the plant enzyme in comparison to the human protein. This could be due to experimental limitations of our approach, or might it indeed reflect a meaningful biological difference. Although we cannot totally exclude the former, we favor the latter as the explanation.

One has to stress that in our experiments we used a 27-aa N-terminal fusion of the AtWRNexo ORF to the calmodulin-binding peptide (CBP) required for protein purification. Our attempts to eliminate this fusion by peptidase digest were always coupled with the precipitation of the enzyme, and therefore we were not able to perform experiments with a protein without the respective tag. However, also in the experiments with hWRN-p a protein fused to a N-terminal hexahistidine tag and additional amino acids from the vector (a total of over 40 aa) was used for the biochemical characterization (9, 19). In contrast to the studies with hWRN-p, we performed characterization of the exonuclease activity of AtWRNexo-p at 22 °C. This is the optimal temperature for growth of *A. thaliana*.

Therefore, we assume that the peculiarities of the plant enzyme are connected with its biological function(s). One has to assume that the small protein interacts with other factors of the repair and recombination machinery in plants and that in the course of these interactions the specificity of the exonuclease activity of AtWRNexo-p might be modulated. Indeed, two-hybrid analysis revealed several proteins involved in DNA repair and recombination that interact with AtWRNexo-p.[2]

Possible Roles of hWRN-p and AtWRNexo-p in DNA Metabolism—Beside its biochemical characterization, a question of utmost importance is the possible role of the exonuclease *in vivo*. Several *in vitro* studies indicated possible *in vivo* functions of hWRN-p. hWRN-p is able to unwind intermediates in DNA replication and repair processes (14), interacts with the human 5′-flap endonuclease/5′ → 3′ exonuclease, and stimulates the cleavage activity of the latter (27, 28). The role of hWRN-p in DNA replication is further supported by the finding of interaction between hWRN-p and other proteins of the DNA replication machinery, such as polymerase δ, proliferating cell nuclear antigen, or replication protein A (13, 29–32). *Xenopus laevis* FFA-1 protein, which is 66% homologous to hWRN-p, is involved together with replication protein A in replication foci formation (33). The hWRN-p exonuclease is also active on the DNA substrates having on the 3′-recessed strand mismatched nucleotides (9, 16). It can be envisaged that misincorporated nucleotides can be removed in manner of a 3′ → 5′ proofreading exonuclease (34).

hWRN-p may also function in the repair of double strand breaks by non-homologous end joining, because it was shown to interact with Ku70/Ku80 (35, 36). Moreover, recent studies revealed an interaction with DNA-PKcs, which forms together with Ku70/Ku80 a complex involved in double strand break repair. Displacement of DNA-PKcs from DNA was achieved by addition of hWRN-p to a Ku·DNA-PKcs·DNA complex, so that the DNA-PKcs could no longer protect DNA ends from the exonucleolytic action of hWRN-p (37). Ku70/Ku80 has been found to stimulate the exonucleolytic activity of hWRN-p and recruit hWRN-p to DNA (26, 36). Involvement of hWRN-p and Ku in the same pathway is further supported by the observation of genetic instability, manifested by chromosomal translocations, DNA breakage, and premature aging in Ku80 knockout mice (38). The coordinated activity of the helicase and the exonuclease of the hWRN protein was investigated on forked

[2] D. Zeuske, H. Plchova, I. Koturbash, F. Hartung, and H. Puchta, unpublished results.

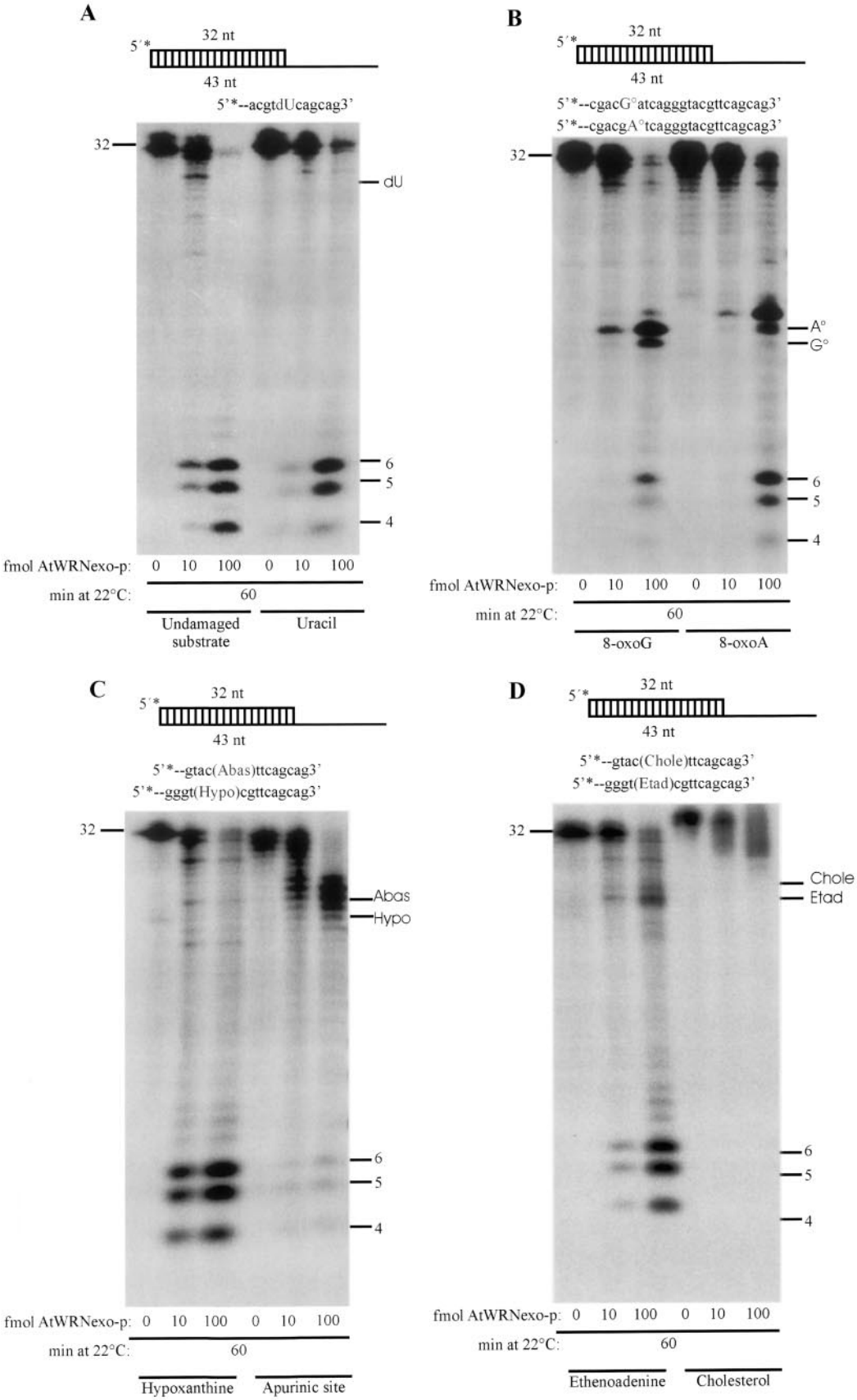


FIG. 6. **Exonucleolytic activity of AtWRNexo-p is markedly inhibited or blocked on substrates containing 8-oxoguanine (8-oxoG), 8-oxoadenine (8-oxoA), apurinic site, or cholesterol adduct.** The DNA substrates (200 fmol) were incubated with indicated amounts of AtWRNexo-p for 1 h at 22 °C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide/7 M urea gel. The DNA sequences contain a modified base (uracil, 8-oxoG, 8-oxoA (*A* and *B*) and hypoxanthine, apurinic site, ethenoadenine, and cholesterol adduct (*C* and *D*)), its respective position is indicated in *light gray*. The positions of the fragments representing the modifications is shown on the *right side* of the figure. The length of degraded fragments is indicated. It was determined using labeled DNA oligonucleotides of known lengths.

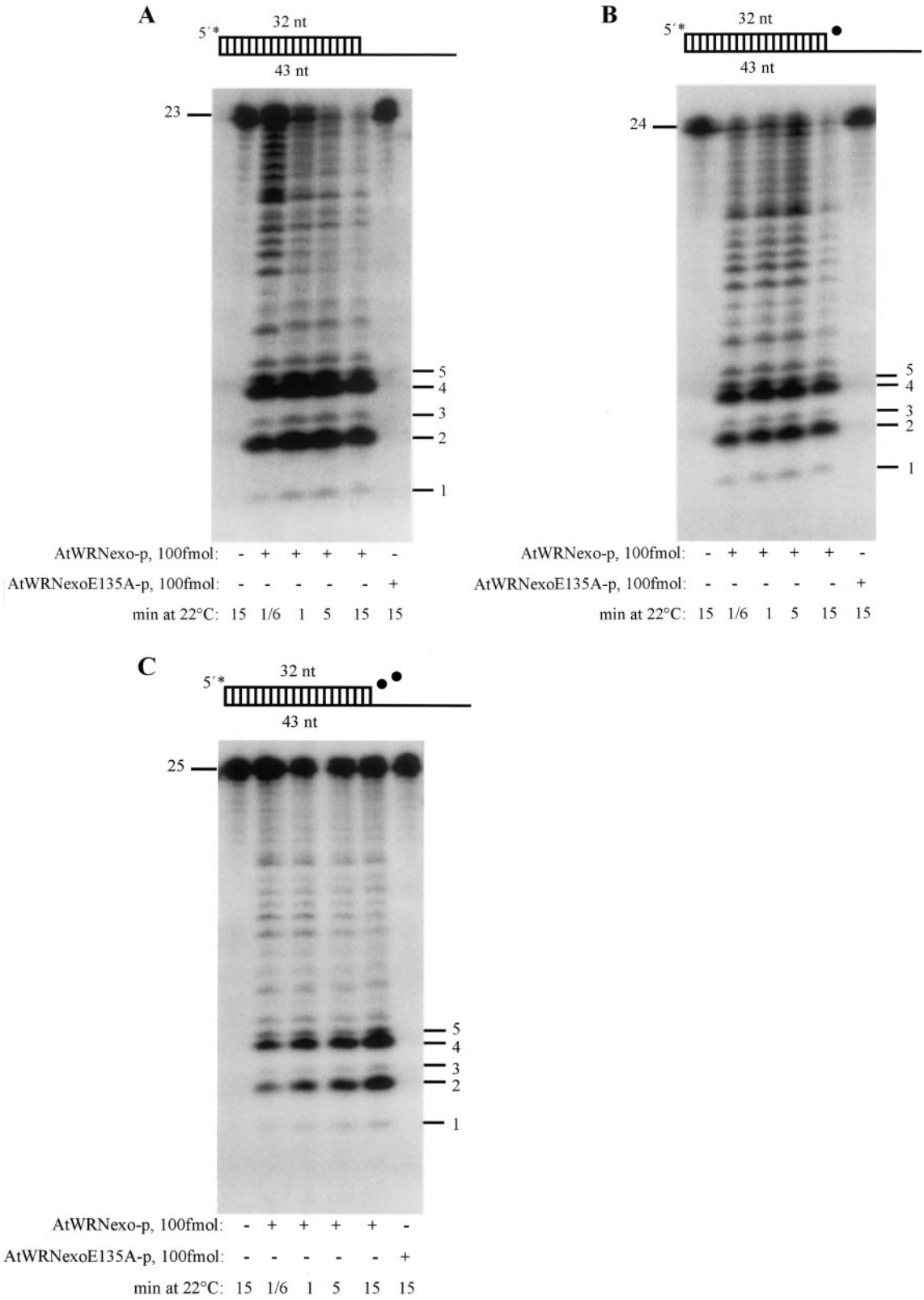
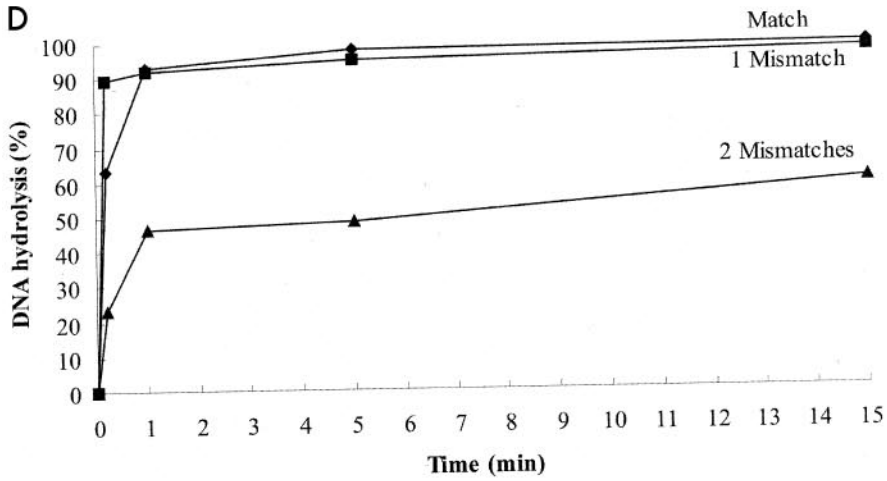
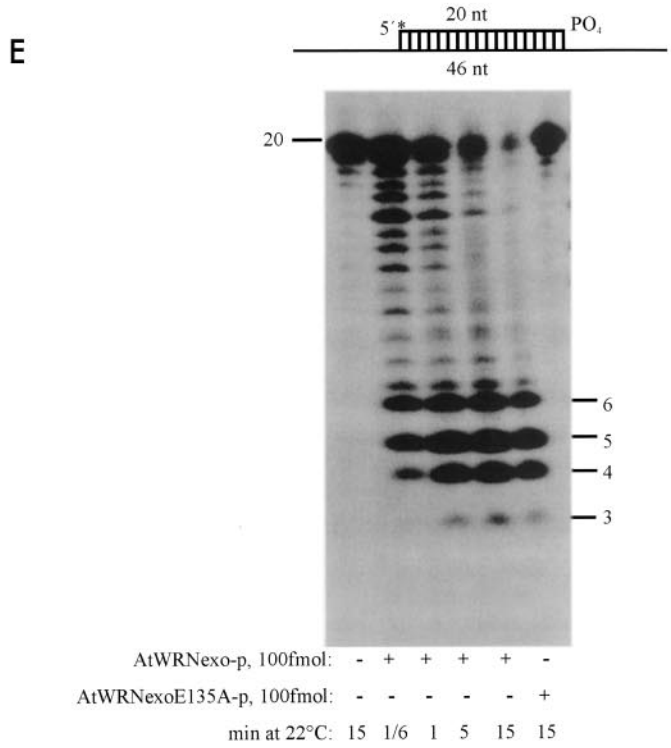


FIG. 7. **Exonucleolytic activity of AtWRNexo-p on DNA substrates with matched, one or two mismatched nucleotides at the recessed 3′ terminus and on DNA terminating with 3′-PO_4 group.** The DNA substrates (*A*, *B*, *C*, and *E*) (200 fmol) were incubated with 100 fmol of either AtWRNexo-p or AtWRNexoE135A-p for the indicated periods of time at 22 °C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide/7 M urea gel, and the ratio between full-length oligonucleotide and degradation products was determined by PhosphorImager analysis (*D* and *F*). The extent of degradation of DNA with 3′-PO_4 group is compared with the DNA terminating with 3′-OH (*D*, see also Fig. 3*A*). Mismatched bases at the 3′ terminus of the recessed DNA strand are indicated as *black points*. The length of degraded fragments is indicated. It was determined using labeled DNA oligonucleotides of known lengths.





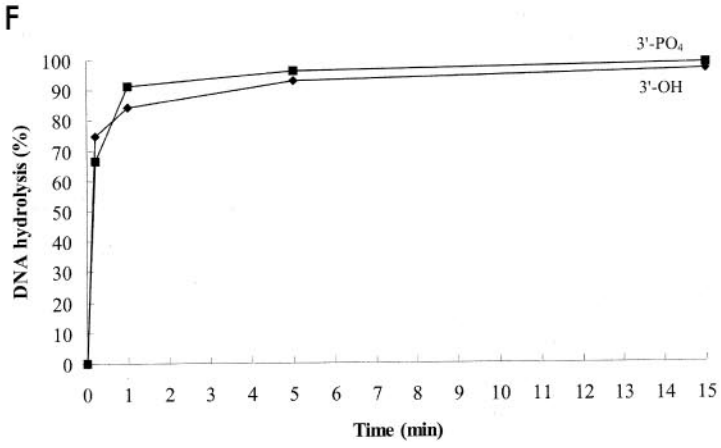


FIG. 7—*continued*

duplexes containing telomeric repeats, and a possible role during removal of recombination intermediates and telomere maintenance was suggested (39). Indeed, a role for the hWRN-p helicase in RAD51-dependent homologous recombination has been defined (40).

There is also some evidence that the hWRN protein might participate in telomere maintenance. The hWRN protein was shown to unwind a large telomere repeat DNA complex in the presence of replication protein A (41). Another report describes unwinding of tetrahelical structures of guanine-rich sequences by Werner syndrome helicase (42). Helicase/exonuclease activities disrupt and degrade D-loops *in vitro* (43). D-loop structures occur in telomeric regions (44, 45) and are formed during recombination. Thus, the loss of hWRN protein may lead to the inability of cells to process D-loops in telomeres and therefore to the loss of telomeric sequences. It was also shown that the yeast WRN homologue Sgs1 is involved in telomere lengthening by a telomerase-independent pathway (46–48). Recent studies described the stimulation of Werner helicase activities by the telomere-binding protein TRF2 (49).

All these findings show that in humans WRN protein plays an important role in the maintenance of genomic stability by its involvement in the different processes of DNA metabolism, such as replication, recombination, repair, or telomere maintenance. The biological role of WRN homologues in plants remains to be determined. The similarity of degradation of DNA substrates with 3′-recessed end, ssDNA, or dsDNA with blunt ends supports the hypothesis that the Werner-like exonuclease activities are a common feature of DNA metabolism in animals and plants. Interactions of the AtWRNexo-p and several members of the AtRecQl helicase family in the two-hybrid assay $(22)^2$ indicate that this exonuclease function is involved in processes that require RecQl-helicase activities. Because this exonuclease is not physically liked to a helicase, one is tempted to speculate that it might be involved also in processes that do not require helicase activities. Beside defining partners of the exonuclease by two-hybrid screens, the analysis of transfer DNA insertion lines will help us to elucidate the function of AtWRNexo-p *in vivo*.

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