# Biochemical characterization of the Werner – like exonuclease from *Arabidopsis thaliana*

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**DISSERTATION**

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## List of abbreviations:

| | |
|---|---|
| aa | Amino acid |
| Abas | Abasic site |
| AD | Activation domain |
| ADP | Adenosine-5'-diphosphate |
| AEBSF | 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride |
| $Amp^r$ | Ampicillin resistance |
| AP | Apurinic/apyrimidinic |
| AT | Ataxia telangiectasia |
| 3-AT | 3-amino-1, 2, 4-triazole |
| ATLD | Ataxia-like disorder |
| ATP | Adenosine-5'-triphosphate |
| ATPγS | Adenosine 5'-[γ-thio]triphosphate |
| AtWRNexo-p | *Arabidopsis thaliana* Werner exonuclease |
| BD | DNA-binding domain |
| BER | Base excision repair |
| BER-LP | Base excision repair long patch |
| bp | Base pair |
| BS | Bloom syndrome |
| BSA | Bovine serum albumine |
| °C | Grad Celsius |
| cAMP | Cyclic 3'-5' adenosine monophosphate |
| CBP | Calmodulin-binding peptide |
| Chole | Cholesterol |
| CPM | Counts per minute |
| CS | Cockayne syndrome |

| | |
|---|---|
| CTP | Cytidine-5'-triphosphate |
| Da | Dalton |
| dATP | Deoxyadenosine-5'-triphosphate |
| dCTP | Deoxycytidine-5'-triphosphate |
| dGTP | Deoxyguanosine-5'-triphosphate |
| D-loop | Displacement loop |
| DMF | Dimethylformamide |
| DNA | Deoxyribonucleic acid |
| DNA-PK | DNA-dependent protein kinase |
| DNA-$PK_{cs}$ | DNA-dependent protein kinase catalytic subunit |
| 5'-dNMP | 5'-deoxy-monophosphate |
| dNTP | Deoxynucleoside 5'-triphosphate |
| DO | Drop out |
| DPS | Decays per second |
| dRP | 5'-deoxyribose phosphate |
| dsDNA | Double-stranded DNA |
| dTTP | Deoxythymidine-5'-triphosphate |
| *E. coli* | *Escherichia coli* |
| ECTR | Extra-chromosomal telomere repeat |
| EGTA | [Ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] |
| EK | Enterokinase |
| EST | Expressed sequence tag |
| Etad | Ethenoadenine |
| f | Femto |
| FA | Fanconi anemia |
| FEN-1 | Flap endonuclease 1 |

| | |
|---|---|
| FFA-1 | Focus forming activity 1 |
| Fig. | Figure |
| g | Gram |
| G | Guanine |
| G4 | Guanine-quadruplex |
| GST | Glutathione S-transferase |
| GTP | Guanosine-5'-triphosphate |
| h | Hour |
| hBLM-p | Human Bloom syndrome protein |
| His | Histidine |
| HRDC | Helicase and RNaseD C-terminal |
| hRECQ4-p | Human Rothmund-syndrome protein |
| hRPA | Human replication protein A |
| hWRN-p | Human Werner syndrome protein |
| Hypo | Hypoxanthine |
| IgG | Immunoglobulin G heavy chain |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| k | Kilo |
| l | Litre |
| LB | Luria-Bertani |
| LIC | Ligation independent cloning |
| m | Mili |
| M | Molarity |
| MBP | Maltose-binding protein |
| MGS1 | Maintenance of Genome Stability 1 |
| min | Minute |

| | |
|---|---|
| mRNA | Messenger RNA |
| mWRN-p | Mouse Werner syndrome protein |
| NBS | Nijmegen breakage syndrome |
| NHEJ | Non-homologous end joining |
| NLS | Nuclear localization signal |
| nt | Nucleotide |
| NTP | Nucleoside 5'-triphosphate |
| OD | Optical density |
| ORF | Open reading frame |
| 8-oxoA | 8-oxoadenine |
| 8-oxoG | 8-oxoguanine |
| p | Piko |
| PEG | Polyethylene glycol |
| pH | Potentia Hydrogenii |
| PCNA | Proliferating cell nuclear antigen |
| PCR | Polymerase chain reaction |
| $P_i$ | Anorganic phosphate |
| PKA | Protein kinase A |
| polβ | Polymerase β |
| polδ | Polymerase δ |
| PTGS | Posttranscriptional gene silencing |
| QDE-3 | Quelling-defective |
| rDNA | Ribosomal DNA |
| RNA pol I | RNA-polymerase I |
| RNA pol II | RNA-polymerase II |
| RNA | Ribonucleic acid |

| | |
|---|---|
| RNaseD | Ribonuclease D |
| RPA | Replication protein A |
| RQC | RecQ family C terminal |
| rRNA | Ribosomal RNA |
| rpm | Revolutions per minute |
| RT | Rothmund-Thomson syndrome |
| s | Second |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | SDS polyacrylamide gel electrophoresis |
| Sgs1 | Slow growth suppressor 1 |
| ssDNA | Single-stranded DNA |
| SUMO-1 | Small ubiquitin-like modifier 1 |
| TEMED | N,N,N',N'-Tetramethylethylenediamine |
| t-loop | Telomere-loop |
| top3 | Topoisomerase III |
| TRDC | Telomere repeat DNA complex |
| TRF1 | TTAGGG repeat binding factor 1 |
| TRF2 | TTAGGG repeat binding factor 2 |
| Tris | Tris (hydroxymethyl) aminomethan |
| TTD | Trichothiodystrophy |
| Ubc9 | Ubiquitin-conjugating 9 |
| UTP | Uridine-5'-triphosphate |
| WHIP | Werner helicase interacting protein |
| WRN | Werner |
| WS | Werner syndrome |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

| | |
|---|---|
| XP | Xeroderma pigmentosum |
| YNB | Yeast nitrogen base |
| μ | Micro |

# 1. Introduction

The maintenance of genome stability is fundamental for cell survival and loss of genome integrity in multicellular organisms is often associated with cancer. Cells have therefore developed a network of DNA repair systems and a signal transduction pathway, which leads to an arrest of cell cycle progression, in order to deal with DNA damage. DNA damage can arise as a result of normal cellular metabolism or exposure to chemical or physical agents in environment (Friedberg et al., 1995).

Mutations in genes involved in defending of genome integrity lead to genome destabilization and elevated mutation rates. In humans, a number of inherited disorders are associated with genomic instability. These disorders include Ataxia telangiectasia (AT), an ataxia – like disorder (ATLD), Nijmegen breakage syndrome (NBS), Fanconi anemia (FA), Xeroderma pigmentosum (XP), Cockayne syndrome (CS), Trichothiodystrophy (TTD), Rothmund – Thomson syndrome (RT), Bloom syndrome (BS) and Werner syndrome (WS).

## 1.1. Werner syndrome (WS)

Werner syndrome is a rare autosomal recessive disorder that is considered a model of accelerating aging (Fig. 1).

**Fig. 1.** Premature aging symptoms in Werner syndrome: Japanese American as a teenager (*left*) looked normal, but by age 48 (*right*), the premature aging was markedly visible.

WS patients usually develop normally until they reach second decade of life. Growth retardation begins to appear typically during puberty and the full symptoms in individuals 20 – 30 years of age. Major age – related symptoms include graying and loss of hair, skin atrophy, atherosclerosis, osteoporosis, bilateral cataracts and diabetes mellitus (type II). Additionally, WS individuals also manifest a high incidence of cancer and other non – age – related symptoms, such as short stature, reduced fertility, hypogonadism, ulceration around the ankles and soft tissue calcification. Patients usually die of either cardiovascular disease or cancer at a medium age of ~ 47 years. Otto Werner originally defined Werner syndrome in 1904 on the basis of bilateral cataracts, scleroderma and premature graying and loss of hair.

The genomic instability is on the cellular level manifested as chromosome breaks, reciprocal chromosomal translocations (Salk et al., 1981), extensive genomic deletions (Fukuchi et al., 1989) and attenuated apoptosis. Furthermore, WS cells are also characterized by defects in DNA replication, DNA recombination, DNA repair, transcription and telomere maintenance.

Werner syndrome is very rare. The estimated incidence is between 1 and 22 cases per million. This desease is more common in Japan, where more than 800 cases were found out of about 1200 reported in the world.

## 1.2. Product of Werner syndrome gene

Aging researches have long wondered what kind of genetic defect could cause the premature aging phenomenon of Werner syndrome. In 1992 Makoto Goto from Tokyo Metropolitan Otsuka Hospital and his colleagues performed a genetic linkage study in order to find out the primary defect of this disorder and had reported that WS mutation is located on the short arm of chromosome 8 (Goto et al., 1992). At that time Gerard D. Schellenberg's group and other teams began to look for the Werner syndrome gene. He and his coworkers identified this gene by positional cloning in 1996 as a first gene associated with aging. The Werner syndrome gene (WRN) is comprised of 35 exons, with the coding sequences beginning in the second

exon (Yu et al., 1997), and encodes a from cDNA predicted protein of 1432 amino acids with similarity to RecQ DNA helicases (Yu et al., 1996).

### 1.2.1. RecQ DNA helicases

The RecQ family of helicases is named after the RecQ protein from *Escherichia coli*. The gene *recQ* was originally identified by genetic analysis of a thymineless death–resistant mutant (Nakayama et al., 1984). The RecQ proteins are evolutionary conserved across species. Homologues have been found also in *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Neurospora crassa, Caenorhabditis elegans, Xenopus laevis, Drosophila melanogaster, Mus musculus* and *Homo sapiens.* In general, *E. coli* and lower eukaryotes such as *S. cerevisiae* and *S. pombe* contain single RecQ helicase, whereas most higher organisms express more RecQ family members – five have been found so far in humans. Mutations of three human RecQ genes give rise to severe genetic disorders: Bloom syndrome (Ellis et al., 1995), Rothmund – Thomson syndrome (Kitao et al., 1999) and Werner syndrome, already described above.

RecQ helicases can be divided into two groups by size. One group, including proteins such as human Werner syndrome protein (hWRN-p), Bloom syndrome protein (hBLM-p), Rothmund – Thomson syndrome protein (hRECQ4-p), Sgs1 from *Saccharomyces cerevisiae*, Rqh1 from *Schizosaccharomyces pombe*, FFA-1 and BLM from *Xenopus laevis*, BLM from *Caenorhabditis elegans* and *Drosophila melanogaster* and finally the biggest RecQ family member QDE-3 from *Neurosporra crassa*, are 1200 – 1900 amino acids in length. The second group includes the RecQ prototype from *E. coli* and other RecQ family members of 400 – 1000 amino acids from different organisms. Selected members are shown in Figure 2.

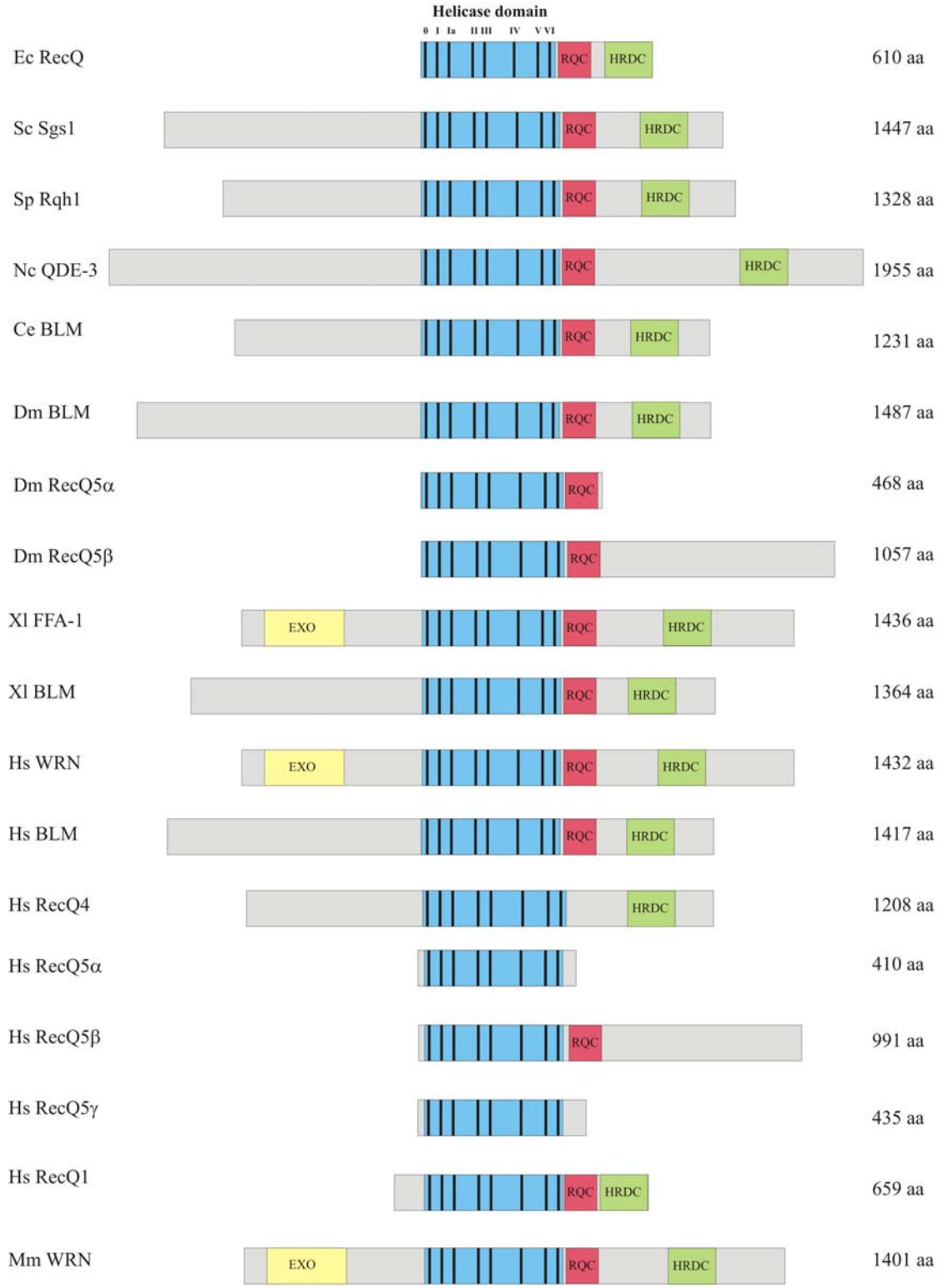



**Fig. 2.** Schematic structure of RecQ proteins of various organisms. Abbreviated names of the organisms are Ec: *Escherichia coli*, Sc: *Saccharomyces cerevisiae*, Sp: *Schizosaccharomyces pombe*, Nc: *Neurospora crassa*, Ce: *Caenorhabditis elegans*, Dm: *Drosophila melanogaster*, Xl: *Xenopus laevis*, Hs: *Homo sapiens*, Mm: *Mus musculus*). The sizes of the proteins (aa: amino acid) are indicated on the right. The proteins were aligned according to the helicase domains (in blue). For the description of the domains see text below.

All RecQ proteins share a highly conserved helicase domain (Figure 2) that comprises approximately 450 amino acids and includes seven sequence motifs (I, Ia, II, III, IV, V and VI) found in a wide variety of DNA and RNA helicases. These motifs are necessary for ATP binding and hydrolysis to obtain energy for nucleic acids unwinding. The eighth motif, motif 0, was identified and characterized very recently by proteolytic mapping of terminal domains of *E. coli* RecQ and it was shown that motif 0 is indispensable for the DNA dependent ATPase and helicase function (Bernstein and Keck, 2003). Nucleotide binding sequence (so – called Walker A and B – boxes) is present in motif I and the for RecQ family characteristic DExH – box in motif II. RecQ helicases can be further distinguished from other helicases by the RQC (RecQ family C terminal) domain located just C–terminal to helicase domain. The RQC domain can be found in most RecQ family members and is supposed to have a role in mediating protein-protein interaction. At the C–terminal region of many RecQ helicases is also present the HRDC (helicase and RNaseD C–terminal) domain (Liu et at., 1999). This auxiliary domain is probably involved in stable binding to DNA or RNA. The lack of this domain doesn't impede catalytic function (Bernstein and Keck, 2003). In certain RecQ enzymes, a nuclear localization signal (NLS) is located near the C – terminus of the protein. Outside of these domains, there is only little sequence similarity amongst the RecQ family members. These non-conserved regions may be required for other activities or interactions with another proteins, which could determine the mode of action of each helicase. Stretches of acidic amino acids similar to those found in transcriptional activators can be also identified within some proteins.

There are also exceptions to this general rule of RecQ helicases organisation. Human Werner syndrome protein (hWRN-p), its orthologue *Xenopus laevis* FFA-1 and mouse Werner syndrome protein (mWRN-p) contain additionally a conserved nuclease domain of approximately 160 amino acids with similarity to bacterial RNaseD, human polymyositis – scleroderma overlap syndrome 100 kDa autoantigen (scleroderma symptoms are prominent in

WS patients) and the 3' → 5' proofreading exonuclease domain of *E. coli* DNA polymerase I in the N – terminal region. This domain spans amino acids 78 – 219 and is composed from 3 highly conserved motifs (I, II and III) (Moser et al., 1997; Mushegian et al., 1997). Additionally, there has been a number of conserved residues outside these motifs identified by sequence analysis to be present in enzymes having proofreading exonucleolytic activities (Shevelev and Hübscher, 2002). Schematic structure of the functional domains of the human Werner syndrome protein is shown in Figure 3.

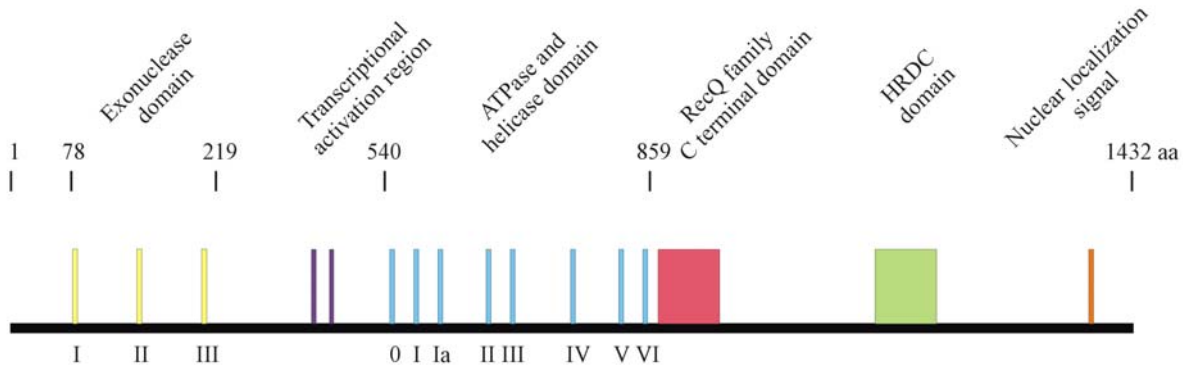



**Fig. 3.** Schematic structure of the functional domains of the human Werner syndrome protein. Arabic numbers represent amino acids of the WRN protein. The functional domains are indicated. The exonuclease domain is composed from 3 highly conserved motifs (I – III). A nonhelicase domain consisting of a direct repeat of 27 amino acids located upstream to the helicase motifs is involved in transcriptional activation (Balajee, 1999). The helicase domain carrying also ATPase function consists of 8 motifs (0 – VI). The nuclear localization signal corresponds to amino acids 1370 – 1375.

## 1.2.2. Mutations in Werner syndrome gene

The studies of DNA samples obtained from WS patients showed that all mutations occur over the WRN gene and lead to truncation of the protein (Figure 4). The known mutations are stop codons, insertions or deletions and splice – site mutations, that result in a frame shift. Interestingly, no missense mutations has been detected in Werner syndrome cases. The loss of nuclear localization signal caused by the truncation of the WRN protein is predicted to result in a failure of the truncated protein to enter the nucleus. This indicates, that the location of the

mutations does not influence the WS phenotype and suggests that Werner syndrome is a result of complete loss of function of the Werner syndrome protein.

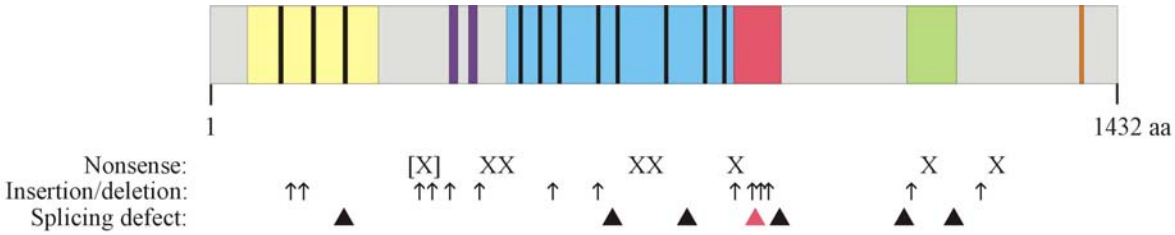



**Fig. 4.** Selected mutations found in Werner syndrome patients. The rectangular boxes indicate the functional domains of the WRN protein (see Figure 3). The nonsense mutations are shown as an X, insertions/deletions, which result in frame shift, as an arrow and splicing mutations as a triangle. The most frequent mutation (R367Stop) seen in Werner syndrome patients of Caucasian origin is indicated by the X in square brackets and the second most common mutation (deletion of exon 26) in Japanese Werner syndrome patients is indicated by the red triangle.

## 1.3. Catalytic activities of human Werner syndrome protein (hWRN-p)

The human Werner syndrome protein is a 1432 amino acid protein with a calculated molecular mass of 160 kDa. Characterization of the motifs of the WRN gene product indicated that hWRN-p should contain 3 catalytic activities: exonuclease, helicase and ATPase. Many researches tried to express the gene in variety of systems, such as in *E. coli*, yeast or in reticulocyte – based protein - synthesizing system *in vitro* and failed to obtain the active enzyme. The first evidence that hWRN-p encodes an active helicase came in 1997 from two independent laboratories. Noriyuki Suzuki from Tokyo Metropolitan Otsuka Hospital and Matthew D. Gray from University of Washington in Seattle and their coworkers demonstrated that the Werner syndrome protein expressed in insect cells using a recombinant baculovirus system has both ATP hydrolysing and DNA unwinding activities (Suzuki et al., 1997; Gray et

al., 1997). One year later, the exonucleolytic activity of hWRN-p was confirmed (Huang et al., Shen et al. and Kamath-Loeb et al., 1998).

### 1.3.1. WRN helicase and ATPase activities

Helicases are found in all living organisms and are required for many processes of nucleic acid metabolism, such as DNA replication, DNA repair, DNA recombination and transcription. Helicases are enzymes that convert double–stranded nucleic acids into single–stranded nucleic acids using the energy derived from the hydrolysis of nucleoside triphosphates (NTP) in the presence of $Mg^{2+}$ ions (Fig. 5). Helicases can be divided into different groups according to their structure and biochemical properties. One classification is according to their directionality of unwinding. They are either 5' → 3' or 3' → 5' helicases with respect to the strand they bind to and move along (Fig. 6 A and B). This classification is actually only possible for those helicases that bind a single–stranded nucleic acid terminus and then translocate along that single–stranded tail to start the unwinding at the single–stranded/double–stranded junction. Helicases can be further classified according to the nucleic acid substrate they unwind – DNA or RNA helicases. Some helicases can unwind both, DNA and RNA. Another method of classification is based on their conserved sequences. They can be divided into families and superfamilies. Finally, helicases can be grouped according to their quarternary and oligomeric structures.

Based on the sequence alignment, the Werner syndrom protein belongs to the RecQ family of DNA helicases. The unwinding of partial duplex DNA proceeds in the 3' → 5' direction (Gray et al., 1997; Shen et al., 1998). The WRN helicase activity is completely dependent on the hydrolysis of nucleoside triphosphates (NTPs or dNTPs). A study done by Shen and his coworkers in 1998 revealed that ATP, dATP, CTP and dCTP can promote strand–displacement. WRN helicase utilizes ATP and dATP preferentially. On the contrary, neither GTP, dGTP, dTTP nor UTP efficiently substitute for ATP.

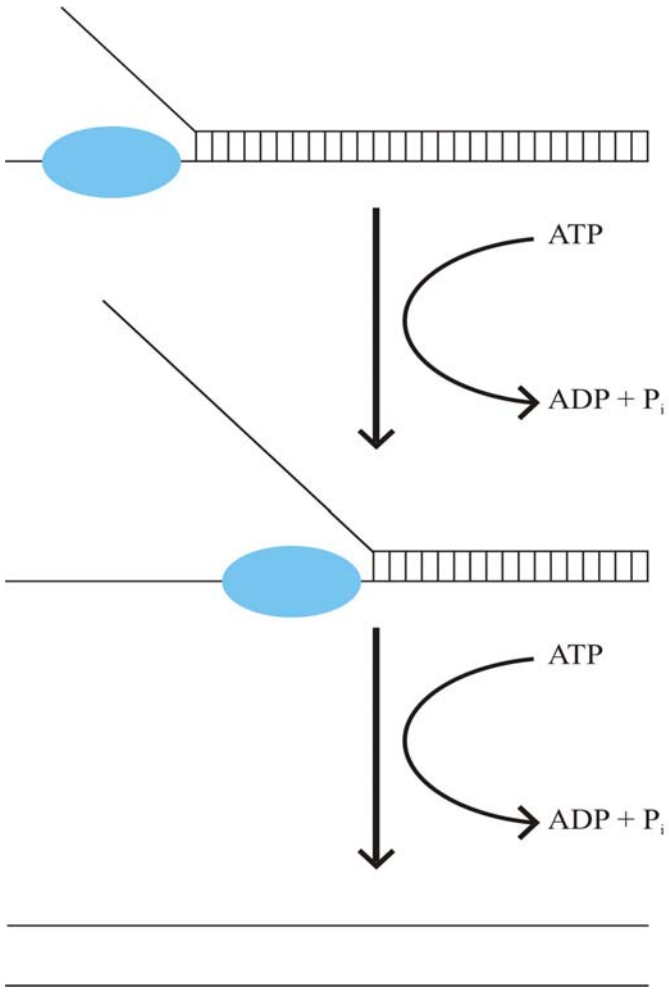



**Fig. 5.** The nucleic acid unwinding by the helicase. The helicase is bound to one of the single strands. The hydrolysis of ATP allows her to move along the bound strand and disrupt the hydrogen bonds holding the two nucleic acid strands together.

The hydrolysis of ATP is completely dependent on the presence of DNA and results in γ-phosphate (Suzuki et al., 1997). The structure of DNA influences the WRN helicase activity. Short 24 nt DNA fragments are more easily unwound than the 40 nt long fragments, but the efficiency of unwinding of 40 nt fragments with additional 10 nt single-stranded ends (forked DNA, see Figure 7) is similar to that determined for 24 nt short fragments. Additionally, the unwinding of RNA/DNA hybrid was observed, although with lower efficiency (Suzuki et al., 1997).

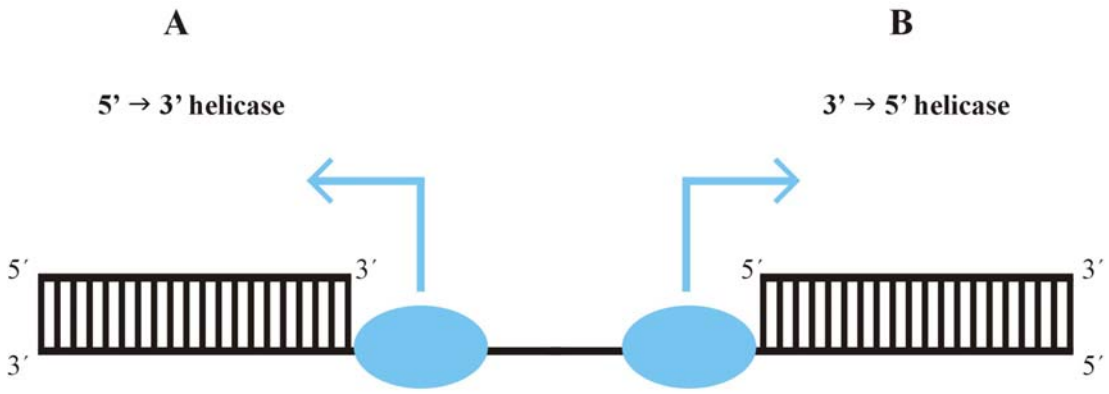



**Fig. 6.** The schematic diagram depicting the directionality of unwinding. The directionality is determined by the strand on which the helicase binds and moves along.

To get a hint of possible *in vivo* roles of Werner syndrome protein the studies on DNA substrate specificity were expanded (summarized in Figure 7 and 8). The first report that hWRN-p is a DNA processing protein for abberant DNA structures was published in 1999. DNA tetraplex, known also as G-quadruplex, is a four–stranded DNA structure containing guanine tetrads stabilized by Hoogsteen hydrogen bonds. G-quadruplex (G4) forming sequences can be found at different G-rich regions such as the fragile X chromosome, telomeric repeats, the immunoglobulin heavy chain switch region and ribosomal DNA (rDNA) gene clusters (Sen and Gilbert, 1988). The WRN helicase can unwind a bimolecular tetraplex structure of the expanded fragile X syndrome repeat sequence $d(CGG)_n$ with either 3' or 5' single-stranded tail more efficiently than double-stranded DNA. On the contrary, hWRN-p fails to unwind blunt-ended bimolecular $d(CGG)_n$ tetraplex, blunt-ended or 5'-tailed bimolecular tetraplex structures of the vertebrate telomeric sequence $d(TTAGGG)_n$ and blunt-ended or 5'-tailed tetramolecular quadruplex structures of an IgG switch region sequence (Fry and Loeb, 1999). Two years later three other synthetic G4 substrates were tested. One substrate containing a single-stranded 3'-DNA tail represented a consensus repeat from the murine immunoglobulin Sγ2b switch region, the other two substrates contained *Oxytricha* telomeric repeat sequence – one of them had again 3'-DNA tail. In these assays hWRN-p was able to efficiently unwind the 3'-tailed G4 substrates. The blunt-ended G-quadruplex DNA

formed from *Oxytricha* telomeric repeat sequence was not unwound. The authors suggested that the lack of WRN helicase activity on tetraplex structures of either IgG switch region sequence or vertebrate telomeric repeats in the previous study is most probably due to the absence of a 3'-DNA tail (Mohaghegh et al., 2001). The ability of some RecQ-helicases, including hWRN-p, to disrupt G-tetraplex DNA structures further inspired researchers to investigate, whether these helicases can be inhibited by ligands binding the telomeric quadruplex DNA, which are potent inhibitors of the telomerase enzyme complex. They showed that di- and trisubstituted acridine ligands are also inhibitors of the unwinding activity of Werner syndrome protein using G-quadruplex and three non-G4 substrates – the synthetic X-junction, the bubble and the forked partial duplex (Li et al., 2001). Moreover, G4 DNA and G-G pairing are expected to exist in the large telomere repeat DNA complex (TRDC) generated by PCR as a model of an extra-chromosomal telomere repeat (ECTR) DNA found in telomerase-negative immortalized cell lines, which consists of non-covalently linked single-stranded telomere repeat units. The hWRN-p is able to dissociate TRDC to single-stranded DNAs in the presence of hRPA (Ohsugi et al., 2000).

Furthermore, the hWRN-p can promote branch migration of Holliday junction recombination intermediate. As mentioned previously, the WRN helicase is unable to unwind DNA fragment longer than 40 bp from duplex DNA. In this report, the unwinding of the recombination intermediates by migration of the junction through more than 2700 bp has been demonstrated. The branch migration by hWRN-p is initiated at the junction. This was shown using RuvA, which has a high affinity for Holliday junctions and can act as a competitor. (Constantinou et al., 2000). Not surprisingly, the WRN helicase unwinds also the synthetic Holliday junction (Mohaghegh et al., 2001).

Another DNA structure that can be unwound by WRN helicase is a triple helix. The third strand is usually polypurine or polypyrimidine and lies in the major groove of double-stranded DNA. The first reported human helicases that can unwind such substrates belong to

the family of RecQ-helicases (Brosh et al., 2001a). The Werner syndrome protein is also capable to unwind DNA triplex with a 3'-single-stranded tail without a requirement for a fork or a long duplex region, but fails to unwind DNA triplexes with a 5'- or no single-stranded tail. On the other hand, the fork is required for the dissociation of duplex DNA substrates.

DNA substrates containing a fork or a single-stranded tail are also unwound by WRN helicase (Brosh et al, 2002a). The length for optimal unwinding of forked DNA duplexes is about 10 nucleotides for both tails and the usual 3' → 5' helicase substrate (partial duplex with a 3'-single-stranded overhang) is unwound relatively inefficiently. To further characterize the requirement for 3'-single stranded overhang, a biotin-strepavidin complex was incorporated into the usual 3' → 5' helicase DNA substrate, either at the terminal 3'-nucleotide of the single-stranded overhang or 6 nucleotides away from the junction in the same DNA strand. The WRN helicase was unable to unwind the DNA containing the block close to the junction, but when the block was incorporated into the forked DNA, the WRN helicase unwinding activity was restored. A similar observation was made for the 5'-single-stranded flap DNA, one of the important intermediates of DNA replication and/or DNA repair. This suggests that hWRN-p doesn't need a 3'-single-stranded overhang to bind to the forked DNA substrate. Most probably, hWRN-p preferentially recognizes the fork-flap structure to initiate the unwinding. The WRN helicase can also unwind synthetic replication forks.

As already described, single-stranded regions are sometimes important for the WRN helicase unwinding of various DNA substrates. What happens, when such single-stranded DNA is centrally located in the blunt-ended DNA substrate (bubble structure)? The WRN helicase can efficiently unwind a 12 nt bubble structure. An 8 nt bubble-containing DNA substrate is unwound to only a limited extent and the substrate with a 4 nt bubble doesn't promote any unwinding (Mohaghegh et al., 2001).

A displacement loop (D-loop) DNA is similar to a bubble structure (Figure 8). Such D-loop is usually formed in an early step of different recombination pathways and in the telomere

maintenance processes. The structure arises by the invasion a single-stranded DNA with a 3'-end into the homologous duplex. It has been demonstrated that the helicase activity of hWRN-p efficiently displaces the invading strand from the substrate, converting the D-loop into a bubble (Orren et al., 2002).

The WRN helicase activity is most efficiently inhibited by two minor groove binders - netropsin and distamycin A (Brosh et al., 2000).

### 1.3.2. WRN exonuclease activity

Exonucleases can degrade nucleic acids either from the 3'-terminus (3' → 5' direction) or from the 5'-terminus (5' → 3' direction). Three reports decribed that WRN exonuclease hydrolyzes the DNA in 3' → 5' direction (Huang et al., Shen et al. and Kamath-Loeb et al., 1998). The substrate specificity studies revealed that its preferred substrate is a recessed strand of a partial DNA duplex. On the contrary, WRN exonuclease cannot degrade 3'-protruding strand, blunt-ended DNA duplex and single-stranded DNA. The DNA degradation results in free 5'-deoxy-monophosphates (5'-dNMP) residues. To investigate, how the modified 3'-terminal nucleotide influences the ability of WRN exonuclease to initiate and further continue the DNA degradation, substrates with 3'-$PO_4$ recessed terminus, with a completely matched and with one or two mismatched nucleotides at the 3'-end have been tested. The DNA substrates terminating with either 3'-OH or 3'-$PO_4$ group are degraded to a similar extent. A single 3'-mismatched nucleotide is removed more efficiently from the DNA than a nucleotide, which is matched. Two 3'-mismatched nucleotides are degraded only barely. The exonuclease is stimulated by ATP, dATP, ATPγS, CTP and dCTP at specific protein to DNA molar ratios (Kamath-Loeb et al., 1998). Interestingly, UTP and dTTP could also enhance the exonuclease activity under the conditions used, but as described already above, these nucleoside triphosphates don't promote a strand-displacement catalyzed by the WRN helicase.

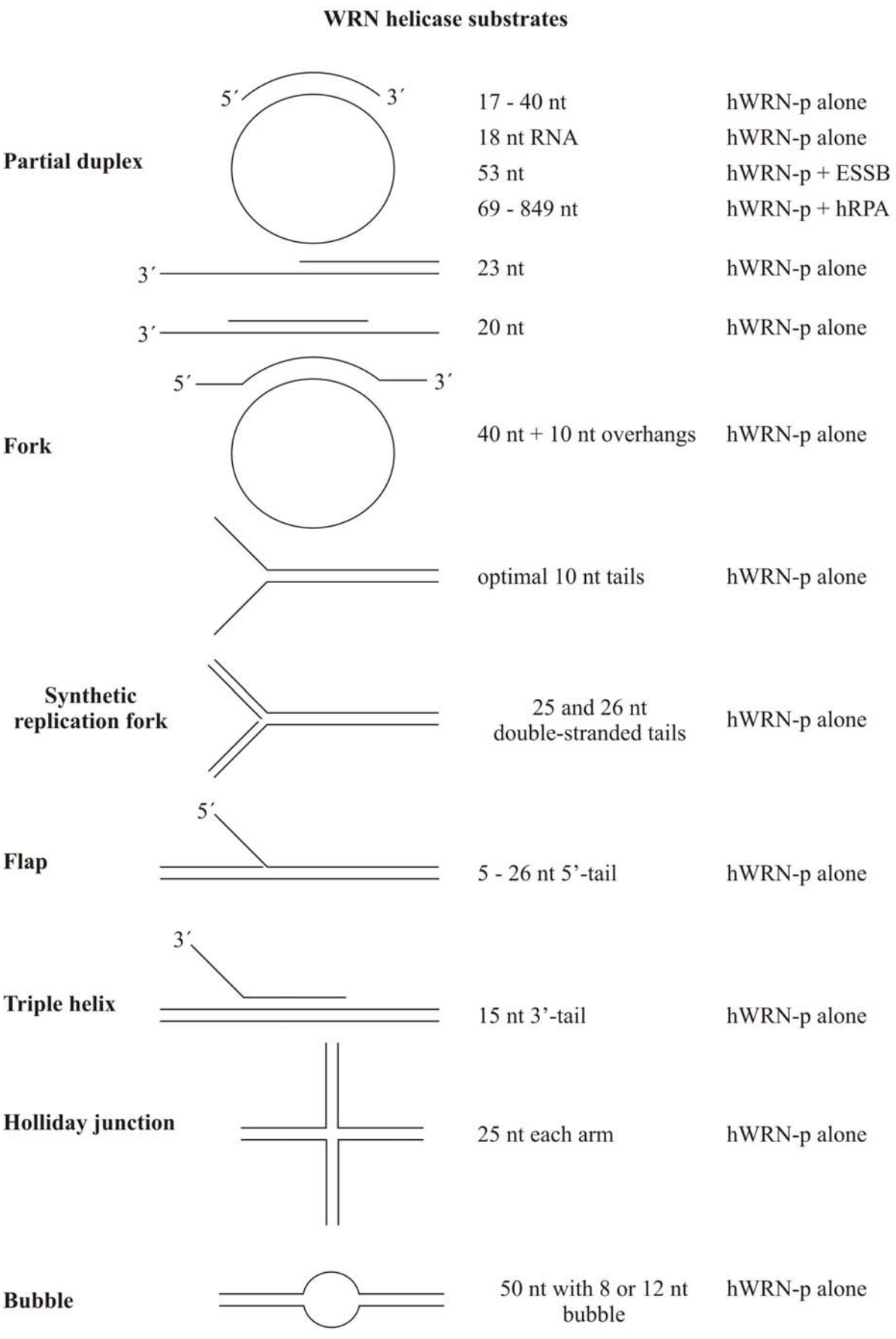



**Fig. 7.** Substrates unwound by WRN helicase.

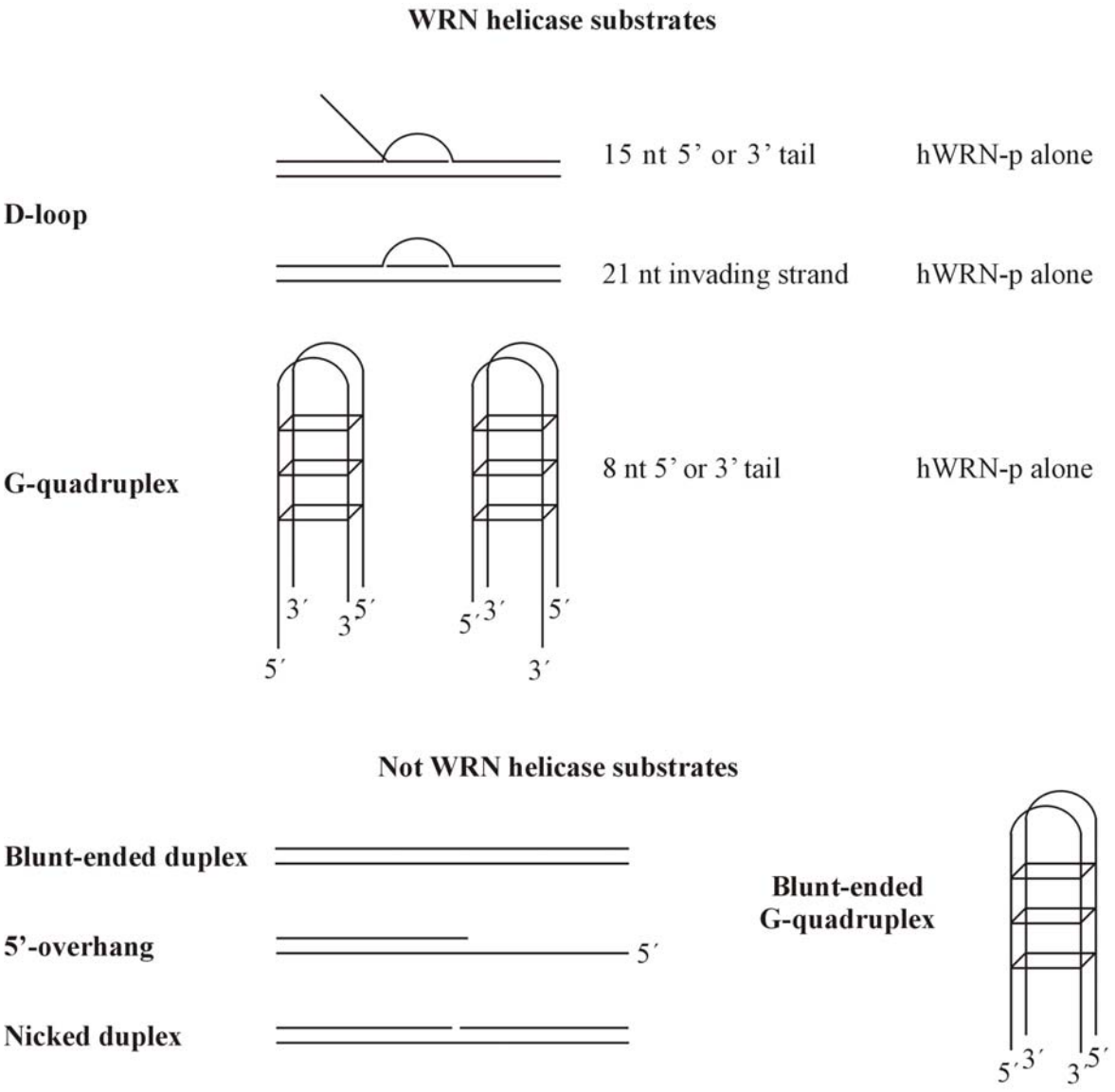



**Fig. 8.** The examples of DNA substrates that are either unwound or not unwound by helicase of hWRN-p.

The secondary DNA structures are not only the substrates for the WRN helicase (summarized in Figure 9). It has been reported that hWRN-p extensively degrades substrates containing a bubble DNA, a stem-loop, a single-stranded loop and a single-stranded loop with a single-stranded nick. These secondary structures together with 3- and 4-way junctions stimulate WRN exonuclease activity on blunt ends (Shen and Loeb, 2000). The 3'-end of the inserted strand of D-loop DNA structure is efficiently digested by hWRN-p (Orren et al., 2002).

The 3' → 5' exonucleolytic activity of hWRN-p is inhibited by DNA modifications such as 8-oxoadenine, 8-oxoguanine, apurinic site and cholesterol adduct. Other DNA modifications

including uracil, hypoxanthine and ethenoadenine don't inhibit the WRN exonuclease (Machwe et al., 2000).

### 1.3.3. Influence WRN 3' → 5' helicase and 3' → 5' exonuclease each other?

The two activities of the Werner syndrome protein can be separated from each other. Two point mutations in the exonuclease domain (D82A and E84A) abolish the 3' → 5' exonucleolytic activity, but do not affect the activity of helicase (Huang et al., 1998). Similarly, the amino acid substitution in the helicase domain (K577M) disables the ATPase and 3' → 5' helicase activities, but retains the activity of the exonuclease (Huang et al. and Shen et al., 1998).

The helicase and exonuclease activities are separable also physically. The N-terminal fragments of hWRN-p, such as 1-333 aa (Huang et al., 1998 and 2000) , 1-368 aa (Shen et al., 1998; Machwe et al., 2000 and 2002) and 70-240 aa (Xue et al., 2002) exhibit the exonucleolytic function. On the contrary, the truncated version of hWRN-p lacking the exonuclease domain (266-1167 aa) displays helicase activity, but no exonuclease activity (Shen et al., 1998). The substrate specificities of the WRN exonuclease and the truncated protein containing only first 333 aa are similar (Kamath-Loeb et al., 1998; Huang et al., 2000). However, the exonucleolytic activity of the N-terminal fragment 1-368 aa on substrates containing an apurinic site or ethenoadenine slightly differs from that of hWRN-p (Machwe et al., 2000). More importantly, this 368 aa – exonuclease is unable to degrade bubble DNA substrates, which are efficiently hydrolyzed by hWRN-p (Machwe et al., 2002).

Although the activities of the helicase and the exonuclease are separable, they influence each other. The helicase dissociates the DNA at the forked end of the substrate, whereas the exonuclease digests at the blunt end. The unwinding of forked duplexes is more rapid than the exonucleolytic digestion. The helicase therefore inhibits to some extent the WRN exonuclease

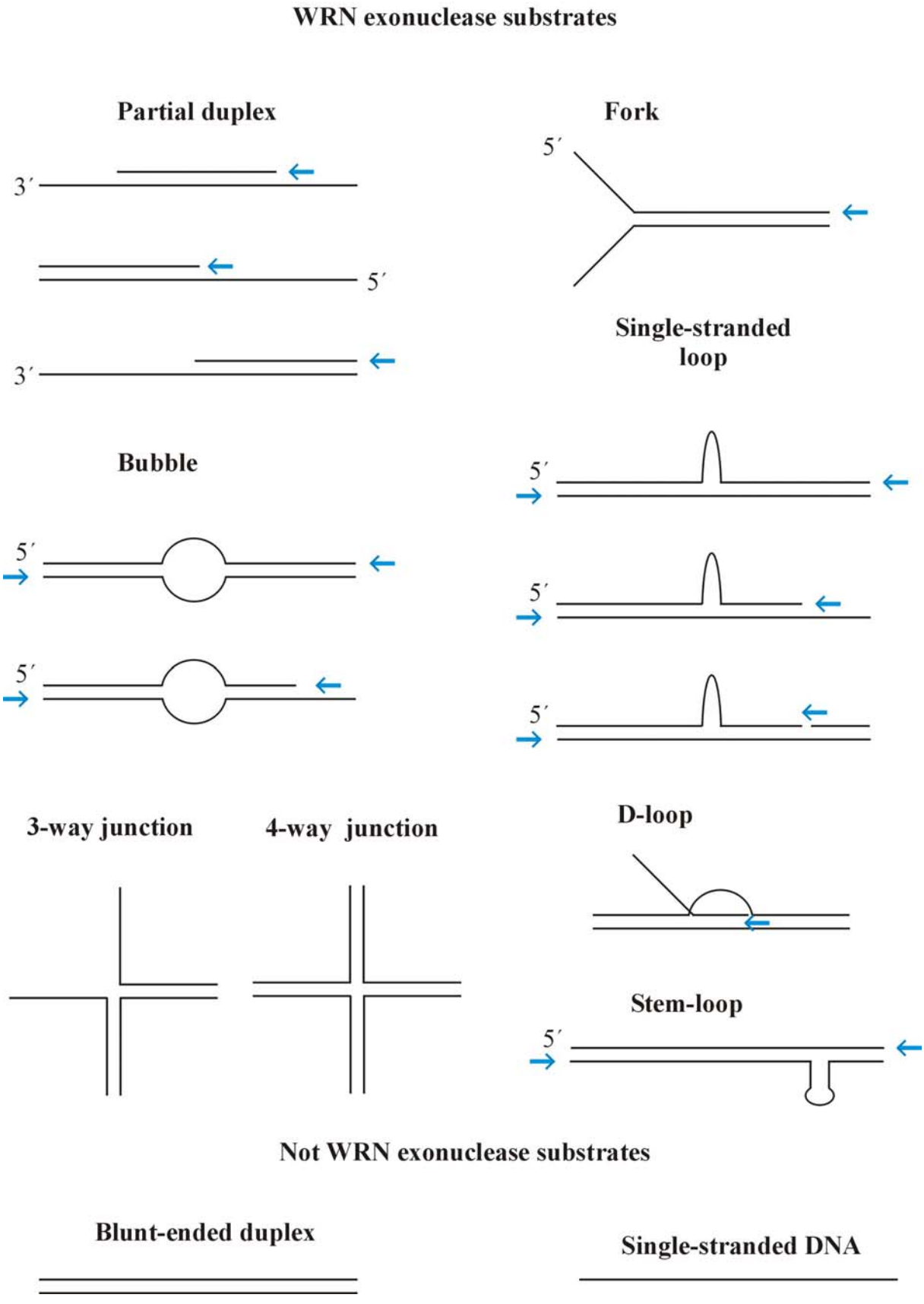



**Fig. 9.** The examples of DNA substrates degraded or not degraded by 3' → 5' WRN exonuclease. Blue arrows indicate the strand and the direction of DNA hydrolysis.

digestion by unwinding the DNA substrate. Both activities together can then remove the DNA strands that are not efficiently unwound by the helicase activity alone (Opresko et al., 2001).

## 1.4. The Werner syndrome homologues from *Mus musculus* and *Xenopus laevis*

It has been shown, that the substrate specificity of the mouse 3' → 5' WRN exonuclease is similar to that of human WRN exonuclease. The exonucleolytic activity of the N-terminal regions of hWRN-p (1-333 aa) and mWRN-p (1-328 aa) were compared. The activity of mouse WRN exonuclease was slightly reduced compared to the human WRN exonuclease (Huang et al., 2000).

Replication foci are discrete sites in the interphase nucleus composed of clusters of small replicons. The focus forming activity 1 (FFA-1) protein from *Xenopus laevis* is a 3' → 5' DNA helicase involved in the assembly of replication foci (Yan et al., 1998). The FFA-1 colocalizes and interacts with the replication protein A (RPA) and the FFA-1 helicase activity is by RPA strongly stimulated (Chen et al., 2001).

## 1.5. Proteins that interact physically and/or functionally with hWRN-p

The broad spectrum of DNA structures that are processed by either WRN helicase or WRN exonuclease activity suggests that hWRN-p may function in diverse DNA metabolism pathways. The identification of hWRN-p-interacting proteins can delineate the pathways in which this protein participates.

### 1.5.1. Proteins involved in transcription

Compared to the wild-type lymphoblastoid cells, the level of transcription in Werner syndrome lymphoblastoid cells is reduced. This transcription reduction has been shown to be

associated with the RNA-polymerase II (RNA pol II), but not with the RNA polymerase I (RNA pol I). The addition of the hWRN-p to the nuclear extract from the normal lymphoblastoid cells resulted in the stimulation of the RNA pol II-specific transcription *in vitro*. Neither the hWRN-p lacking the transcriptional activation region (see Figure 3) nor the hWRN-p mutant K577M could significantly stimulate the transcription (Balajee et al., 1999). Interestingly, WS fibroblasts are characterized by a reduction of rRNA transcription mediated by RNA polymerase I compared with the wild-type cells. The hWRN-p is associated with an RNA pol I subunit RPA40. Furthermore, the expression of hWRN-p restores the rRNA transcription in WS fibroblasts to that of normal cells (Shiratori et al., 2002).

## 1.5.2. Proteins involved in DNA replication

1.5.2.1. Human replication protein A

The human replication protein A (hRPA) is a single-strand binding protein composed of three subunits 70, 32 and 14 kDa. hRPA is required for DNA replication, recombination and repair. The WRN helicase is strongly stimulated by hRPA (Shen et al., 1998). The hWRN-p alone is unable to unwind a partial duplex longer than 40 bp, but in the presence of hRPA the unwinding of long partial duplex DNA substrates up to 849 bp has been detected. This functional interaction between hWRN-p and hRPA appears to be specific. The stimulation of WRN helicase activity by hRPA is neither due to enhanced ssDNA-dependent ATPase activity of hWRN-p nor due to hRPA-binding to the single DNA strands resulting from DNA duplex unwinding. Taken together with the experiments with a DNA competitor, the data suggest that hRPA does not increase the processivity of hWRN-p. Furthermore, a direct interaction between hWRN-p and hRPA has been demonstrated. (Brosh et al., 1999). Human RPA stimulates also the unwinding of a telomere repeat DNA complex (TRDC), as described already above (Ohsugi et al., 2000). In addition, the hWRN-p is recruited from the nucleoli to the nucleoplasm and co-localizes with hRPA to the sites of stalled replication that is caused

by depletion of the cellular dNTP pool after hydroxyurea treatment (Constantinou et al., 2000).

1.5.2.2. DNA polymerase δ

The DNA polymerase δ is an eukaryotic polymerase participating in DNA replication and DNA repair. Cells from WS individuals display DNA replication defects. The hWRN-p selectively stimulates the polymerization activity of DNA polymerase δ in the absence of proliferating cell nuclear antigen (PCNA) (Kamath-Loeb et al., 2000). The physical interaction of hWRN-p with the p50 subunit of DNA polymerase δ was confirmed by yeast two-hybrid analysis. The interaction is mediated by the C-terminus of hWRN-p. The hWRN-p coimmunoprecipitates with the p50 subunit. The catalytic subunit p125 of DNA polymerase δ is also present in the precipitate. Both subunits co-localize with hWRN-p in the nucleolus, indicating that hWRN-p recruits DNA polymerase δ to the nucleolus to facilitate the DNA replication progression probably by resolving unusual DNA secondary structures (Szekely et al., 2000). Hairpin and bimolecular $d(CGG)_n$ tetraplex DNA structures severaly impede DNA synthesis by three eukaryotic DNA polymerases - α, δ and ε. The addition of hWRN-p into the primer-extension reaction allows DNA polymerase δ to traverse the template secondary structures and generate full-length DNA products, but doesn't facilitate the DNA synthesis performed by polymerase α and δ (Kamath-Loeb et al., 2001).

1.5.2.3. PCNA and topoisomerase I

The eukaryotic proliferating cell nuclear antigen (PCNA) is a trimeric protein that acts as a sliding clamp to increase the processivity of DNA polymerization. The mouse WRN-p (mWRN-p) co-purifies with the 17S multiprotein DNA replication complex and co-immunoprecipitates with a major component of this complex – PCNA. Pull down

experiments revealed that the N-terminal part of mWRN-p is responsible for the interaction (Lebel et al., 1999). Furthermore, the hWRN-p also co-immunoprecipitates with PCNA (Huang et al., 2000) and binds PCNA at physiological protein concentrations in normal fibrobloblasts. Both PCNA and hWRN-p co-localize to sites of active DNA replication (Rodriguez-Lopez et al., 2003). A 70-240 aa N-terminal fragment of hWRN-p interacts with PCNA (Xue et al., 2002).

The eukaryotic topoisomerase I is a monomeric enzyme that has been found to be also a component of the 17S replication complex. The interaction between the hWRN-p and topoisomerase I was confirmed by immunoprecipitation experiments (Lebel et al., 1999).

1.5.2.4. Flap endonuclease 1

The flap endonuclease 1 (FEN-1) is a structure specific endo- and 5'→3' exonuclease involved in DNA replication, recombination and repair. It has been shown that the hWRN-p binds with its C-terminal part to FEN-1 and stimulates FEN-1 cleavage activity. The stimulation is independent of hWRN-p activities - the WRN mutant K577M lacking the ATPase and helicase activities and the exonuclease mutant E84A are able to stimulate FEN-1 incision. The hWRN-p can stimulate not only the FEN-1 cleavage of 1 nt 5'-flap, but also the cleavage of longer 5'-flap of 5 nt or an incision of the nicked DNA substrate (Brosh et al., 2001c). The FEN-1 cleavage of DNA replication intermediate, flap substrate with a terminal ribonucleotide, longer 5'-flaps (such as 26 or 80 nt), that may arise during the lagging strand synthesis or pseudo-Y forked structures, is efficiently stimulated by hWRN-p. The optimal stimulation of FEN-1 cleavage requires that WRN helicase doesn't unwind the 5'-flap structure (Brosh et al., 2002b).

1.5.2.5. Werner helicase interacting protein

The mouse Werner syndrome protein interacts with a so called Werner helicase interacting protein (WHIP), which is evolutionary conserved (Kawabe et al., 2001). In *Saccharomyces cerevisiae* was the WHIP also named as a Maintenance of Genome Stability 1 (MGS1). The protein is a DNA-dependent ATPase with a similarity to the eukaryotic replication factor C3 and to *E. coli* Holliday junction branch migration protein RuvB. A mutation in *Saccharomyces cerevisiae* Sgs1 gene, which encodes for only member of RecQ DNA helicase family in this organism, suppresses the slow growth phenotype of a *top3* (type-IA topoisomerase) mutant. The effect of Mgs1 mutation on the slow growth defect of *top3* mutant is opposite. Opposite to the Sgs1 DNA unwinding function, the Mgs1 reveals a single-strand annealing activity. It suggests, that all three proteins together, Mgs1, RecQ and DNA topoisomerase, may participate in the maintenance of genome stability during DNA replication (Hishida et al., 2001).

## 1.5.3. Proteins involved in a maintenance of genome stability

1.5.3.1. p53

The tumor suppressor protein p53 functions as a guardian of the genome integrity via its involvement in various DNA metabolic pathways. The fibroblasts from Werner syndrome patients have an attenuated p53-mediated apoptotic response. The immunoprecipitation analysis and *in vitro* binding assays revealed that hWRN-p interacts with p53 and binds to its C-terminus. Moreover, the expression of hWRN-p in WS cells restores the p53-mediated apoptosis to normal levels. (Spillare et al., 1999). For the specific interaction with p53 is responsible the C-terminus of hWRN-p. The p53 activates the sequence-specific transcription of target genes. The hWRN-p enhances the transcriptional activity of p53 driven from different p53-specific promoters (Blander et al., 1999). On the other hand, p53 suppresses the expression of hWRN-p (Yamabe et al., 1998). Furthermore, p53 inhibits the WRN

exonuclease activity by a direct interaction with the C-terminus of hWRN-p. The binding of p53 to the DNA substrate may contribute to the WRN exonuclease inhibition. The helicase and ATPase function of hWRN-p are not affected by p53 on the short 28 bp M13 partial DNA duplex substrate (Brosh et al., 2001b), but the helicase is inhibited by p53 on the synthetic X-junction. The phosphorylation of p53 at Ser378 reduces the WRN helicase inhibition (Yang et al., 2002). Similar to hRPA, p53 co-localizes with hWRN-p in the nucleoplasm after the treatment of cells with hydroxyurea (Brosh et al., 2001b).

1.5.3.2. TRF2

The TTAGGG repeat binding factor 2 (TRF2) has been shown to induce the invasion of 3'-single-stranded tail of TTAGGG repeat into duplex telomeric DNA, forming a structure called telomeric loop (t-loop) (Greider, 1999). This structure is believed to protect telomeric ends. The TRF2 binds with a high affinity to hWRN-p primarily via RecQ C-terminal domain and stimulates WRN helicase activity, but doesn't influence the activity of WRN exonuclease. Moreover, the helicase activity of hWRN-p together with RPA is not inhibited by TRF2 bound on the DNA substrate (Opresko et al., 2002).

1.5.3.3. DNA-PK and Ku

The DNA-dependent protein kinase (DNA-PK) is composed of a DNA-dependent catalytic subunit (DNA-$PK_{CS}$) and the Ku70/80 heterodimer. This complex is required for DNA double-strand break repair by non-homologous end joining (NHEJ). The Ku70/80 heterodimer binds to broken DNA ends and then associates with DNA-$PK_{CS}$. There are some conflicting data concerning the role of hWRN-p in association with DNA/DNA-PK complex and the role of DNA-PK in regulation of hWRN-p activity. It has been shown that the hWRN-p stabilizes the DNA/DNA-PK complex and is phosphorylated *in vivo* by DNA-PK (Yannone et al., 2001). On the contrary, another report demonstrated that hWRN-p displaces DNA-$PK_{CS}$

from the DNA/Ku70/80 complex. For this displacement both Ku binding sites on hWRN-p are required (Li and Comai, 2002). The WRN exonuclease activity is not influenced by DNA-$PK_{CS}$ (Karmakar et al., 2002a; Li and Comai, 2002). On the other hand, the inhibition of both activities of hWRN-p by DNA-$PK_{CS}$ has been reported. The presence of Ku70/80 reduced the inhibition of hWRN-p (Yannone et al., 2001). The opposite effect of Ku70/80 on hWRN-p activity has been observed - most probably due to the phosphorylation of hWRN-p by DNA-$PK_{CS}$ in the presence of Ku heterodimer (Karmakar et al., 2002a).

The WRN exonuclease is strongly stimulated by Ku70/80, but the helicase and ATPase activities remain unaffected (Cooper et al., 2000). The stimulation requires both Ku70 and Ku80 (Li and Comai, 2000). Ku70/80 binds N-terminal and C-terminal domain of hWRN-p (Li and Comai, 2000, 2001; Karmakar et al., 2002b). The presence of Ku70/80 alters the substrate specificity of WRN exonuclease. The WRN exonuclease is not only stimulated on the usual 3'-recessed DNA substrate, but it can also cleave substrates previously unhydrolyzable, such as blunt-ended or 3'-protruding DNA (Li and Comai, 2000). Furthermore, this stimulation is seen with 1-388 aa N-terminal fragment of hWRN-p. A degradation of single-stranded DNA by this fragment has been described (Li and Comai, 2001). The Ku heterodimer facilitates digestion also through regions containing 8-oxoadenine and 8-oxoguanine modifications (Orren et al., 2001).

#### 1.5.3.4. DNA polymerase β

The DNA polymerase β is a protein involved in both short- and long-patch base excision repair (BER). This repair pathway removes DNA damages, such as deaminated, oxidized, alkylated and fragmented bases with the help of several proteins (Wilson and Thompson, 1997). It has been shown that DNA polymerase β interacts with the C-terminal part of hWRN-p and has no effect on hWRN-p activities. On the contrary, hWRN-p stimulates a strong strand displacement synthesis by DNA polymerase β, which is dependent on the WRN

helicase activity. Interestingly, the WRN helicase can unwind a BER substrate, an oligonucleotide containing a nick, in the presence of an uracil-DNA glycosylase and an apurinic/apyrimidinic endonuclease. The data suggest, that hWRN-p may participate in long-patch BER (Harrigan et al., 2003).

#### 1.5.3.5. Bloom syndrome protein

The human Bloom syndrome protein (hBLM-p) belongs also to the RecQ family of helicases (see 1.2.1. and Figure 2) and displays structure-specific 3' → 5' helicase activity similar to that of the Werner syndrome protein (Brosh et al., 2001a; Karow et al., 1997, 2000; Mohaghegh et al., 2001; Sun et al., 1998). The interaction beween hWRN-p and hBLM-p is direct and is mediated by both N-terminal and RecQ family C-terminal domains.The binding of hBLM-p to the N-terminus of hWRN-p (aa 1-120) inhibits the WRN exonuclease activity. No synergistic unwinding by hWRN-p and hBLM-p has been observed (von Kobbe et al., 2002).

### 1.5.4. Other proteins

#### 1.5.4.1. Protein kinase A

Cyclic 3'-5' adenosine monophosphate (cAMP) dependent protein kinase A (PKA) is an important enzyme in signal transduction. The enzyme is composed from two regulatory (R) and two catalytic (C) subunits. In the absence of cAMP the regulatory subunits are bound to the active site of the catalytic subunits, inactivating them. When cAMP is present, it binds to the regulatory subunits releasing the two catalytic subunits in an active stage. In dividing cells the human Werner syndrome protein is localized in the nucleolus. An increased level of cAMP or an expression of the regulatory subunit RIβ of PKA inhibits the nuclear redistribution of hWRN-p caused by serum withdrawal. Moreover, direct interaction of hWRN-p with RIβ has been detected (Nguyen et al., 2002).

1.5.4.2. Ubc9 and SUMO-1

The degradation of many proteins is mediated by ubiquitilation – a conjugation of ubiquitin to other cellular proteins. Ubiquitin is a highly conserved protein of 76 amino acids. The ubiquitin-conjugating enzyme Ubc9 is involved in the conjugation of an ubiquitin-related protein so called small ubiquitin-like modifier SUMO-1 (Pichler and Melchior, 2002; Tatham et al., 2003). The two hybrid analysis revealed that the mouse Werner syndrome protein interacts directly with Ubc9, which mediates further the interaction with SUMO-1. Furthermore, the hWRN-p has been found to be covalently associated with SUMO-1 (Kawabe et al., 2000).

## 1.6. Pathways in which the Werner syndrome protein may participate

The biochemical characterization of WRN-p and the growing evidence of interactions between the Werner syndrome protein and other proteins involved in various pathways of DNA metabolism suggests that the WRN-p may participate in these processes. The putative pathways, in which the WRN-p may take a part are summarized in Figure 10.

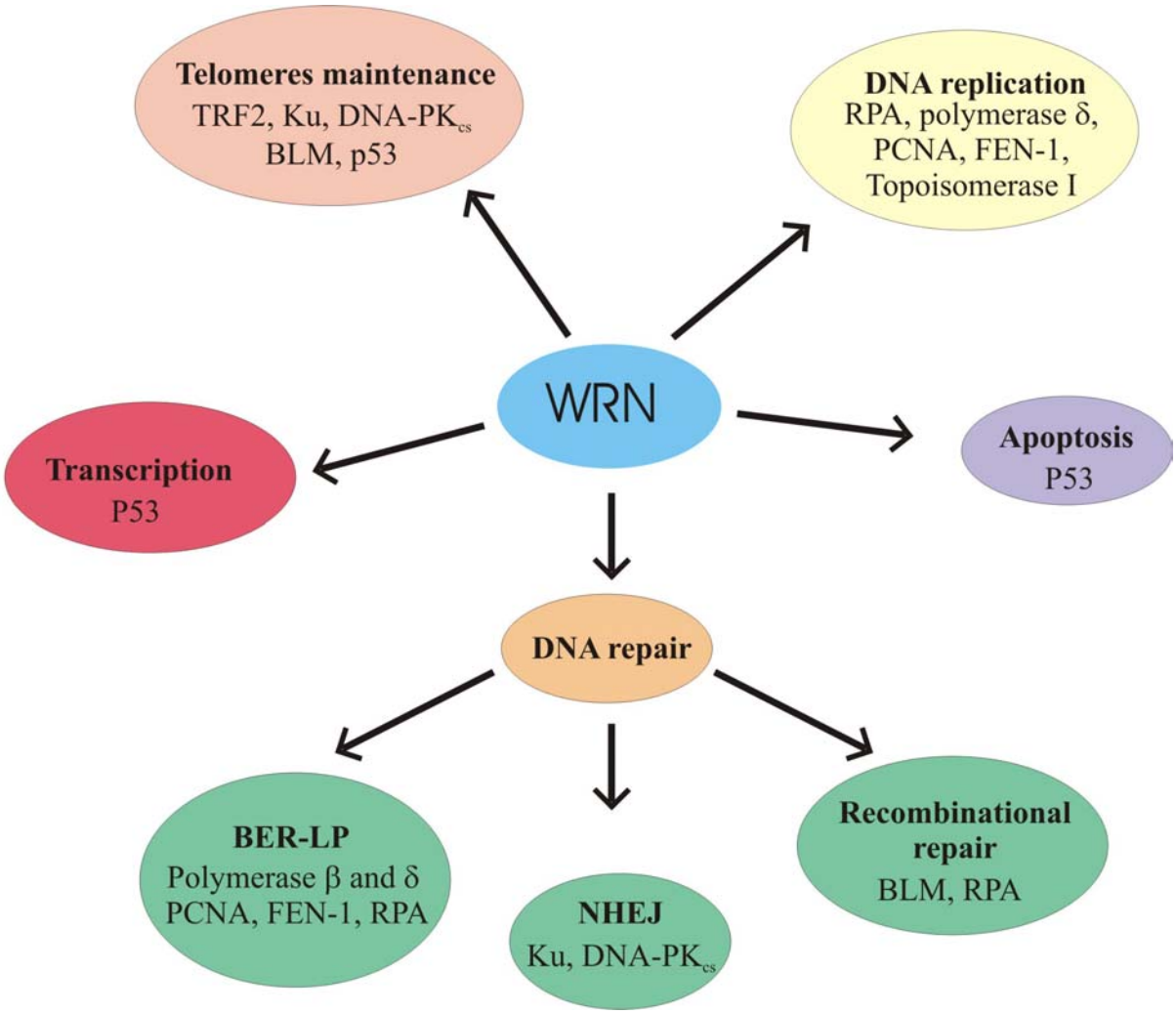



**Fig. 10.** Possible pathways, where the Werner syndrome protein may participate. The WRN-p-interacting proteins are written in the bubbles together with the name of the pathway (in bold black characters), in which they are involved. BER-LP: Base excision repair – long patch, NHEJ: Non-homologous end joining.

## 2. Aim of the work

The isolation and characterization of factors involved in DNA recombination and DNA repair from *Arabidopsis thaliana* allows us to understand the biological functions of such proteins in plants.

The intention of this study was the characterization of the Werner syndrome exonuclease homologue (AtWRNexo-p) from *Arabidopsis thaliana*. The first goal was to establish the molecular structure of the AtWRNexo gene. The second goal was to determine the activity and substrate specificity of AtWRNexo-p and compare it to the exonuclease activity of human Werner syndrome protein (hWRN-p). The third goal was to identify plant proteins interacting with AtWRNexo-p.

## 3. Material

### 3.1. *Escherichia coli* strains

DH5α: recA1, endA1, gyrA96, thi-1, hsdR17, ($r_K$-$m_K$+), relA1, supE44, [u80ΔlacZΔM15, Tn10, ($Tet^r$)]$^c$ — Gibco BRL, Eggenstein

BL21(DE3) : B $F^-$ *dcm ompT hsdS*($r_B$-$m_B$-) *gal* λ (DE3) — Novagen, Madison, USA

### 3.2. *Saccharomyces cerevisiae* strains

Y190: *MATa*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *112*, *gal4*Δ, *gal80*Δ, *cyh*$^r$*2*, *LYS2::GAL1*$_{UAS}$-*HIS3*$_{TATA}$-*HIS3*, *MEL1* *URA3::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ* — Clontech, Heidelberg

EGY48: MATα, *ura3*, *his3*, *trp1*, *LexA*$_{op(x6)}$-*LEU2* — Clontech, Heidelberg

### 3.3. Plasmids

| | |
|---|---|
| pCAL-n-FLAG | $Amp^r$ (Stratagene) |
| pGILDA | $Amp^r$, *HIS3* (Clontech) |
| pB42AD | $Amp^r$, *TRP1* (Clontech) |
| p8oplacZ | $Amp^r$, *URA3* (Clontech) |
| pGAD GH | $Amp^r$, *LEU2* (Clontech) |
| pAS2-1 | $Amp^r$, *TRP1* (Clontech) |

### 3.4. Clones for two-hybrid systems

AtRecQl1/pAS2-1, AtRecQl2/pAS2-1, AtRecQl3/pAS2-1, obtained from Dr. Frank Hartung

AtWRNexo/pGAD GH

AtKu70/pGILDA, AtRad51/pGILDA, AtUbc9/pGILDA, obtained from Ihor Koturbash

AtMLH1/pGILDA, AtKu70/pB42AD, AtRad51/pB42AD,

AtUbc9/pB42AD, AtMLH1/pB42AD

## 3.5. Primers and oligonucleotides

Primers for amplification of AtWRNexo ORF for cloning into pCAL-n-FLAG:

HP40 5’ - GACGACGACAAGATGTCATCGTCAAATTGGATC – 3’

HP41 5’ – GGAACAAGACCCGTTTATGAGCCACTGACAGCA – 3’

Primers for amplification of AtWRNexo ORF for cloning into pAS2-1:

HP21 5’ - CAT GGA ATT CAT GTC ATC GTC AAA TTG G – 3’

WRN-Sal1-AS21 5’ - ACG CGT CGA CTT ATG AGC CAC TGA CAG – 3’

Primers for amplification of AtWRNexo ORF for cloning into pGILDA and pB42AD:

HP21 5’ - CAT GGA ATT CAT GTC ATC GTC AAA TTG G – 3’

HP22 5’ - CGA ACT CGA GTT ATG AGC CAC TGA CAG C – 3’

Primers for introduction of a mutation into AtWRNexo ORF:

HP150 5’ - GTT GGC TTG GAT ATT GCG TGG AGA CCA AGT TTT AG – 3’

HP151 5’- CTA AAA CTT GGT CTC CAC GCA ATA TCC AAG CCA AC – 3’

Primers for amplification of ORFs of AtWRNexo interaction partners:

AtRecQl1 for cloning into pGILDA:

RQ5-5S-Xma1 5’- ACT ATC CCG GGG ATG AAG GAT CAA GAT C-3’

RQ5-3S-BamH1 5’-GCT AGG GAT CCT CAG CTT CTT TCA CAG T-3’

AtRecQl3 for cloning into pGILDA:

HP7 5’-ACG CCT CGA GAT GAA GAA ATC GCC G-3’

HP59 5’-ACG CCT CGA GTT ATT GAA GCT TCA TTA TCT T-3’

AtRecQl4a for cloning into pGILDA:

RQl2-5S-Xma1 5’-GCT ATC CCG GGT ATG AGT AGG AGT CAC C-3’

RQl2-3S(BamH1) 5’-GCT AGG GAT CCT CAC CAC GGT AAT ACT C-3’

AtRecQl5 for cloning into pGILDA:

HP69 5’-GCT AGG GAT CCG TAT GAC GTC TTC TTC C-3’

HP70 5’-CGA ACT CGA GTT ATG TAG CTA AAG CTG-3’

AtRecQsim (First half) for cloning into pGILDA:

RQ3-5S(XmaI) 5’-GCT ATC CCG GGT ATG GAT TTG TCT TCT G-3’

HP60 5’-ACG CGC TAG CTT TTT CCC G-3’

AtRecQsim (Second half) for cloning into pGILDA:

HP61 5’-ACG CGC TAG CAG TAA TCT CA-3’

RQ3-3S(XhoI) 5’-CGA ACT CGA GTT ATT CTT TGC TTC TCC-3’

AtKu80 for cloning into pGILDA:

HP79 5’-CAT GGA ATT CAT GGC ACG AAA TCG G-3’

HP80 5’-GCT AGG GAT CCT TAG CTC TCG AGC ATT-3’

AtKu80 for cloning into pB42AD:

HP79 5’-CAT GGA ATT CAT GGC ACG AAA TCG G-3’

HP81 5’-CAT GGA ATT CTT AGC TCT CGA GCA TT-3’

Primers for PCR screening:

PCAL-n-FLAG:

pET3-18mer 5’-CTAGTTATTGCTCAGCGG-3’

pCAL-n5-21mer 5’-GAATTTCATAGCCGTCTCAGC-3’

pAS2-1:

pAS 2-1 up 5’-CAT CGG AAG AGA GTA GTA AC-3’

pAS 2-1 down 5’-AGC GAC CTC ATG CTA TAC C -3’

pGILDA

HP9 5’-GTCAGCAGAGCTTCACCATT-3’

HP10 5’-AGCGACCTCATGCTATACCT-3’

pB42AD

| | |
|---|---|
| HP11 | 5’-CAGCCTCTTGCTGAGTGGA-3’ |
| HP12 | 5’-CGTATCTACCAACGATTTGAC-3’ |

Primers for sequencing:

AtRecQl1:

| | |
|---|---|
| RQ5-1 | 5’-CAT TGG ATG ATC TTG AAT TTG-3’ |
| RQ5-R4 | 5’-CCT GGT AAC ACA AAC TTT TCC-3’ |
| RQ5-R2 | 5’-CTT GAA GCC CGT CCC TTC-3’ |

AtRecQl3:

| | |
|---|---|
| BL4 | 5’-CAA CCT GTG ATG ACT TGT CT-3’ |
| RQ1-5 | 5’-GAA GGT TCT GGA TGC CGA AG-3’ |
| BLrev2 | 5’-GCT CAT CTC TGC TTG CGG T-3’ |
| BLrev5 | 5’-ACA GTA CTC CTC ATT TTA CT-3’ |

AtRecQl4a:

| | |
|---|---|
| RQL2-(-3) | 5’-AGT GCA ATG AAT GTT TCA GG-3’ |
| RQL2-(-1) | 5’-GCA GCT CGA GAA CCA TAT CC-3’ |
| RQL2-2 | 5’-TTC GAC CTA ACC AGA GAG AA-3’ |
| RQL2-3 | 5’-GAT GAA GCT CAT TGT GTG AG-3’ |
| RQL2-4 | 5’-GCC TGA TGT CCG CTT TGT TA-3’ |
| RQL2-R1 | 5’-CCA AGG CCA TTG ATT TCA AG-3’ |
| RQL2-R3 | 5’-ACT ACT CAA CAA TTA TCA CAC-3’ |
| RQL2-R6 | 5’-AAT AAG AGA CAC AAG TGG AGA-3’ |
| RQL2-R7 | 5’-TAG ATT CGA ACT GCT CAG CTG-3’ |

AtRecQl5:

| | |
|---|---|
| RQ7-1a | 5’-GAA CTT AAA TGG CAA GAG AGG-3’ |
| RQ7-1 | 5’-ATG GTG TTT CGT AGC AAC G-3’ |
| RQ7-2 | 5’-TCT CTC TGC TAC CAG ATT CC-3’ |

| | |
|---|---|
| RQ7-3 | 5’-GAC TTG ACA AGG GAG ATG TAG-3’ |
| RQ7-4 | 5’-GCG GTT GTC ATA TTG TCA TCT C-3’ |
| RQ7-R1 | 5’-CAA CTC CGT GCA TTA TTC TC-3’ |
| RQ7-R2 | 5’-GTG TGC AAG ACT CCG AAG C-3’ |
| AtRecQsim: | |
| RQ3-1a | 5’-GTC GTA ATT TCG CCT TTG AT-3’ |
| RQ3-2 | 5’-GAG CTG GAA GAG ATG GCG-3’ |
| RQ3-3 | 5’-GAA CAC TTC ACA ATG TCG G-3’ |
| RQ3-R1 | 5’-GAA CTA AAT TCC TCC CCA AAG-3’ |
| RQ3-R2 | 5’-CAA TGC GCT TCA TCA ATT GC-3’ |
| RQ3-R1a | 5’-CGC CAT CTC TTC CAG CTC-3’ |
| AtKu80: | |
| Ku80-2 | 5’-AGT ATG ATG AAG TTG GAA TTG-3’ |
| Ku80-R1 | 5’-ATT GGG TTT GCA TCT CCG AC-3’ |
| Ku80-R2 | 5’-GTG GGA AGT CTC TCC TCA G-3’ |

Oligonucleotides for the exonuclease assays:

| Oligo No. | Name | Nucleotide sequence |
|---|---|---|
| 1 | 20merT | 5’-CGCTAGCAATATTCTGCAGC-3’ |
| 2 | 19merNP | 5’-Pho-CGCTAGCAATATTCTGCAG-3’ |
| 3 | 46merT | 5’-TTTTTTTTTTTTTGCTGCAGAATATTGCTAGCGTTTTTTTTTTTTT-3’ |
| 4 | T21 | 5’-TTTTTTTTTTTTTTTTTTTTT-3’ |
| 5 | BLU1 | 5’-CGCTAGCAATATTTTTTTTTTT-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 | 20merP | 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’ |
| 21 | 20merNP | 5’-CGCTAGCAATATTCTGCAGC-3’ |
| 22 | 20merR | 5’- CGCUAGCAAUAUUCUGCAGC-3’ |
| 23 | 46merR | 5’-UUUUUUUUUUUUUGCUGCAGAAUAUUGCUAGCGUUUUUUUUUUUUU-3’ |

Oligos 8, 9, 17, 18, 19 and 20 carry a DNA modification: 8-oxoG (8-oxoguanine), 8-oxoA (8-oxoadenine), ABAS (abasic site), CHOLE (cholesterol), ETAD (ethenoadenine), HYPO (hypoxanthine). “Pho” in oligonucleotide 2 indicates a phosphate group on the 5’-end.

## 3.6. DNA, proteins, systems and kits

| | |
|---|---|
| Smart – ladder | Eurogentec, Seraing, Belgium |
| Oligonucleotide sizing markers | Amersham, Braunschweig |
| SigmaMarker High Range | Sigma, München |
| *PfuTurbo* DNA polymerase | Stratagene, Heidelberg |
| Bacteriophage T4 Polynucleotide kinase | New England Biolabs, Beverly, MA |
| Klenow fragment of *E. coli* DNA polymerase I | New England Biolabs, Beverly, MA |
| Lysozyme | Roche, Mannheim |
| ExTaq Polymerase | TaKaRa, Alpen |
| Qiaex II kit, Qiagen Plasmid Isolation Kit | Qiagen, Hilden |
| QuikChange Site-Directed Mutagenesis Kit | Stratagene, Heidelberg |
| MATCHMAKER Two-Hybrid System 2 | Clontech, Heidelberg |
| MATCHMAKER LexA Two-Hybrid System | Clontech, Heidelberg |
| Affinity LIC Cloning and Protein Purification Kit | Stratagene, Heidelberg |
| High Pure PCR Product Purification Kit | Roche, Mannheim |
| ABI Prism$^{TM}$ DNA Sequencing Kit | Applied Biosystems, Weiterstadt |

## 3.7. Chemicals

| | |
|---|---|
| Isopropyl-β-D-thiogalactopyranoside (IPTG), | Stratagene, Heidelberg |

| | |
|---|---|
| Carrier DNA | |
| Benzamidine, Leupeptine, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), D(+) Galactose (<0,01% glucose), 3-amino-1, 2, 4-triazole (3-AT), Triton X-100 | Sigma, München |
| [$\gamma$–$^{32}$P]ATP, [$\alpha$-$^{32}$P]dCTP | Amersham, Braunschweig |
| Adenosine 5 -[$\gamma$-thio]triphosphate (ATP$\gamma$S) | |
| Ammonium persulphate, (TEMED) | Bio-Rad, München; AGS Heidelberg |
| Urea | |
| D(+) Raffinose pentahydrate, Dithiothreitol (DTT), N,N-dimethylformamide (DMF), Glucose, Disodium hydrogenphosphate heptahydrate, Adenosine triphosphate (ATP), Caseine hydrolysate, Lithium acetate dihydrate, Ammonium sulphate | Fluka, Deisenhofen |
| Bacto agar, Yeast nitrogen base (YNB) | Difco, Detroit, MI, USA |
| Peptone 140 selected | Gibco BRL, Eggenstein |
| Yeast extract Servabacter, $\beta$-mercaptoethanol, Zink chloride, Manganese (II) chloride tetrahydrate, Ethidiumbromide | Serva, Heidelberg |
| Tryptone, Carbenicillin | Duchefa, Haarlem, NL |
| Drop out (DO) Supplements: -Ura, -Trp, -Leu, -Ura/-Trp, -His/-Ura, -Leu/-Trp, - His/-Trp/-Ura, -His/-Trp/-Leu, -His/-Trp/ -Ura/-Leu, -His/-Leu, -Ura/-Trp/-Leu, -Ura/-His/-Leu | Clontech, Heidelberg |

| | |
|---|---|
| Sodium chloride, Sodium dihydrogenphosphate, monohydrate, Glycerol, 5-bromo-4-chloro-3-indolyl -β-D-galactopyranoside (X-gal), Polyethylene glycol (PEG 3350), Calcium chloride, Tris, Sodium hydroxide, Hydrogenchloride, Potassium chloride | Roth, Karlsruhe |
| Magnesium chloride, Bovine serum albumin (BSA) | New England Biolabs, Beverly, MA |
| Magnesium sulphate heptahydrate | Merck, Haar |
| Agarose (Sea-Kem) | Biozym, Hessisch. Oldendorf |

## 3.8. Consumed material

| | |
|---|---|
| MicroSpin™ G-50 Sepharose columns | Amersham, Braunschweig |
| Gel temperature indicator, Ready gel (10 well Tris-HCl, 10%), Ready gel (10 well TBE, 15%), Poly-Prep Chromato-graphy columns | Bio-Rad, München; AGS Heidelberg |
| Gel sealing tape | Gibco BRL, Eggenstein |
| Blue Slick | Serva, Heidelberg |
| Rotiszint®eco plus, Mini Vials C | Roth, Karlsruhe |
| Whatman 3MM-filter paper | Bender&Hobein, Karlsruhe |

## 3.9. Laboratory tools

| | |
|---|---|
| Mini-Wipptisch WT | Biometra, Göttingen |
| Sonopuls HD2070, Lärmschutzbox LS5 | Schütt, Göttingen |
| Edelstahl (Hubtisch) | |
| Model S2 Sequencing Gel Electrophoresis | Gibco BRL, Eggenstein |

| Apparatus | |
|---|---|
| DNA Gel-electrophoresis tanks | Bio-Rad, München; AGS Heidelberg |
| Spectrophotometer Spectronic 1201 | Milton Roy, Rochester, NY, USA |
| pH-meter Hanna-Hi 9321 | Schütt, Göttingen |
| Shaker (Vortex Genie 2$^{TM}$), Megafuge 1.0R | Heraeus, Osterode |
| ABI PRISM DNA sequencer, WINSpectal | Perkin Elmer, Wellesley, USA |
| WALLAC LSC, Perkin Elmer Gene Amp PCR System 2400 | |
| Waterbath | Haake, Karlsruhe |
| Weight | Sartorius, Göttingen |
| Centrifuge 5415 R, Thermomixer 5436 | Eppendorf, Hamburg |
| Gel-drier Model PH-t 60 | Biotec-Fischer, Reiskirchen |

## 3.10. Media, buffers and solutions

| | |
|---|---|
| Bio-Safe Coomassie Stain, Nucleic Acid Sample Loading Buffer, 5X TBE-Urea Sample Buffer, Laemmli Sample Buffer, 10X Tris/Glycine/SDS Buffer, 10X TBE Buffer, Acrylamide and Bis-Acrylamide solutions, Protein assay | Bio-Rad, München; AGS Heidelberg |

| | |
|---|---|
| Carbenicillin | 50mg/ml $H_2O$ |
| LB broth | 10g NaCl, 10g Tryptone, 5g Yeast extract for 1l (pH 7.0) |
| LB-carbenicillin broth | LB broth, 100µg/ml carbenicillin (pH 7.0) |
| LB-Carbenicillin Agar | LB broth, 20g agar, 100µg/ml carbenicillin (pH 7.0) |
| $NZY^+$ broth | 10g Casein hydrolysate, 5g Yeast extract, 5g NaCl, 2.5g $MgCl_2$ · |

| | |
|---|---|
| | $6H_2O$, 3g $MgSO_4 \cdot 7H_2O$, 4g Glucose |
| SOC medium | 20g Tryptone, 5g Yeast extract, 0.5g NaCl, 2g $MgCl_2 \cdot 6H_2O$, 2.5g $MgSO_4 \cdot 7H_2O$, 4g Glucose |
| $CaCl_2$ binding buffer | 750mM NaCl, 50mM Tris-HCl, pH 8.0, 2mM $CaCl_2$, 0.1%Triton X-100, 10mM ß-mercaptoethanol |
| Washing buffer 1 | 750mM NaCl, 50mM Tris-HCl, pH 8.0, 2mM $CaCl_2$, 10mM ß- mercaptoethanol |
| Washing buffer 2 | 500mM NaCl, 50mM Tris-HCl, pH 8.0, 2mM $CaCl_2$, 10mM ß-mercaptoethanol |
| Elution buffer | 500mM NaCl, 50mM Tris-HCl, pH 8.0, 2mM EGTA, 10mM ß-mercaptoethanol |

Protease inhibitors:

| | |
|---|---|
| 1000X Leupeptine | 2mg/ml $H_2O$ |
| 100mM Benzamidine | 1.57mg/ml $H_2O$ |
| 420X AEBSF | 50mg/ml $H_2O$ |

In the protein isolation procedure the final working concentrations were 2µg/ml leupeptine, 1mM benzamidine and 0.1mg/ml AEBSF.

| | |
|---|---|
| Reaction buffer 1 | 40mM Tris-HCl, pH 8.0, 4mM $MgCl_2$, 5mM dithiothreitol, 1mM ATP, and 0.1mg/ml bovine serum albumine |
| Reaction buffer 2 | 40mM Tris-HCl, pH 7.4, 4mM $MgCl_2$, 5mM dithiothreitol, 1mM ATP, and 0.1mg/ml bovine serum albumine |

Solutions for Two-hybrid assays:

| | |
|---|---|
| 10X glucose(20%) | 22g glucose monohydrate/100ml $H_2O$ |
| 10X $(NH_4)_2SO_4$ | 5g /100ml $H_2O$ |
| 10X YNB | 1,7g /100ml $H_2O$ |
| 10X DO | 0.7g/100ml $H_2O$ |

| | |
|---|---|
| 125X adenine sulphate | 5mg/ml |
| 1M LiAc | 20.4g $LiAc \cdot 2H_2O$/ 200ml $H_2O$, adjust pH 7.5 with diluted acetic acid |
| 2mg/ml carrier DNA | add 200 µl carrier DNA (10mg/ml) to 800 µl $H_2O$, heat 5 min at 100°C and put quickly on ice |
| 50% (w/v) PEG 3350 | 40g /80ml $H_2O$ |
| X-gal | 20mg /ml DMF |
| 10X BU salts (1l) | 30.8g $Na_2HPO_4.2H_2O$<br>39.66g $NaH_2PO_4.H_2O$, adjust pH 7 with NaOH lens |
| 5X galactose (10%) | 100g /l $H_2O$ |
| 20X raffinose (20%) | 23.6g raffinose pentahydrate/100ml $H_2O$ |
| 1M 3-amino-1, 2, 4-triazole (3-AT) | |
| Z-buffer (1l) | 16.1g $Na_2HPO_4 \cdot 7H_2O$, 5.5g $NaH_2PO_4 \cdot H_2O$, 0.75g KCl, 0.246g $MgSO_4 \cdot 7H_2O$ |
| Z/X-buffer | 100ml Z-buffer, 0.27ml β-mercaptoethanol, 1.67ml X-gal |

Mediums for yeast transformation:

| | |
|---|---|
| YPAD (1l) | 10g Yeast extract, 20g Peptone, 100ml 10x glucose, 4 ml 125X adenine sulphate |
| DO liquid medium (1l) | 100ml 10X glucose, 100ml 10X $(NH_4)_2SO_4$, 100ml 10X YNB, 100ml 10X DO, rest $H_2O$ |
| DO medium/ agar (1l) | DO liquid medium, 20g agar |

Mediums for GAL4 protein-protein interaction assay:

| | |
|---|---|
| DO/3-AT (1l) | 100ml 10X glucose, 100ml 10X $(NH_4)_2SO_4$, 25ml 1M 3-AT, 100ml 10X YNB, 100ml 10X DO, rest $H_2O$<br>(DO = -Trp/-Leu/-His) |

Mediums for LexA protein-protein interaction assay:

| | |
|---|---|
| DO/X-gal/BU salts (1l) | 100ml 10X glucose, 100ml 10X $(NH_4)_2SO_4$, 100ml 10X YNB, 100ml 10X DO, 100ml 10X BU salts, 4ml X-gal, rest $H_2O$ (DO = -Ura/-His/-Trp or -Ura/-His/-Trp/-Leu) |
| DO/galactose/raffinose (1l) | 200ml 5X galactose, 50ml 20X raffinose, 100ml 10X YNB, 100ml 10X $(NH_4)_2SO_4$, 100ml 10X DO, rest $H_2O$ (DO = -Ura/-His/-Trp/-Leu) |
| DO/X-gal/BU salts/galactose/raffinose (1l) | 200ml 5X galactose, 50ml 20X raffinose, 100ml 10X YNB, 100ml 10X $(NH_4)_2SO_4$, 4ml X-gal, 100ml 10X DO, rest $H_2O$, 100ml 10X BU salts (DO = -Ura/-His/-Trp or -Ura/-His/-Trp/-Leu) |

## 3.11. Software

The DNA and protein sequence data were processed using the program “DNA-star”. The evaluation of pictures from Phosphoimager was done with the program “TINA”.

# 4. Methods

## 4.1. Cloning of AtWRNexo into vectors for protein expression and two-hybrid assay

The AtWRNexo open reading frame (ORF) was amplified using the *PfuTurbo* DNA polymerase and combination of primers HP40/HP41 (for pCAL-n-FLAG), HP21/WRN-Sal1-AS21 (for pAS2-1) and HP21/HP22 (for pGILDA and pB42AD). The plasmid pGAD GH containing the AtWRNexo ORF (obtained by Dr. Frank Hartung) was used as a template in the amplification reaction. For the amplification of AtWRNexo to clone it into the two-hybrid vectors, following PCR program was run: 5' 95°C; 5X: 20'' 95°C/15'' 50°C/1' 72°C; 20X: 20'' 95°C/15'' 60°C/1' 72°C; 2' 72°C; 4°C. The amplified products were purified using the High Pure PCR Product Purification Kit according to the manufacturer. The following gel electrophoresis, restriction and ligation were performed according to Sambrook *et al*, 1989. The transformation of *E. coli* (DH5α) was done using the heat shock procedure. The *E. coli* cells carrying the plasmid were selected on LB-carbenicillin plates. The amplification of AtWRNexo for the expression vector was done by following PCR program: 5' 95°C; 17X: 20'' 95°C/15'' 56°C/1' 72°C; 2' 72°C; 4°C. The PCR product was then purified using the High Pure PCR Product Purification Kit according to the manufacturer and cloned into the pCAL-n-FLAG vector using the Affinity LIC Cloning and Protein Purification Kit. The ligation-independent cloning (LIC) method doesn't need any restriction or ligation enzymes (Figure 11). The LIC reaction was then transformed into Epicurian Coli SoloPack Gold cells as described in the manufacturer's protocol. The positive clones (both for the protein expression and for the two-hybrid system) were identified by PCR screening using the primers for the respective vector. The amplified products were sequenced using PCR screening primers and ABI PRISM DNA sequencer. The mutation free clones were identified

with the "DNA-star" software and the plasmid DNA (pCAL-n-FLAG-AtWRNexo) was isolated using Qiagen Plasmid Isolation Kit.

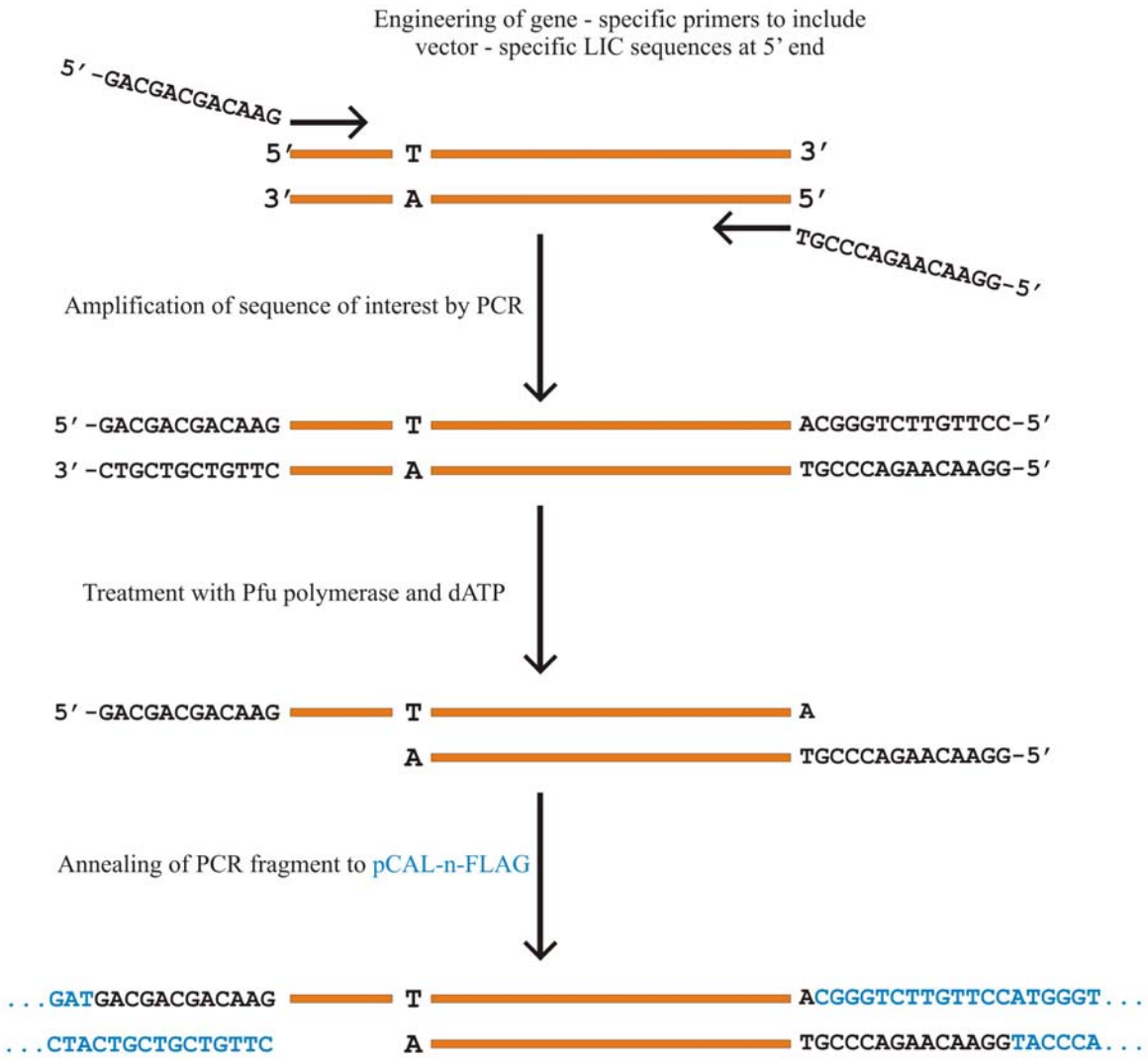



**Fig. 11.** Ligation independent cloning of the protein – coding sequence to produce CBP-FLAG fusion proteins. PCR primers are engineered to include the 5' end vector specific sequence shown in the figure. The sequence of interest is than amplified by PCR. Treatment of the DNA with *Pfu* DNA polymerase and dATP results in a degradation of the 3' ends until the first adenine base (A) is reached (the DNA is cleaved also in the inserted sequence of interest). The treated PCR fragment is annealed to the single-stranded overhangs (12- and 13-nts) of the pCAL-n-FLAG vector (this vector is restriction-digested and pretreated with *Pfu* DNA polymerase in the presence of dTTP) and the single-stranded gap is repaired *in vivo* following transformation of SoloPack Gold supercompetent cells with the annealed DNA.

## 4.2. 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 according to the manufacturer's protocol using primers HP150 and HP151, carrying the mutation.

This method was performed using *PfuTurbo* DNA polymerase and a temperature cycler. The PCR mixture containing the plasmid pCAL-n-FLAG with inserted AtWRNexo ORF was given into the thermal cycler and following PCR program was run: 30'' 95°C; 12X: 30'' 95°C/1' 60°C/14' 68°C; 4°C. The incorporation of the mutated primers generated a mutated plasmid (pCAL-n-FLAG-AtWRNexoE135A) containing nicks. The parental methylated DNA (DNA isolated from almost all *Escherichia coli* strains is dam methylated) was then digested with endonuclease *Dpn* I, which is specific for methylated and hemimethylated DNA. The nicked vector DNA containing the desired mutation was transformed into XL1-Blue supercompetent cells, which repaired the nick. The cells were propagated on LB-carbenicillin plates. The positive clones were identified by PCR screening. The amplified products were then sequenced using PCR screening primers and ABI PRISM DNA sequencer. Mutation-free clones were identified with the "DNA-star" software and the plasmid DNA was isolated using Qiagen Plasmid Isolation Kit.

## 4.3. Expression and purification of AtWRNexo-p and AtWRNexoE135A-p

The pCAL-n-FLAG-AtWRNexo and pCAL-n-FLAG-AtWRNexoE135A were transformed into the *E. coli* protein expression strain BL21(DE3) from Novagen according to the Novagen's protocol and propagated on the LB-carbenicillin plates. 5 ml of LB-carbenicillin broth was then inoculated with a single colony and was grown at 37°C till the optical density $OD_{600}$ of 0.6-1.0 was reached. The culture was centrifuged at 6000 x rpm for 15 minutes at 4°C. The used LB-carbenicillin medium was replaced by new one and 5 x 100 ml LB-carbenicillin broth was inoculated with 1 ml of the cells. The cells were grown at 37°C till

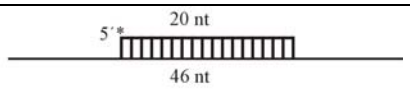
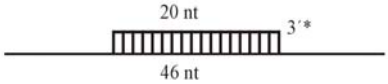
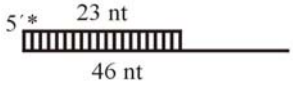
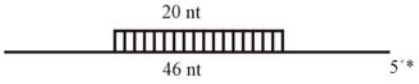
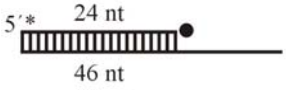
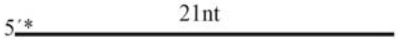
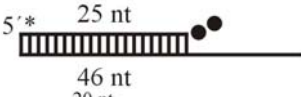
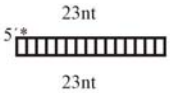
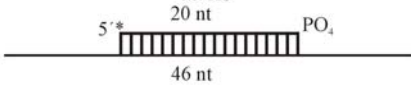
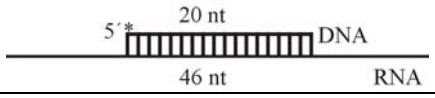
$OD_{600}$ of 0.6-1.0. After a centrifugation at 6000 x rpm for 15 minutes at 4°C the LB-carbenicillin medium was replaced again. The CBP-FLAG-AtWRNexo protein was then overexpressed by 1mM isopropyl-β-D-thiogalactopyranoside induction for 6h at 25°C. After the induction, the cells with the expressed protein were centrifuged at 4000 x rpm for 30 minutes at 4°C and after the removal of the medium were frozen in a liquid nitrogene and stored at -80°C until further use. The purification procedure followed the instructions of Affinity LIC Cloning and Protein Purification Kit manufacturer with some modifications. The frozen cells were thawed and resuspended in $CaCl_2$ binding buffer (5ml/g) and a mixture of protease inhibitors (leupeptine, benzamidine, AEBSF). Lysozyme (200μg/ml) was added and the suspension was incubated with gently shaking on ice for 20 minutes. The cells were further disrupted by sonication and centrifuged at 13000 x rpm for 20 minutes at 4°C. The lysate was then put on a 2ml-CaM-resin PolyPrep Chromatography column equilibrated in $CaCl_2$ binding buffer. The column was extensively washed with ten volumes of $CaCl_2$ binding buffer and two volumes of washing buffers 1 and 2 supplemented with proteases inhibitors. Proteins were eluted in elution buffer. Elution fractions of 0.5 ml were collected and analysed by SDS-PAGE. The protein concentration was determined by the Bradford assay using bovine serum albumine (BSA) as a standard. The purified AtWRNexo-p was supplemented with 100μg/ml BSA and stored in elution buffer containing 25% glycerol in 20 μl aliquots at -80°C. The activity of AtWRNexo-p remained stable for at least 6 months. AtWRNexoE135A-p was expressed and purified in an identical fashion as used for AtWRNexo-p.

## 4.4. Oligomers labeling and substrate preparation

Single-stranded DNA and RNA oligomers (7 pmol) were 5'-end-labeled with [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase according to the manufacturer. The oligonucleotides ABAS, ETAD, HYPO and CHOLE were desalted before labeling using

Qiaex II kit. To produce DNA and RNA substrates (see Table below), labeled oligomers (in Table below marked with asterisk) were annealed to the complementary strand in a 1:2 molar ratio in 70mM Tris-HCl buffer, pH 7.6, 10mM $MgCl_2$ by incubation at 95°C for 5 min and then slow cooling to room temperature. The 3'-end-labeled substrate was produced by the annealing of 19-mer DNA (19merNP) to the 46-mer (46merT) oligonucleotide (1:2) and labeling the 3'-end of 19-mer using [$\alpha$-$^{32}$P]dCTP and Klenow fragment of *E. coli* DNA polymerase I according to the manufacturer. Unincorporated labeled nucleotides were removed from labeling reactions using MicroSpin™ G-50 columns. DNA substrates were stored at 4°C. RNA substrates were stored at – 20°C.

DNA and RNA substrates used in this study

| Oligonucleotides | DNA or RNA substrate | Oligonucleotides | DNA or RNA 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 |  |
| *21 + 23 |  | *22 |  |

## 4.5. DNA and RNA substrates concentration determination

The DNA substrate (1µl) was mixed with 4ml of scintilation liquid Rotiszint®eco plus and analyzed by WINSpectal liquid scintillation counter. From measured counts per minute (CPM), the concentration of DNA substrate in terms of DNA molecules was calculated according following equation:

(a) The measured counts per minute (CPM) were recalculated to obtain decays per second (DPS), which represent the real value of decays (normalized by an efficiency of measurement of the scintillation counter).

DPS = CPM/ (60 * 0.9) = CPM/ 54

The value 60 means 60 s.

The value 0.9 is an efficiency of measurement (90%, experimentally determined).

(b) The decays per second of 1µl of [$\gamma$-$^{32}$P]ATP (3.33 pmol) at the day of measurement (N) were calculated as follows.

$N = N_0 * e^{-\lambda\tau} / 86400 = (31968 * 10^6 * e^{-0,0484615\tau}) / 86400$

The value $N_0$ represents decays per day (DPD) of 1µl of [$\gamma$-$^{32}$P]ATP at the day of calibration and corresponds to 31968 * $10^6$ (DPD).

$\tau$ is a time distance (in days) from the day of calibration.

$\lambda$ is a decay constant calculated under the assumption that $N = N_0/2$ and a decay half time $\tau_{1/2}$ is 14.3 days.

(c) The determined values DPS and N were used to calculate the concentration (C) of the probes (in fmol/µl):

C = (3.33 * DPS * 1000)/ N

## 4.6. Exonuclease assay

Reactions (10µl) were performed in reaction buffer 1, for investigation of cation requirements reaction buffer 2 supplemented with respective cations was used as indicated in Figure 21. For the amounts of labeled DNA substrates (DNA amounts are expressed in terms of molecules) and AtWRNexo proteins used see figure legends. The reactions were performed at

22°C for 1h, unless otherwise indicated and terminated by addition of an equal volume of denaturing loading buffer, immediately heated at 98°C for 3 min, analysed on 20% polyacrylamide 1XTBE - 7M urea gel using a Model S2 sequencing gel electrophoresis apparatus and visualized by autoradiography.

## 4.7. Cloning of AtWRNexo interaction partners into vectors for two-hybrid assay

ORFs of AtWRNexo interacting partners were amplified using the *PfuTurbo* DNA polymerase and combination of primers RQ5-5S-XmaI/RQ5-3S-BamHI (for AtRecQl1 into pGILDA), HP7/HP59 (for AtRecQl3 into pGILDA), RQl2-5S-XmaI/RQl2-3S-(BamHI) (for AtRecQl4a into pGILDA), HP69/HP70 (for AtRecQl5 into pGILDA), HP79/HP80 (for AtKu80 into pGILDA) and HP79/HP81 (for AtKu80 into pB42AD). The plasmid pGEM-T containing the respective ORF (obtained by Dr. Frank Hartung) was used as a template in the amplification reaction. For the amplification following PCR program was run: 5' 95°C; 5X: 20'' 95°C/15'' 46°C/Y' 72°C; 20X: 20'' 95°C/15'' 60°C/Y' 72°C; 2' 72°C; 4°C. "Y" indicates the time for amplification as follows: 4' for the amplification of AtRecQl1, AtRecQl3, AtRecQl5 and AtKu80 and 8' for the amplification of AtRecQ4a. The amplified products were purified using the High Pure PCR Product Purification Kit according to the manufacturer. The following gel electrophoresis, restriction and ligation were performed according to Sambrook *et al*, 1989. The transformation of *E. coli* (DH5α) was done using the heat shock procedure. The *E. coli* cells carrying the plasmid were selected on LB-carbenicillin plates.

AtRecQsim was cloned into pGILDA using primers RQ3-5S(XmaI)/HP60 (for the first half of AtRecQsim) and HP61/RQ3-3S(XhoI) (for the second half of AtRecQsim). cDNA (obtained by Dr. Frank Hartung) was used as a template in the amplification reaction. For the

amplification following PCR program was run: 5' 95°C; 5X: 20'' 95°C/15'' 46°C/2' 72°C; 20X: 20'' 95°C/15'' 60°C/2' 72°C; 2' 72°C; 4°C. The amplified products were purified using the High Pure PCR Product Purification Kit according to the manufacturer. The following gel electrophoresis, restriction and ligation were performed according to Sambrook *et al*, 1989. Both halfs containing common NheI restriction site were ligated together and cloned into the pGILDA plasmid. The transformation of *E. coli* (DH5α) was done using the heat shock procedure. The *E. coli* cells carrying the plasmid were selected on LB-carbenicillin plates.

The positive clones were identified by PCR screening using the primers for the respective vector. The amplified products were sequenced using PCR screening primers, primers specific for the respective ORF and ABI PRISM DNA sequencer. The mutation free clones were identified with the "DNA-star" software and the plasmid DNA (pCAL-n-FLAG-AtWRNexo) was isolated using Qiagen Plasmid Isolation Kit.

## 4.8. Two-hybrid assay

The identification of interacting partners with AtWRNexo protein was performed using the Two-Hybrid GAL4 and LexA System according to the protocols of the manufacturer. In GAL4 system, AtWRNexo fused to the activating domain was tested for the possible interactions with AtRecQl1, AtRecQl2 and AtRecQl3, which were fused to the DNA binding domain (obtained from Dr. Frank Hartung). Single clones were transformed into Y190 yeast strain via "Lazy bones" plasmid transformation procedure (Elble, 1992). The contransformation of both clones together was done following the High-efficiency transformation protocol (Gietz and Schiestl, 1995). After the transformation, the yeast cells carrying single clones were propagated on –Trp or –Leu plates, the double-transformants were grown on –Trp/-Leu plates at 28°C. To investigate the protein-protein interactions, the double-transformants were transferred on –Trp/-Leu/-His plates supplemented with 25mM 3-

amino-1, 2, 4-triazole (3-AT) to screen for an activation of HIS3 reporter gene. Additionally, an activation of the second reporter gene lacZ was tested by colony-lift filter assay according to the Clontech protocol. The clones were also tested for self-activation by growing on –His medium with 25mM 3-AT and by the same X-gal staining procedure. As a positive interaction control, SNF1 fused to the DNA binding domain and SNF4 fused to the activation domain was used.

In the LexA system the interactions between AtWRNexo protein fused to the activation domain and AtRecQl1, AtRecQl3, AtRecQl4a, AtRecQl5, AtRecQsim, AtWRNexo, Ku80, Ku70, Ubc9, Rad51 and MLH1 proteins, which were fused to the DNA binding domain, were tested. Moreover, the AtWRNexo protein fused to the DNA binding domain and Ku80, Ku70, Ubc9, Rad51 and MLH1 protein bound to the activation domain were investigated. The yeast strain EGY48 was firstly transformed using High efficiency transformation procedure (Gietz and Schiestl, 1995) with the plasmid p8oplacZ carrying lacZ reporter gene. This yeast was then propagated on –Ura medium at 28°C. The single and double transformants were then obtained by transforming the EGY48/p8oplacZ with respective clones using the same transformation protocol. The single transformants were then plated either on –Ura/-His or on –Ura/-Trp medium and the double transformants on –Ura/-His/-Trp medium and let grow at 28°C. To investigate the protein-protein interactions, double transformants were incubated at 28°C on following mediums: –Ura/-His/-Trp/galactose/raffinose/X-gal/BU salts, –Ura/-His/-Trp/-Leu/galactose/raffinose/X-gal/BU salts and –Ura/-His/-Trp/galactose/raffinose. An induction of expression of target proteins should take place only on medium containing galactose. If there is an interaction between tested proteins, the LEU2 and lacZ genes will be activated. As a positive control for interacting proteins the murine p53 protein fused to the DNA binding domain and SV40 large T-antigen fused to the activation domain were used. The clones were also tested for the self-activation by growing on –Ura/-His/galactose/raffinose/X-gal/BU salts, –Ura/-His/-Leu/galactose/raffinose/X-gal/BU salts, -

Ura/-His/-Leu/galactose/raffinose, -Ura/-Trp/galactose/raffinose/X-gal/BU salts, -Ura/-Trp/-Leu/galactose/raffinose/X-gal/BU salts and -Ura/-Trp/-Leu/galactose/raffinose. That the positive interactions are a result of the expression of the tested proteins was checked by plating the transformed yeast on following plates containing glucose: –Ura/-His/X-gal/BU salts, –Ura/-His/-Leu/X-gal/BU salts, –Ura/-Trp/X-gal/BU salts, –Ura/-Trp/-Leu/X-gal/BU salts, –Ura/-His/-Trp/X-gal/BU salts and –Ura/-His/-Trp/-Leu/X-gal/BU salts.

# 5. Results

## 5.1. RecQ homologues from *Arabidopsis thaliana*

Seven RecQ homologues have been identified in *Arabidopsis thaliana* by a database search using homologies to known genes of other organisms (Hartung et al., 2000). Six of these proteins are named RecQ-like (AtRecQl1, 2, 3, 4A, 4B and 5, respectively). The seventh one is named RecQsim (similar), because of an insertion of approximately 160 aa between helicase domains III and IV. The schematic structure of the AtRecQ-like proteins is shown in Figure 12.

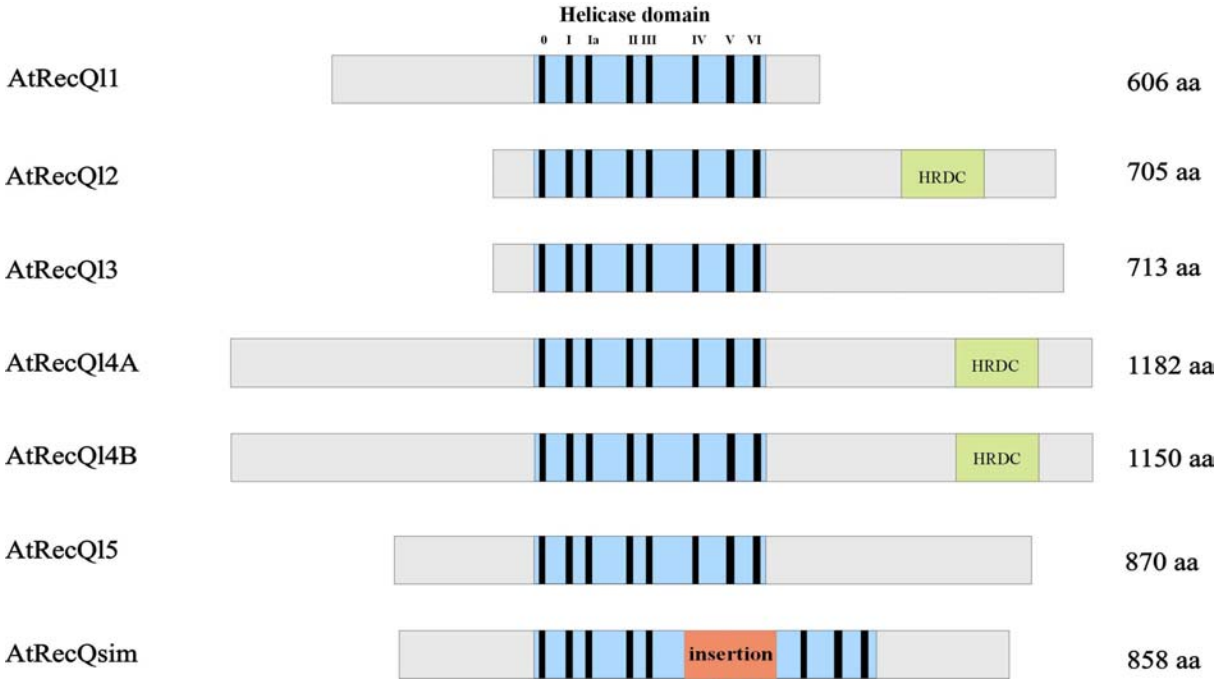



**Fig. 12.** The schematic structure of the *Arabidopsis thaliana* RecQ proteins. The helicase domain with 8 conserved motifs and the HRDC domain is indicated. The insertion between the motif III and IV of the helicase domain by AtRecQsim is shown in red.

The members of *Arabidopsis thaliana* RecQ family of helicases can be divided into two groups according to their size. There are five RecQ proteins, which are small in size: AtRecQl1 (606 aa), AtRecQl2 (705 aa), AtRecQl3 (713 aa), AtRecQl5 (870 aa) and AtRecQsim (858 aa). The second group comprises two bigger proteins AtRecQl4A (1182 aa) and AtRecQl4B (1150 aa). These two proteins most probably arose by a recent sequence

duplication. They are highly homologous with ~70% identity at both nucleic acid and protein level. There has been, additionally to the usual RecQ domains, other domains in some RecQ proteins identified (Bagherieh-Najjar et al., 2003).

## 5.2. WRN-like gene (AtWRNexo)

The RecQ homologues are conserved across species. Surprisingly, using the homologies to the Werner syndrome protein, no gene encoding the complete homologue of WRN protein could be identified. Instead, a small gene (AtWRNexo) with a striking homology to only the exonuclease domain of human Werner syndrome protein has been detected. This gene is present on chromosome 4 and is free of any RecQ-like helicase domain. Further analysis revealed that AtWRNexo consists of six exons and encodes a protein with predicted 285 aa. The schematic intron/exon structure is shown in Figure 13.

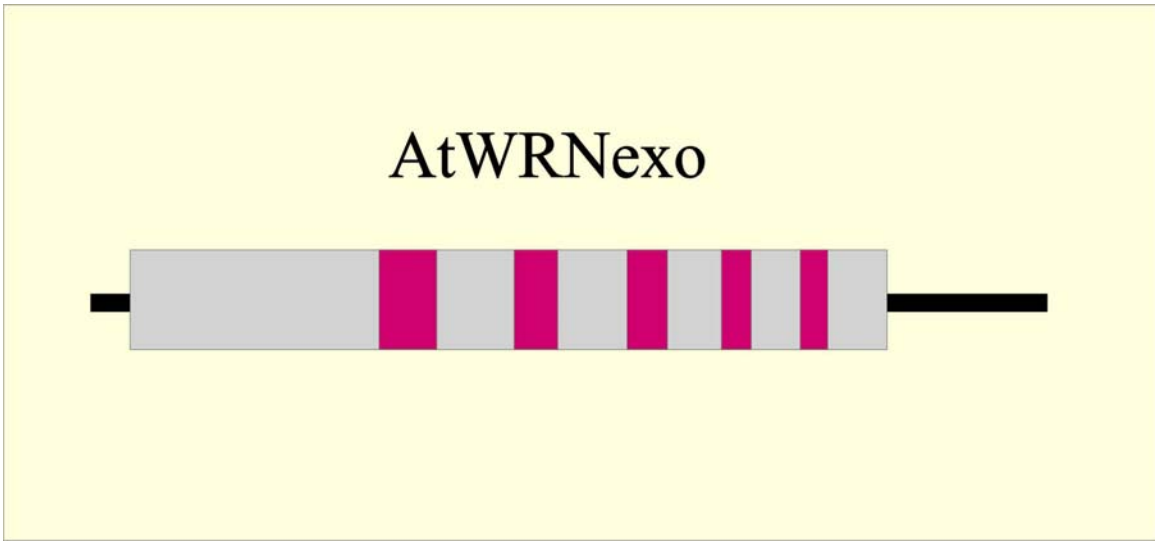



**Fig. 13.** Schematic intron/exon structure of the Werner-like gene from *Arabidopsis thaliana*. The five introns are shown in dark red colour.

## 5.3. WRN-like protein (AtWRNexo-p)

An alternative splicing event of intron 5 in AtWRNexo results in production of two cDNAs. One cDNA corresponds to the protein of 285 aa. The second cDNA is 3 aa longer. The sequence of both proteins is shown in Figure 14.

MSSSNWIDDAFTEEELLAIDAIEASYNFSRSSSSSSSAAPTVQATTSVHGHEEDPNQIPNNI
RRQLPRSITSSTSYKRFPLSRCRARNFPAMRFGGRILYSKTATEVDKRAMQLIKVLDTKRDE
SGIAFVGLDIEWRPSFRKGVLPGKVATVQICVDSNYCDVMHIFHSGIPQSLQHLIEDSTLVK
VGIGIDGDSVKLFHDYGVSIKDVEDLSDLANQKIGGDKKWGLASLTETLVCKELLKPNRIRL
GNWEFYPLSKQQLQYAATDAYASWHLYK**VLK**DLPDAVSGS

**Fig. 14.** The sequence of AtWRNexo gene products. The 3 extra amino acids present in the slightly longer Werner-like protein are depicted in red.

### 5.3.1. The identification of the amino acid residues in AtWRNexo-p, critical for the exonucleolytic activity

The amino acid sequence of the longer 288 aa AtWRNexo-p was compared with the N-terminal part of the human Werner syndrome protein. In human, the amino acids predicted to be critical for the exonuclease activity are D82, E84, 143D, 212Y and 216D. The comparison of the protein sequences revealed conserved positions of all five amino acids D133, E135, D194, Y263, D267 in the *Arabidopsis thaliana* sequence. All five residues lie within the 3 highly conserved motifs present in the exonuclease domain (see also Figure 3). 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. (Figure 15).

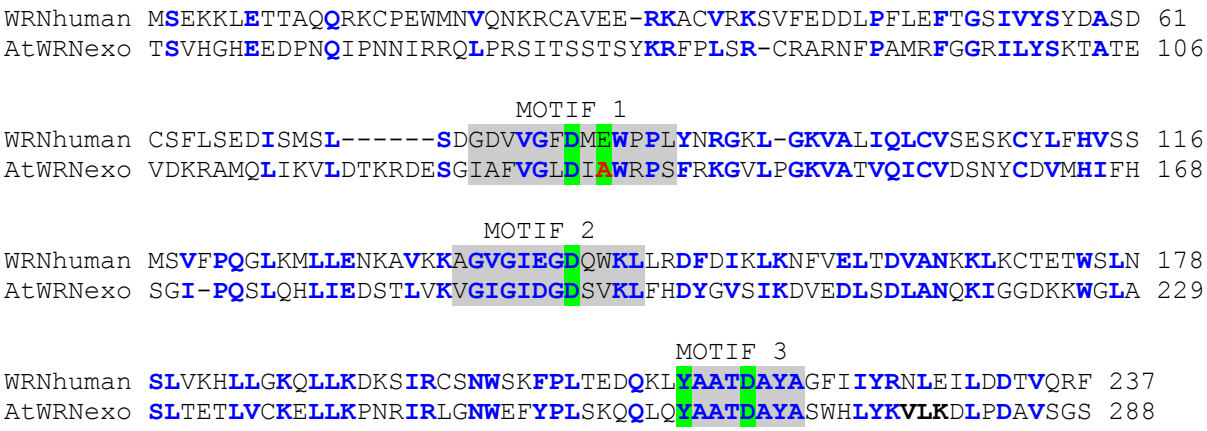



**Fig. 15.** Amino acid sequence alignment of AtWRNexo-p with the N-terminus of the WRN protein from human. The three extra amino acids resulting from an alternative splicing event of intron 5 of AtWRNexo gene are shown in bold black characters. Identical and conserved amino acids are shown in bold blue characters, highly conserved motifs within the exonuclease domain are represented as gray shaded boxes. The five amino acids thought to be critical for exonuclease activity are green shaded. The point mutation E135A that abolishes the exonucleolytic activity of AtWRNexo-p is shown in red.

### 5.3.2. The construction of the CBP-tagged AtWRNexo-p and CBP-tagged AtWRNexoE135A-p

An ORF encoding this larger version of AtWRNexo was cloned into a bacterial vector pCAL-n-FLAG by ligation independent cloning (see Figure 11). 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 (Huang et al., 1998), was introduced into the AtWRNexo ORF by site-directed PCR mutagenesis to generate pCAL-n-FLAG-AtWRNexoE135A. The sequences of cloned fragments of AtWRNexo and AtWRNexoE135A were checked by sequencing. AtWRNexo was free of any mutation and AtWRNexoE135A revealed only one desired codon substitution GAG to GCG corresponding to the amino acid change E135A. The ORFs were inserted in frame into the vector to allow the production of calmodulin-binding peptide (CBP) fusion proteins (Figure 16).

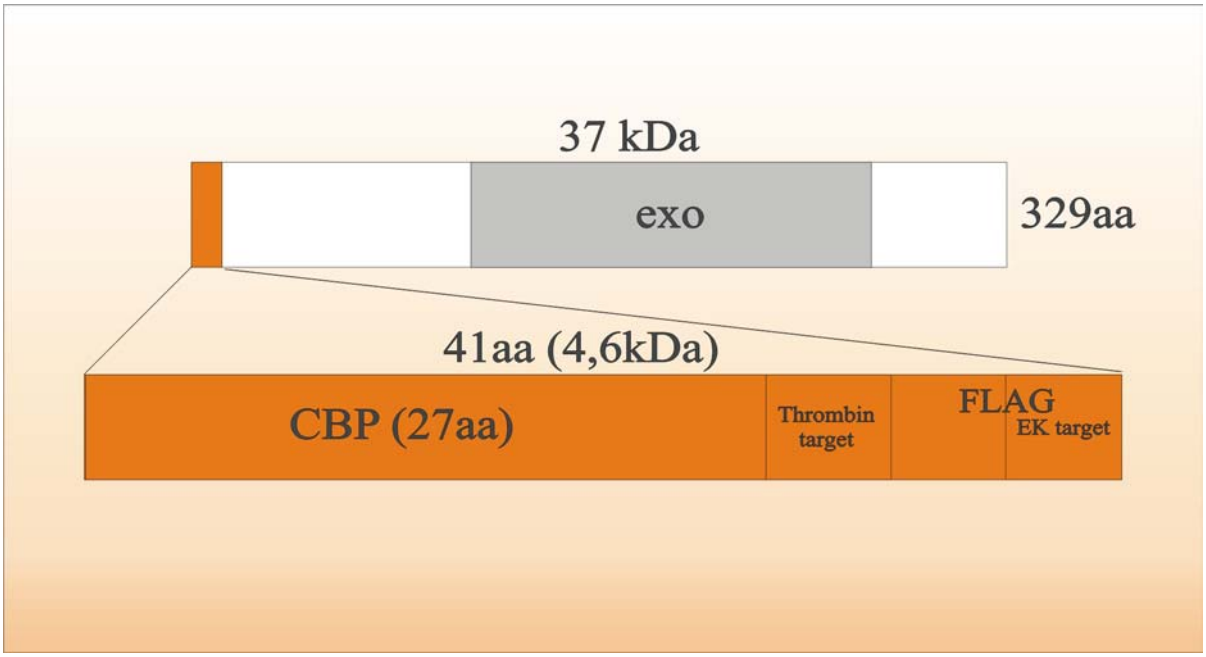



**Fig. 16.** The schematic structure of the AtWRNexo protein. The protein is N-terminally fused to the calmodulin-binding peptide (CBP) purification tag followed by the FLAG epitope, an antibody recognition site. The thrombin and enterokinase (EK) cleavage sites are indicated. The whole tag comprises 41 aa and the tagged AtWRNexo protein is 329 aa in length.

### 5.3.3. Expression and purification of AtWRNexo-p and AtWRNexoE135A-p

Recombinant proteins were overexpressed in *E. coli* and purified by one step CaM-affinity chromatography. The temperature is one of the factors determining whether the expressed protein will accumulate in inclusion bodies. The expression of AtWRNexo proteins was therefore performed at 25°C to enhance the fraction of soluble proteins. The small size of the 4 kDa CBP affinity tag allows production of soluble target proteins of a larger size than those that are commonly achieved with glutathione S-transferase (GST, 26 kDa) or maltose-binding protein (MBP, 40kDa) and is also less likely to affect the function of the protein of interest. The CBP-tagged proteins bind to CaM resin in the presence of low concentrations of calcium and elute in the presence of 2mM EGTA at neutral pH. The CBP-fusion is therefore an important alternative to the use of the histidine-fusion that requires harsh elution conditions, that might harm the protein.

The purification procedure was optimized to reach the optimal purity of AtWRNexo proteins. Purified AtWRNexo-p and AtWRNexoE135A-p had a molecular weight 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 (Figure 17).

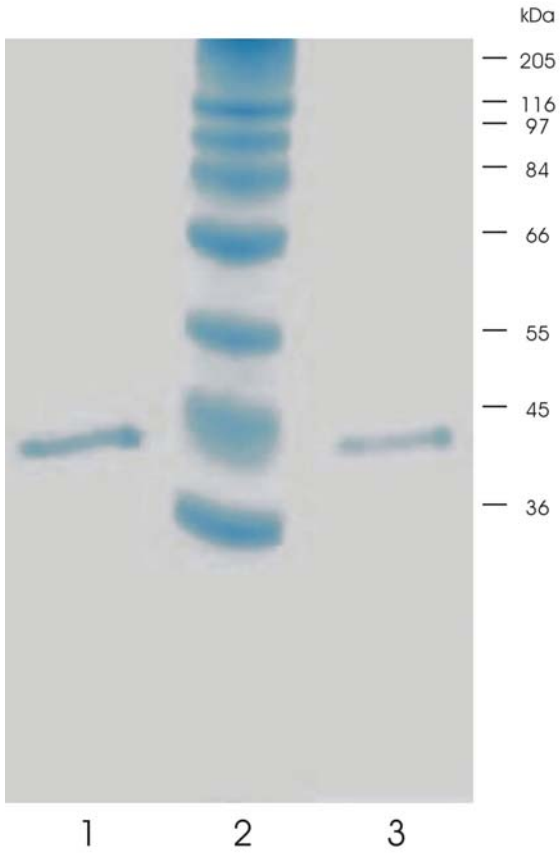



**Fig. 17.** SDS-PAGE analysis of bacterially expressed and purified AtWRNexo-p and AtWRNexoE135A-p. Both proteins, AtWRNexo-p (*lane 1*) and AtWRNexoE135A-p (*lane 3*) were purified by one step calmodulin (CaM) – affinity chromatography, electrophoresed (0.3μg) by 10% SDS-PAGE and stained with Bio-Safe Coomassie blue. *Lane 2* represents molecular markers, positions of molecular size marker proteins are indicated.

## 5.3.4. AtWRNexo-p has 3' → 5' exonuclease activity

To determine whether AtWRNexo-p has an 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, the DNA substrates (200 fmol) with increasing amounts of AtWRNexo-p were incubated. 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. 18, *A* and *B*). It was possible to observe an accumulation of 2 – 6 nt fragments already after 15min 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. 18*B*). Fragments of 3 – 6 nt could be the result of the fact that at this length the DNA duplex may dissociate under the conditions applied yielding non-digestible single strands (see section 5. 3. 5.).

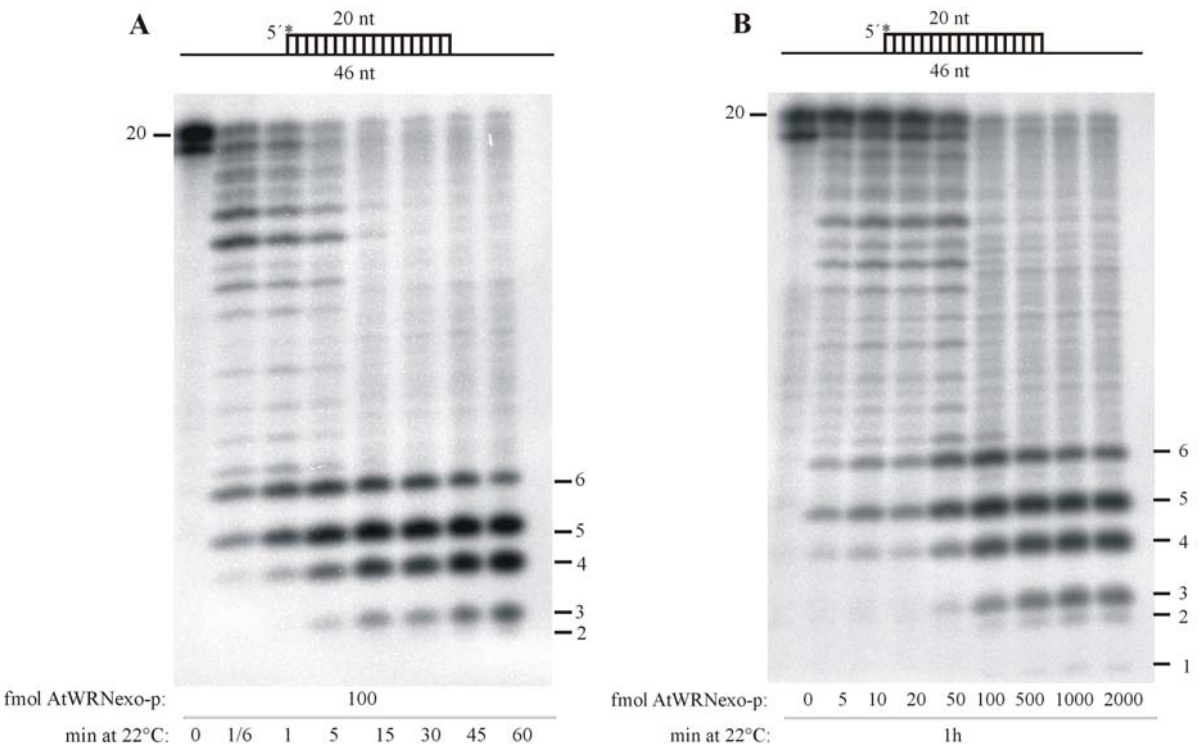



**Fig. 18.** AtWRNexo-p hydrolyzes DNA in 3' → 5' direction. DNA substrates (200 fmol), $^{32}$P-labeled at the 5'-end of 20-mer, were incubated with 100 fmol of AtWRNexo-p for the indicated periods of time (*A*) or with indicated amounts of AtWRNexo-p for 1h at 22°C (*B*). The length of single-stranded DNA present on each side of the duplex region is 13nt. 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.

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. 19, *A* and *B*). 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.

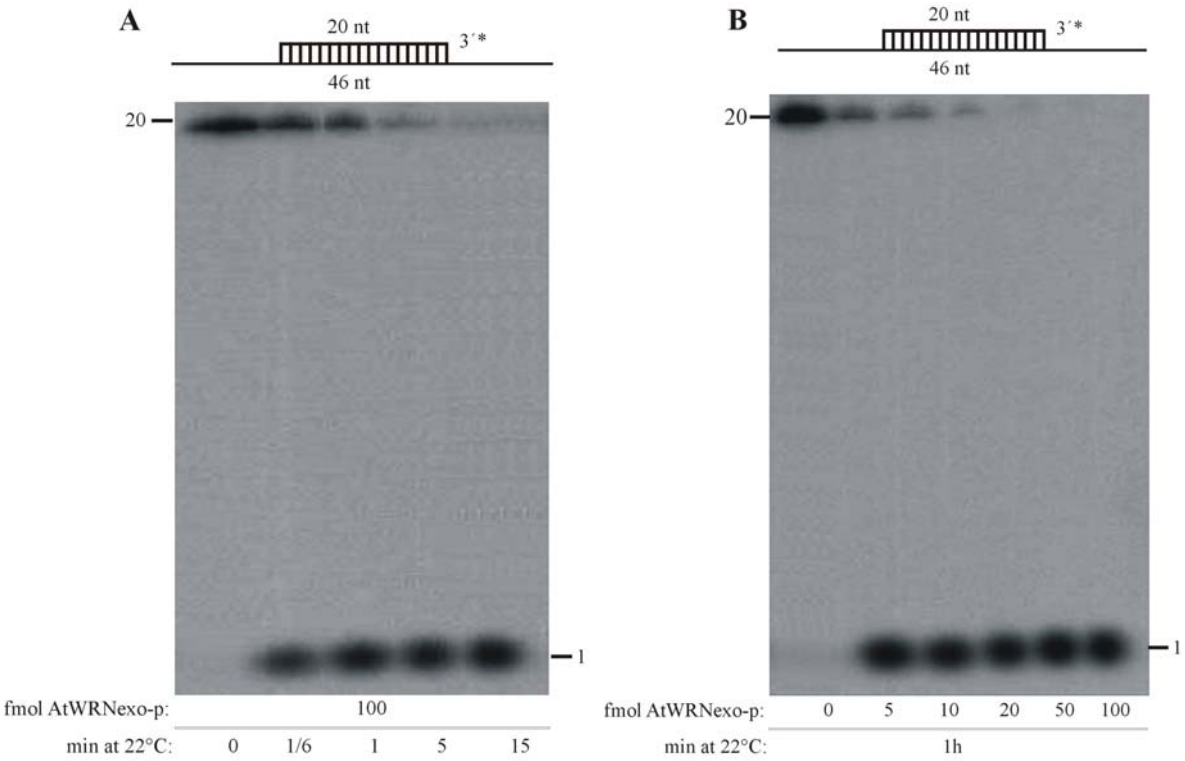



**Fig. 19.** AtWRNexo-p hydrolyzes DNA in 3' → 5' direction. DNA substrates (200 fmol), $^{32}$P-labeled at the 3'-end of 20-mer, were incubated with 100 fmol of AtWRNexo-p for the indicated periods of time (*A*) or with indicated amounts of AtWRNexo-p for 1h at 22°C (*B*). The length of single-stranded DNA present on each side of the duplex region is 13nt. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide – 7 M urea gel. The length of degraded fragment is indicated. It was determined using labeled DNA oligonucleotides of known lengths.

## 5.3.5. Activity of AtWRNexo-p on different DNA substrates

AtWRNexo-p was assayed on different DNA substrates to determine its substrate requirements (Fig. 20). AtWRNexo-p hydrolyzes the recessed strand of DNA duplexes (see

Fig. 18 and 19), as does hWRN-p (Kamath-Loeb et al., 1998). The efficiency of degradation increases, when the reaction is performed at 37°C (data not shown).

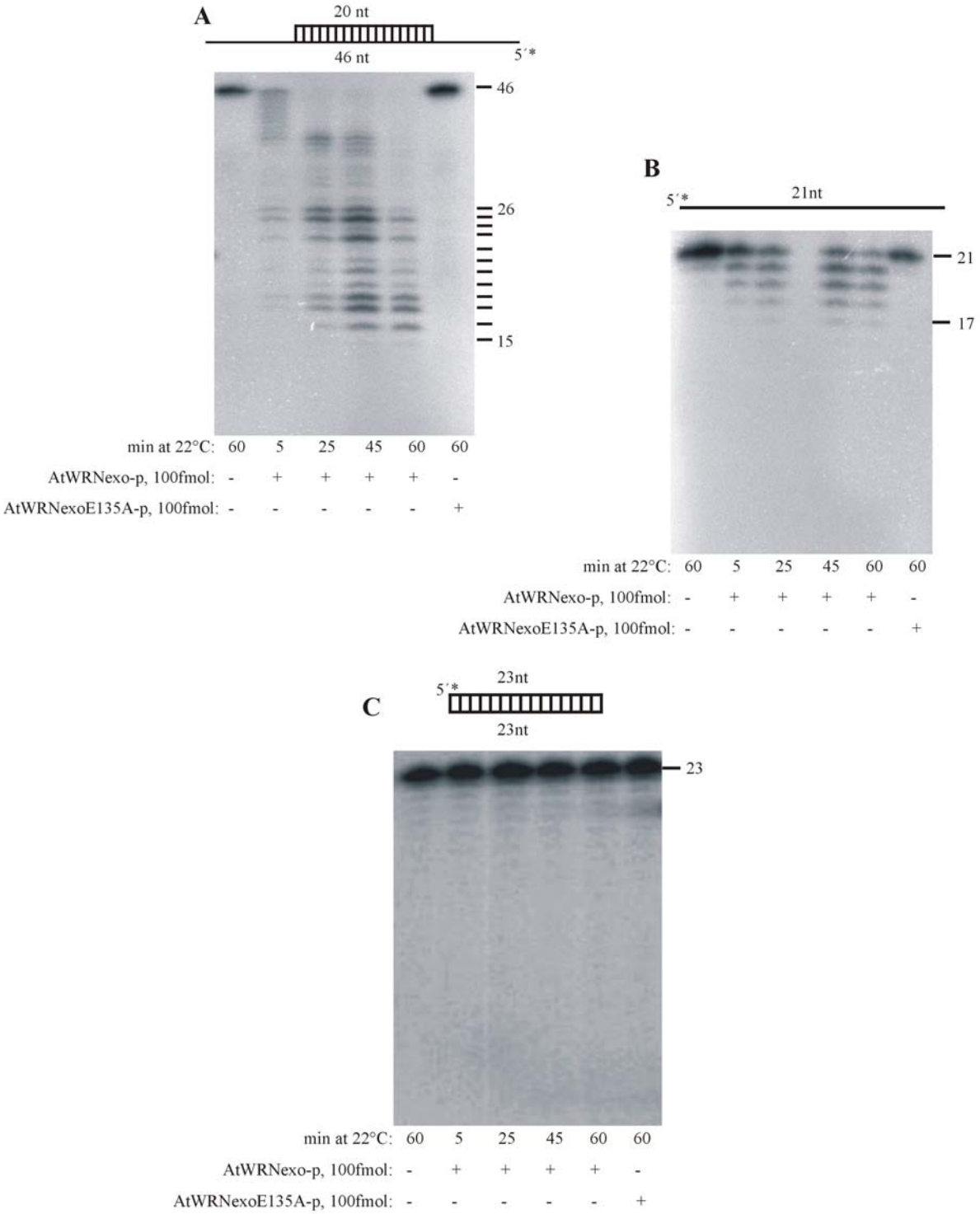



**Fig. 20.** AtWRNexo-p hydrolyzes recessed and protruding strands in DNA duplexes. DNA substrates (200 fmol), were incubated with 100 fmol of either AtWRNexo-p or AtWRNexoE135A-p for the indicated periods of time at 22°C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide – 7M 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.

Notably, AtWRNexo-p is able to degrade also the protruding strand of the same DNA substrate (Fig. 20*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 (Kamath-Loeb et al., 1998).

Similarly to hWRN-p, single-stranded DNA and double-stranded DNA with blunt ends are hardly degraded by AtWRNexo-p (Fig. 20, *B* and *C*). The mutant protein AtWRNexoE135A-p is 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.

### 5.3.6. Requirement of cations and ATP for the reaction catalyzed by AtWRNexo-p

The concentration and nature of divalent cations is influencing the specificity and efficiency of various exonuclease reactions. Therefore the activity of AtWRNexo-p was assayed in the presence of different divalent cations (Fig. 21, see also section 4. 6.). 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 – 16mM $Mg^{2+}$). $Mg^{2+}$ can be partially substituted by $Mn^{2+}$ at lower concentrations (62 – 66% of DNA substrate degraded in the presence of 2 – 6mM $Mn^{2+}$). 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 4mM $Zn^{2+}$). In 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 $Mg^{2+}$.

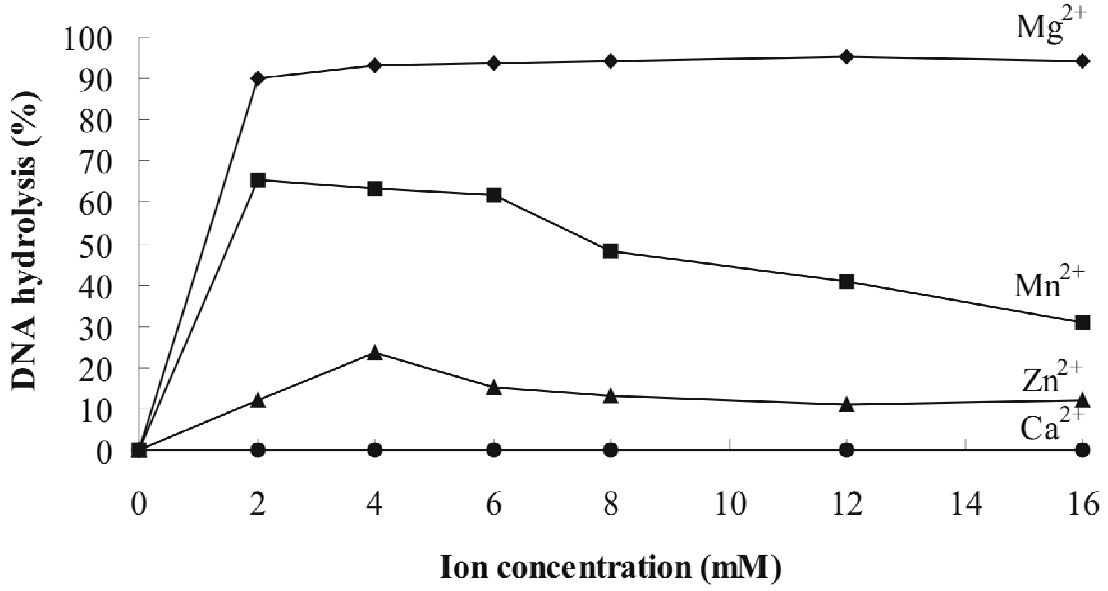



**Fig. 21.** AtWRNexo-p needs $MgCl_2$ or $MnCl_2$ for exonucleolytic digestion. The duplex DNA (oligonucleotides *1 + 3, 200 fmol; see section 3. 4. and and 4. 4.) was incubated with 100 fmol of AtWRNexo-p in the presence of indicated concentrations of $MgCl_2$, $MnCl_2$, $CaCl_2$ or $ZnCl_2$ for 15min at 22°C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide – 7M urea gel and the ratio between full-length oligonucleotide and degradation products was determined by PhosphorImager analysis.

It was reported for hWRN-p that ATP stimulates the WRN exonuclease activity on duplexes with 3'-recessed ends (Kamath-Loeb et al., 1998). ATP is required for structure dependent binding of hWRN-p to DNA, while its hydrolysis allows the exonucleolytic degradation (Shen and Loeb, 2000). 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 (Machwe et al., 2002). When AtWRNexo-p was incubated with duplex DNA with a 3'-recessed end and the influence of ATP and its analogue with increased metabolic stability, ATPγS, on exonuclease activity was tested, no difference in DNA substrate degradation has been observed. Quantification of the ratio between full-length oligonucleotides and degradation products revealed no significant differences. Between 6.5 – 7 % of substrate remained undigested in all cases (Figure 22).

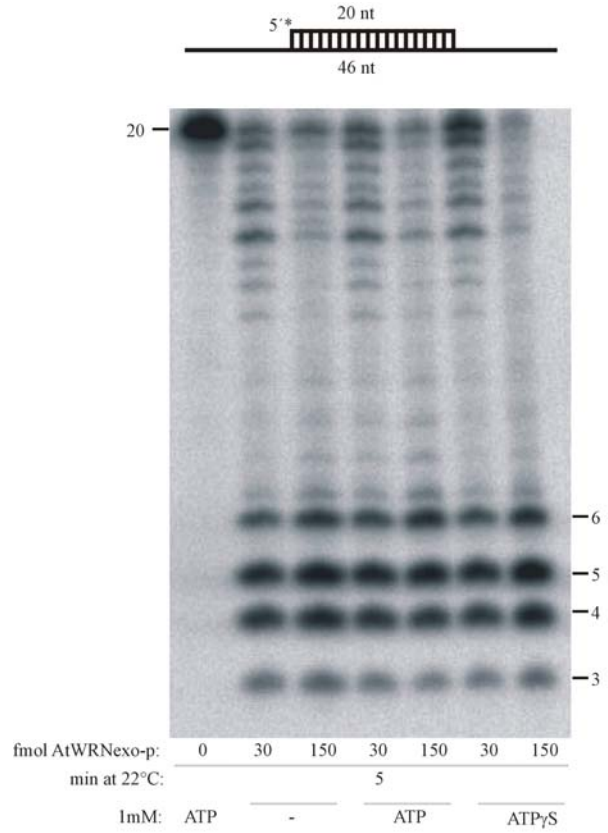



**Fig. 22.** AtWRNexo-p is not stimulated by ATP or ATPγS. The partial duplex DNA (200 fmol) was incubated with indicated amounts of AtWRNexo-p either without ATP or in the presence of 1mM ATP or ATPγS, respectively. Reactions were carried out at 22°C for 5 minutes. Products of DNA hydrolysis were resolved by electrophoresis through a 20% polyacrylamide – 7M urea gel. The length of degraded fragments is indicated. It was determined using labeled DNA oligonucleotides of known lengths.

### 5.3.7. 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 (Machwe et al., 2000). To investigate a possible function of AtWRNexo-p in DNA damage processing and to compare its activities with those of hWRN-p, the influence of several types of DNA modifications on the exonucleolytic

degradation was tested. Double-stranded DNA substrates with a 5'-protruding tail, that contained in the labeled strands modifications, were used. These modifications included helix-distorting lesions (apurinic site and cholesterol adduct), minor base modifications (8-oxoguanine, 8-oxoadenine and ethenoadenine) and unusual bases (uracil and hypoxanthine) (Figure 23). The sequences of oligonucleotides and the positions of respective modifications are shown in section 3.5..

AtWRNexo-p is not inhibited by uracil and hypoxanthine (Fig. 24*A* and Fig. 25*A*). Only weak inhibition at the position 3' to the modification could be observed using the substrate with ethenoadenine (Fig. 25*B*). On the contrary, the exonuclease is strongly inhibited by an apurinic site and completely blocked by a cholesterol adduct (Fig. 25, *A* and *B*). AtWRNexo-p is also inhibited by 8-oxoguanine (8-oxoG) and 8-oxoadenine (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. 24*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.

### 5.3.8. Activity of AtWRNexo-p on modified recessed 3'-DNA ends

For hWRN-p it was reported that its exonuclease, which is homologous to the 3' → 5' proofreading domain of *E. coli* DNA polymerase I (Mian, 1997), preferentially digests the 3'-recessed strand with a single 3'-mismatched nucleotide (Kamath-Loeb et al., 1998), suggesting a possible proofreading activity of hWRN-p during DNA replication. Therefore was AtWRNexo-p tested on DNA substrates containing matched and one or two mismatched nucleotides on the 3'-recessed end (Fig. 26 *A*, *B* and *C).* DNA substrate (200 fmol) was incubated 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

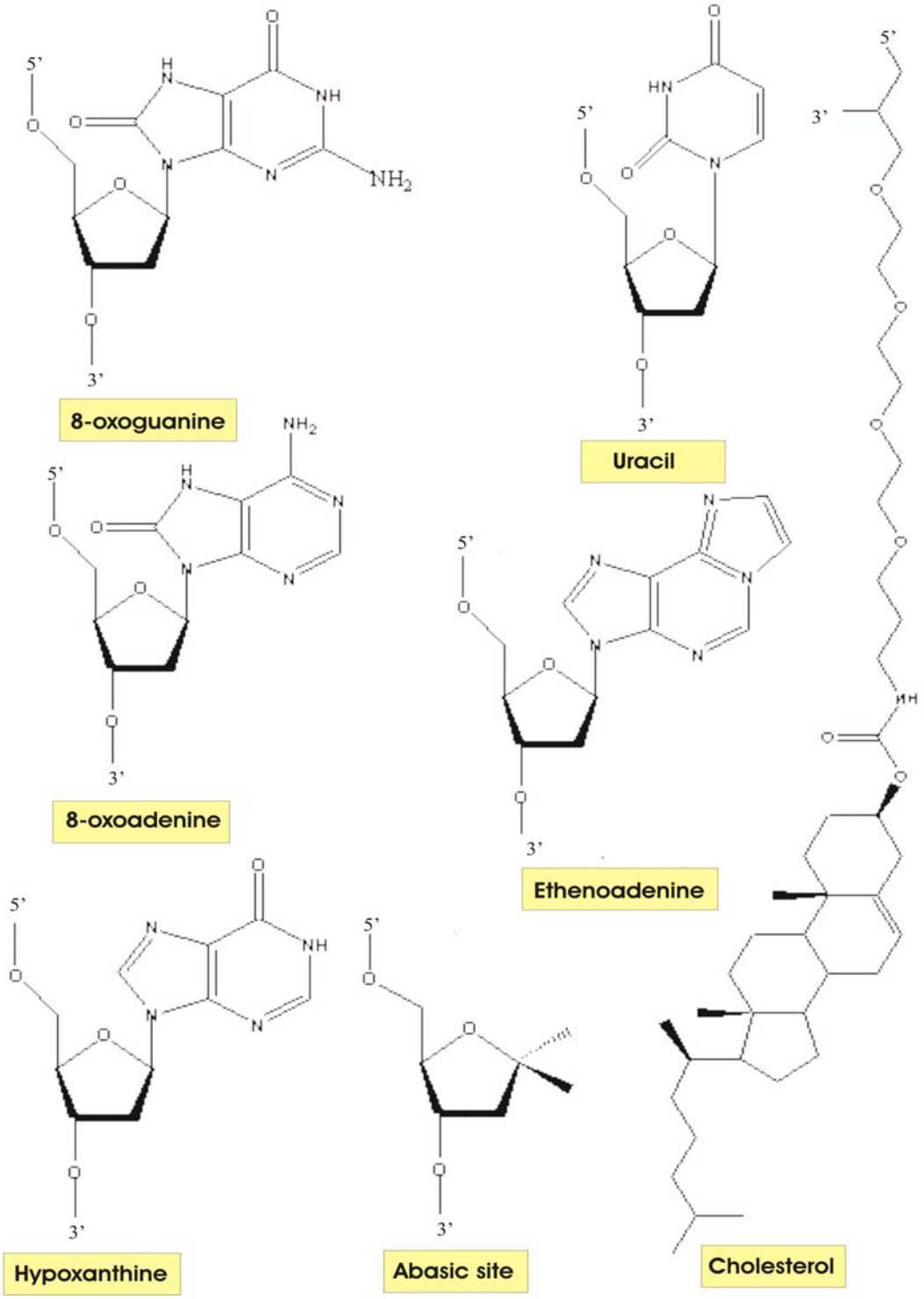



**Fig. 23.** DNA modifications, which were present in DNA substrates used for a determination of the AtWRNexo-p role in DNA repair.

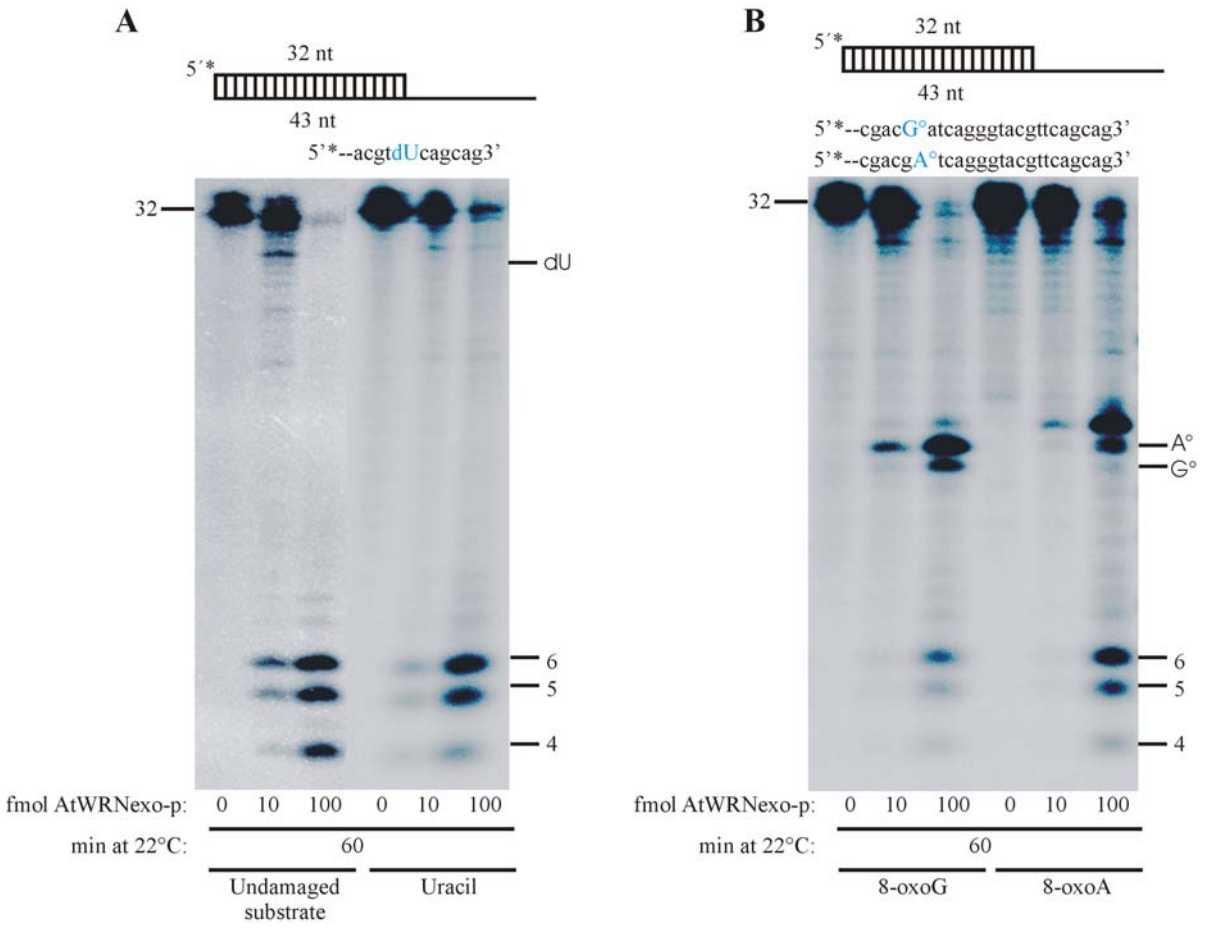



**Fig. 24.** Exonucleolytic activity of AtWRNexo-p is markedly inhibited on substrates containing 8-oxoguanine (8-oxoG), 8-oxoadenine (8-oxoA). The DNA substrates (200 fmol) were incubated with indicated amounts of AtWRNexo-p for 1h at 22°C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide – 7M urea gel. The DNA sequences contain a modified base **[**uracil (*A*), 8-oxoG, 8-oxoA (*B*)**],** its respective position is indicated in blue. The positions of the fragments representing the modifications is shown on the right site of the figure. The length of degraded fragments is indicated. It was determined using labeled DNA oligonucleotides of known lengths.

the first 10sec about a third higher than that of the complementary terminal nucleotide. Further time intervals revealed 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 15min of the reaction only about 60% of the DNA substrate was hydrolyzed (Fig. 27). 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.

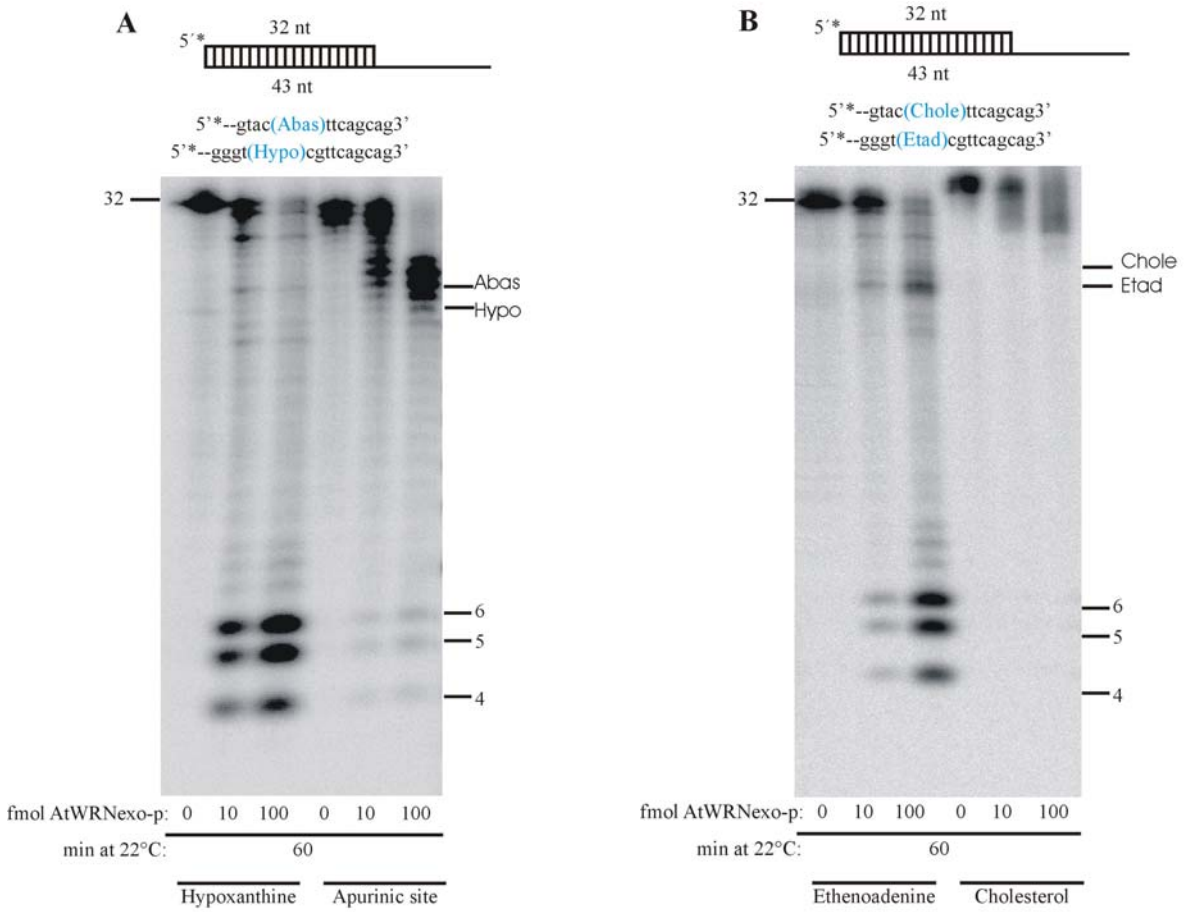



**FIG. 25.** Exonucleolytic activity of AtWRNexo-p is markedly inhibited or blocked on substrates containing apurinic site or cholesterol adduct. The DNA substrates (200 fmol) were incubated with indicated amounts of AtWRNexo-p for 1h at 22°C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide – 7M urea gel. The DNA sequences contain a modified base **[**hypoxanthine, apurinic site (*A*), ethenoadenine and cholesterol adduct (*B*)**],** its respective position is indicated in blue. The positions of the fragments representing the modifications is shown on the right site of the figure. The length of degraded fragments is indicated. It was determined using labeled DNA oligonucleotides of known lengths.

To determine the ability of AtWRNexo-p to start the digestion also from 3'-$PO_4$ termini, the DNA substrate containing a 3'-$PO_4$ recessed strand was incubated with 100 fmol of either AtWRNexo-p or AtWRNexoE135A-p for the indicated periods of time at 22°C (Fig. 28). The hydrolysis of the substrate by AtWRNexo-p is similarly efficient for both types of termini (Fig. 29). The same has been reported for hWRN-p (Kamath-Loeb et al., 1998).

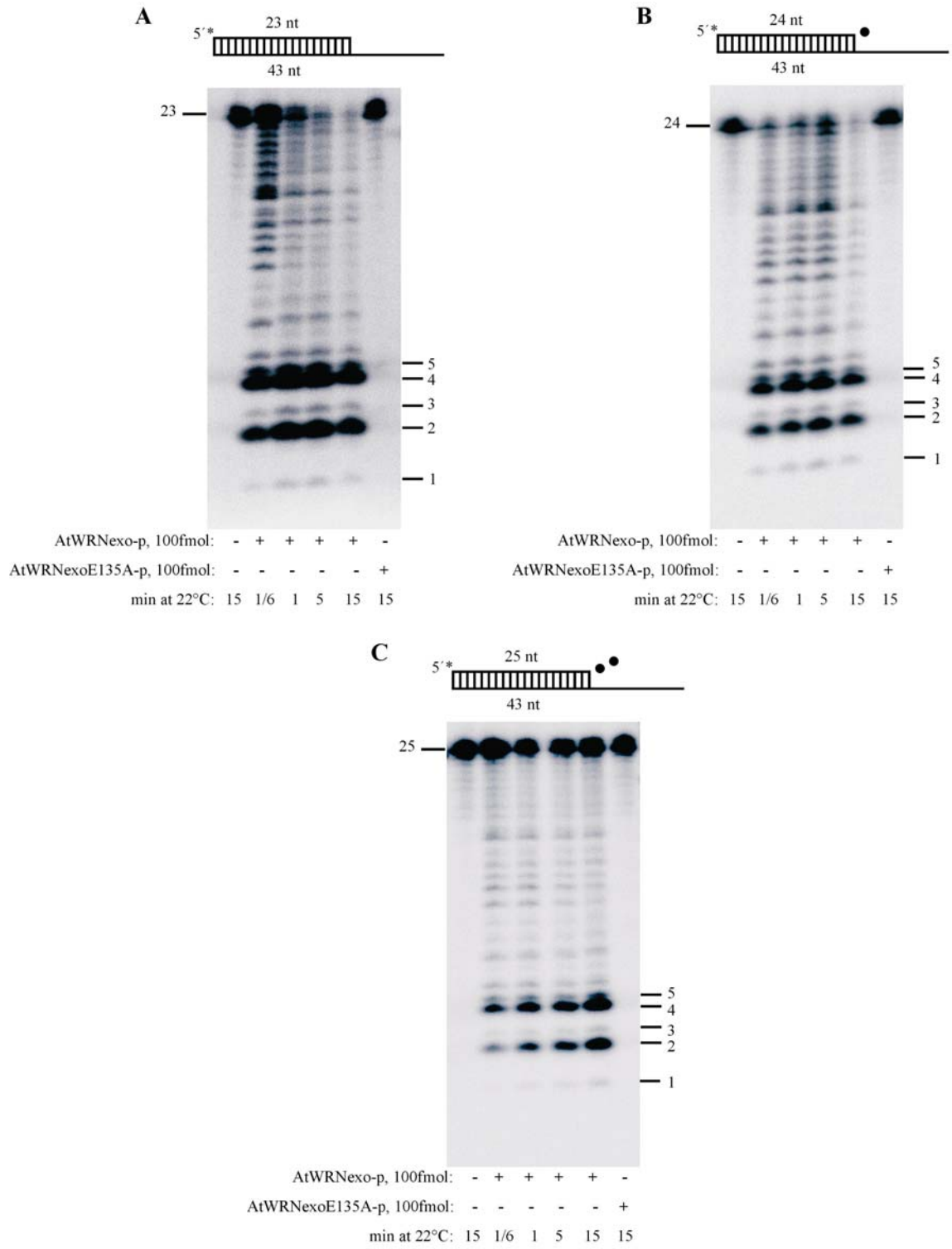



**Fig. 26.** Exonucleolytic activity of AtWRNexo-p on DNA substrates with matched, one or two mismatched nucleotides at the recessed 3'-terminus. The DNA substrates (200 fmol) were incubated with 100 fmol of either AtWRNexo-p or AtWRNexoE135A-p for the indicated periods of time at 22°C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide – 7M urea gel. 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.

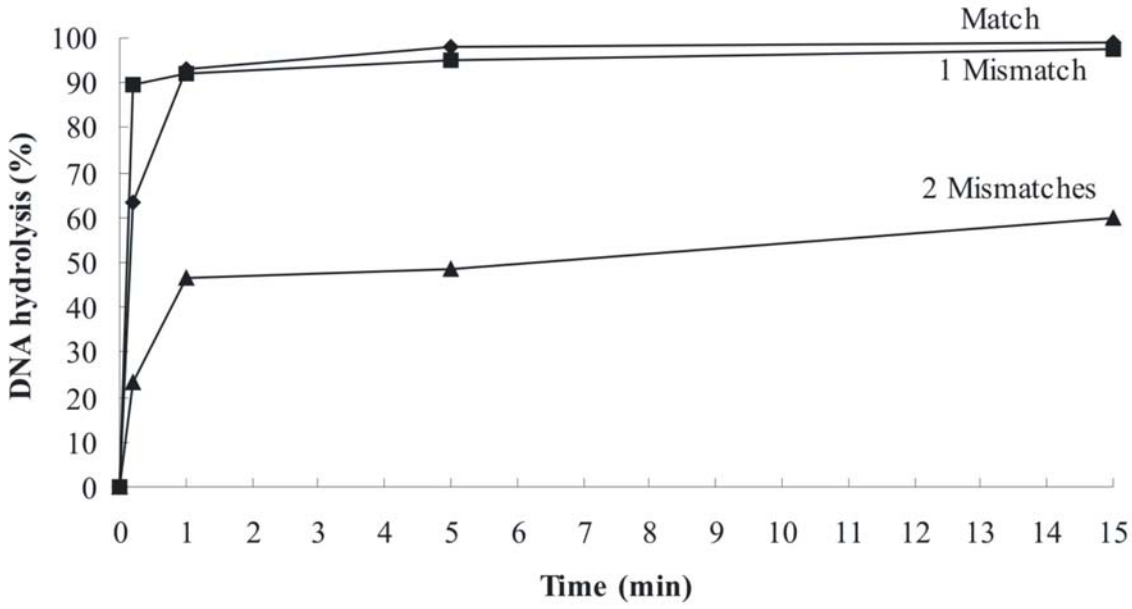



**Fig. 27.** Exonucleolytic activity of AtWRNexo-p on DNA substrates with matched, one or two mismatched nucleotides at the recessed 3'-terminus. The ratio between full-length oligonucleotide and degradation products was determined by PhosphorImager analysis (see also Figure 26).

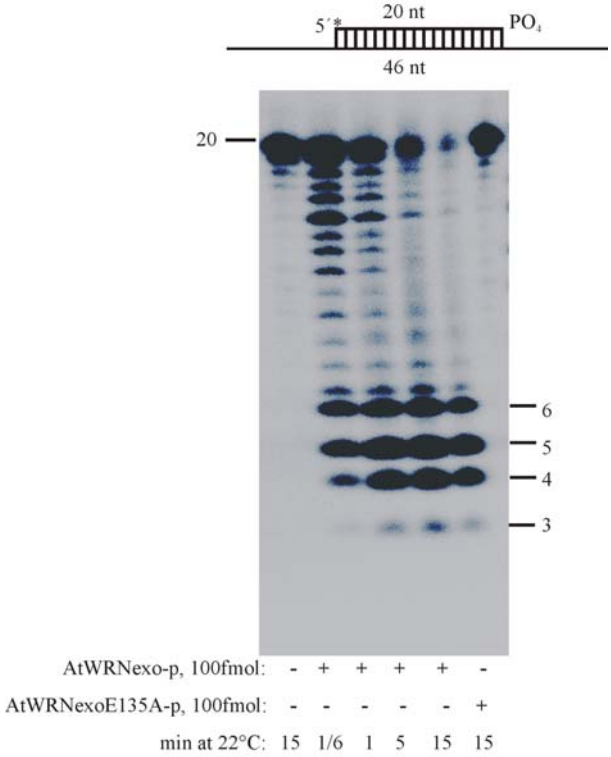



**Fig. 28.** Exonucleolytic activity of AtWRNexo-p on DNA terminating with 3'-$PO_4$ group. The DNA substrate (200 fmol) was incubated with 100 fmol of either AtWRNexo-p or AtWRNexoE135A-p for the indicated periods of time at 22°C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide – 7M urea gel. The length of degraded fragments is indicated. It was determined using labeled DNA oligonucleotides of known lengths.

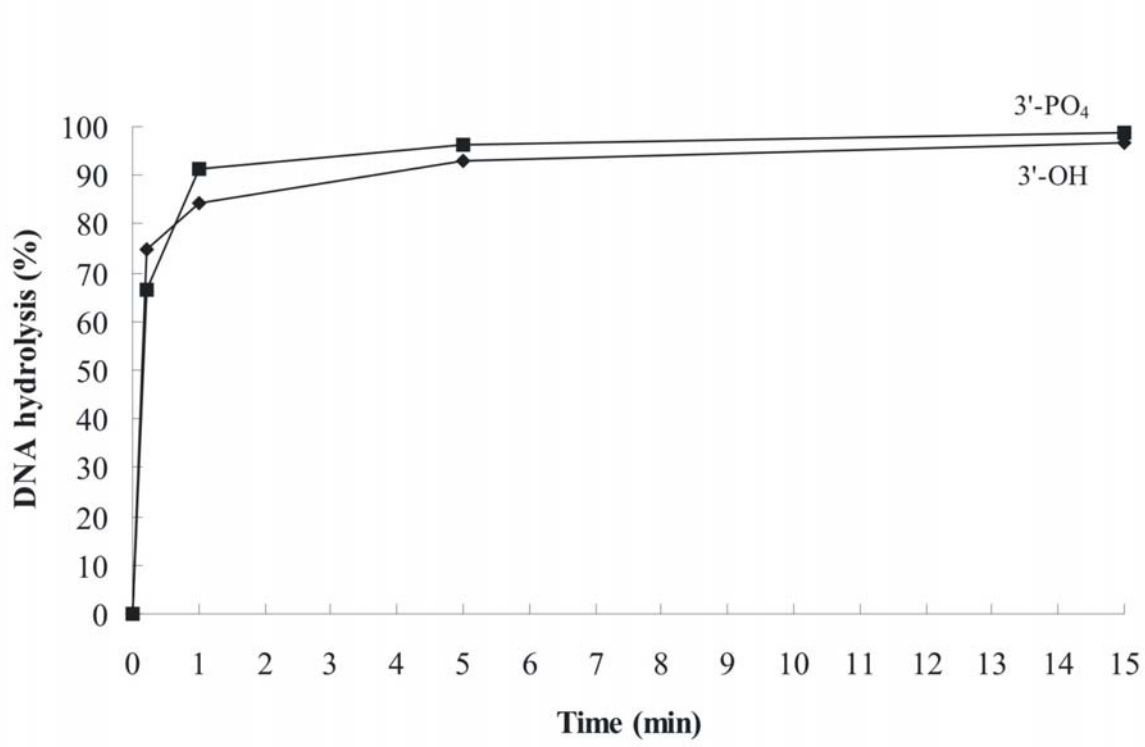



**Fig. 29.** Comparison of the extent of degradation of DNA with 3'-$PO_4$ group and the DNA terminating with 3'-OH (see also Figure 18*A*).

## 5.3.9. Activity of AtWRNexo-p on substrates containing RNA

It has been reported that WRN helicase unwinds RNA/DNA heteroduplexes (Suzuki et al., 1997) and the N-terminal fragments of human and mouse Werner syndrome protein degrade the 3'-recessed DNA strands in DNA/RNA heteroduplexes (Huang et al., 2000). AtWRNexo-p was therefore tested, whether it can also proceed such substrates. The assay revealed that AtWRNexo-p digests the 3'-recessed DNA strand of DNA/RNA heteroduplex in a time dependent manner. On the contrary, the mutant AtWRNexoE135A-p had no activity on this substrate (Figure 30).

Furthermore, because of the similarity of the N-terminal region of Werner syndrome protein to the nuclease domain of bacterial RNaseD (Mian, 1997), AtWRNexo-p was incubated with single-stranded RNA. As shown in the Figure 30, the activity of AtWRNexo-p on single stranded RNA is poor, suggesting that for its degradation a complementary strand might be needed. AtWRNexoE135A-p is not able to digest the RNA, therefore the observed

exonucleolytic activity belongs only to AtWRNexo-p and this purified protein is free of any contaminating single-strand specific RNases.

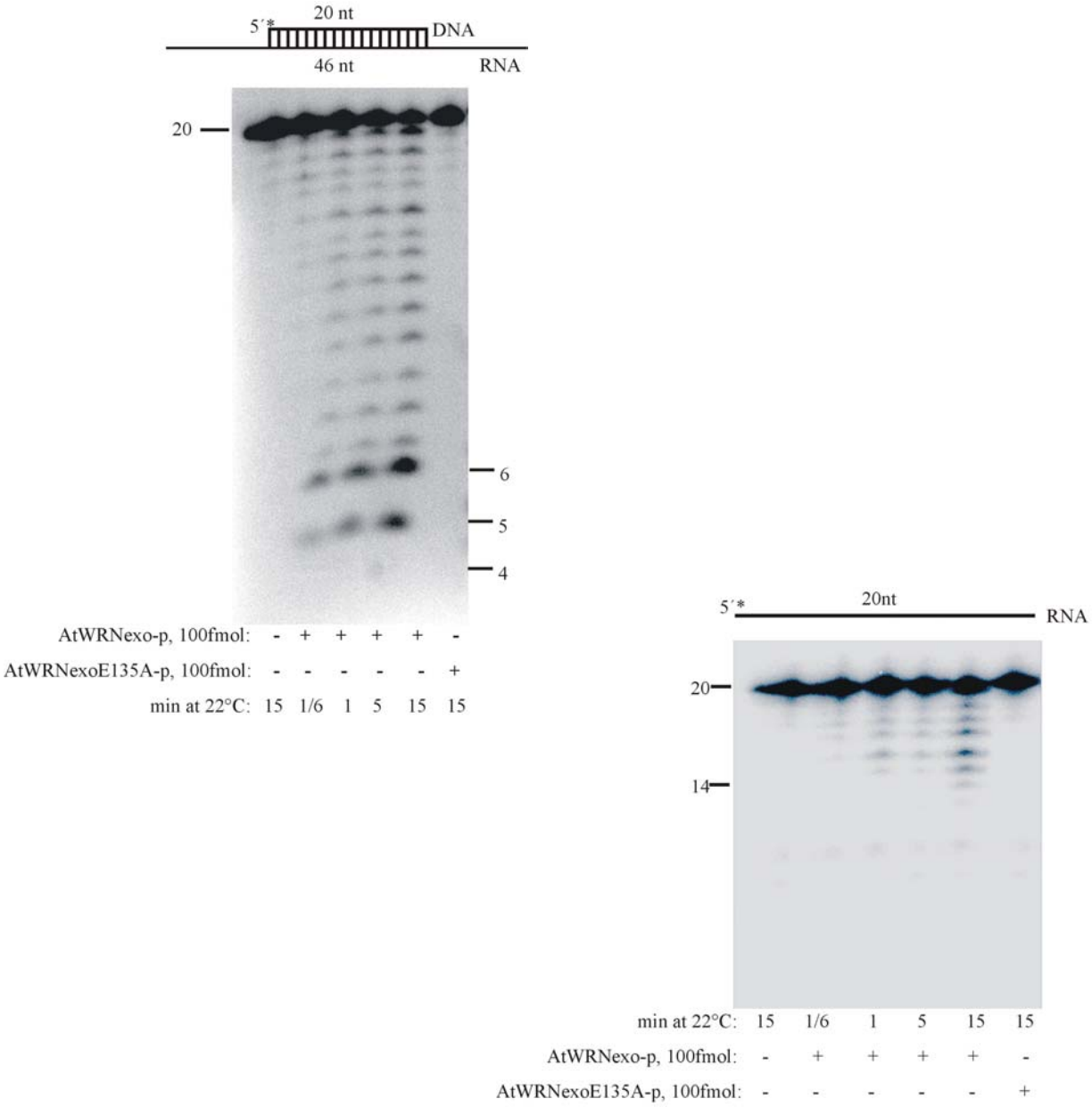



**Fig. 30.** Exonucleolytic activity of AtWRNexo-p on DNA/RNA heteroduplex and single-stranded RNA. The DNA/RNA or RNA substrate (200 fmol) was incubated with 100 fmol of either AtWRNexo-p or AtWRNexoE135A-p for the indicated periods of time at 22°C. Products of DNA hydrolysis were resolved by electrophoresis on a 20% polyacrylamide – 7M urea gel. The length of degraded fragments is indicated. It was determined using labeled DNA oligonucleotides of known lengths.

## 5.4. Identification of interacting partners of AtWRNexo protein

Many eukaryotic transcription factors are composed of physically separable, functionally independent domains – DNA-binding domain (BD) and activation domain (AD). Both are required to activate a transcription of target genes and normally are part of the same protein. When separated from each other, they do not directly interact and thus cannot activate the target genes. However, if they are brought into close physical proximity in the promoter region, the transcription is activated. The genes encoding proteins that potentially interact with each other are cloned into two different vectors to generate hybrid proteins fused either to the DNA binding-domain or to the activation domain. An interaction of tested proteins brings both BD and AD together and the transcription of target genes can occur. This is a principal of the two-hybrid assays, which were used to identify the interacting partners of AtWRNexo protein. We used two different two-hybrid systems in order to detect more protein-protein interactions. It is possible that some types of protein-protein interaction are not detected in a GAL4-based system, but may be detectable in a LexA system and *vice versa*. As the AtWRNexo gene doesn't posses any RecQ-like sequence motifs, the question arises, whether AtWRNexo protein may interact with any of the smaller AtRecQl proteins to form the functional analogue of the human Werner syndrome protein. Therefore at that time, AtRecQl1, AtRecQl2 and AtRecQl3 proteins fused to the DNA binding domain and AtWRNexo protein fused to the activation domain were tested for the possible interaction in GAL4 two-hybrid system. A strong interaction between AtWRNexo protein and AtRecQl2 protein already after 4 h X-gal staining was detected. On the contrary, no interaction between AtWRNexo protein and AtRecQl1 or AtRecQl2 proteins was observed, even when the staining time was prolonged to 12 h (Figure 31).

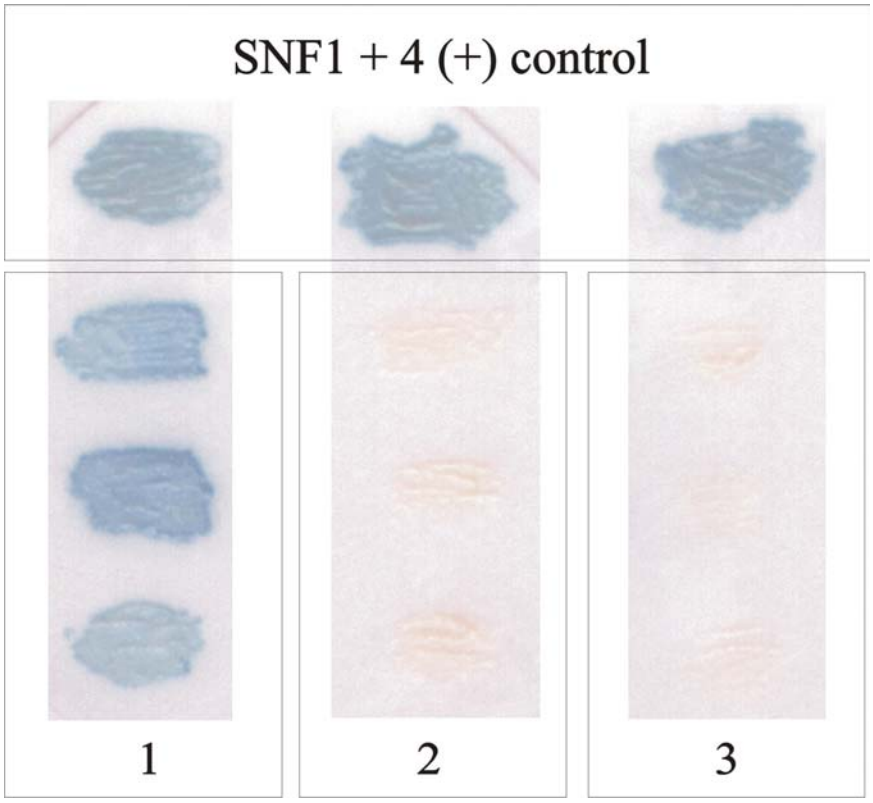



**Fig. 31.** The protein-protein interaction analysis using the GAL4 two-hybrid system. AtWRNexo protein showed a strong interaction with AtRecQl2 protein after 4 h X-gal staining (panel 1). The panels 2 and 3 with analysis of an interaction between AtRecQl1 and AtWRNexo proteins or AtRecQl3 and AtWRNexo proteins were X-gal stained for 12 h without resulting in a positive signal. As a positive control the SNF1 + 4 interaction is shown in the upper part of the figure.

During the time further factors involved in DNA recombination and DNA repair from *Arabidopsis thaliana* were identified in our group. Based on the broad spectrum of human Werner syndrome protein interactions (see section 1.5.), these factors were tested for the possible interactions with AtWRNexo protein using LexA two-hybrid system. Opposite to the GAL4 two-hybrid system, where the hybrid proteins are expressed constitutively, this system allows an inducible expression of fusion proteins and thus to analyze potentially toxic proteins. Proteins, which were tested in this system and the results of the analysis are summarized in Figure 32.

| pGILDA/pB42AD | AtWRNexo |
|---|---|
| AtRecQl1 | - |
| AtRecQl3 | - |
| AtRecQl4a | Self activation of AtRecQl4a |
| AtRecQl5 | Blue colour after 42 h |
| AtRecQsim | - |
| AtWRNexo | Blue colour after 18 h |
| AtKu80 | - |
| AtKu70 | - |
| AtUbc9 | - |
| AtRad51 | - |
| AtMLH1 | Blue colour after 18 h |
| pB42AD/pGILDA | AtWRNexo |
| AtKu80 | Blue colour after 24 h |
| AtUbc9 | Blue colour after 42 h |
| Positive control | p53 |
| SV40 large T-antigen | Blue colour after 18 h |

**Fig. 32.** The protein-protein interactions tested in LexA two-hybrid assay. In the red colour are written the respective vectors, in which the genes encoding the tested protein were cloned. The positive control (written in green) represents the interaction of indicated proteins. No interaction is depicted as "minus". A different degree of a blue colour shows a different strength of interactions. Weaker interactions are represented as a light blue colour, very strong interactions as a very dark blue colour. The time needed for blue colour development is indicated.

A very strong interaction was found between proteins AtWRNexo and AtMLH1 or AtWRNexo. Already after 18 h of incubation with X-gal substrate the yeast colonies turned blue as in the positive control. These two interactions activated also the second reporter gene LEU2 and grew comparable as a positive control on the mediums lacking leucine. A weaker interaction showed proteins AtWRNexo and AtRecQl5. For the colour development 42 h of X-gal staining were needed to obtain blue colour of lower intensity than that by the positive control. A second reporter gene LEU2 was also weaker activated. AtRecQl4a fused to the BD revealed strong self-activation of both reporter genes. The growth and colour development was same as by the positive control. It suggests that AtRecQl4a posseses itself a transcription activation domain. Similarly, the self-activation phenomenon was observed also by AtWRNexo protein, but this self-activation was very weak (slightly blue colour after 48 h of X-gal staining).

Interestingly, further interactions were detected, when the proteins were connected to the opposite fusion than they had before and tested for the interaction. It can be due to the fact, that the fused DNA-binding or activation domain occlude the site of interaction or that the hybrid protein folds improperly. The intensity of the staining indicates that the interaction of proteins AtWRNexo and AtUbc9 is similar to the interaction of AtWRNexo and AtRecQl5. The colour caused by the interaction of AtWRNexo with AtKu80 was visible after 24 h indicating that this interaction is stronger than that between AtWRNexo and AtRecQl5 or AtUbc9 proteins, but weaker than between AtWRNexo and AtWRNexo or AtMLH1 proteins. That the proteins interact was also supported by an activation of the second reporter gene LEU2, which correlated with colour development.

# 6. Discussion

## 6.1. Comparison of the exonucleolytic activities of AtWRNexo-p and hWRN-p

The seven RecQ homologues were identified in *Arabidopsis thaliana*. One RecQ homologue differs from the others by another arrangement of the helicase domain (Figure 12). Additionally to the usual RecQ domains, other domains in some AtRecQ proteins have been identified (Bagherieh-Najjar et al., 2003). Instead of the complete homologue of WRN protein a small ORF (AtWRNexo) with striking similarity to only the exonuclease domain of the human Werner syndrome protein is present in the *Arabidopsis* genome (Hartung et al., 2000). To investigate whether it encodes the functional exonuclease and to determine its substrate requirements, this ORF was cloned into the expression vector and the protein was produced. The product of the ORF possesses indeed exonuclease activity (see Figure 18). 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 a 3' → 5' exonuclease activity cleaving 3'-recessed ends of partial dsDNA molecules. AtWRNexo-p additionally digests also protruding strands of duplexes. Single-stranded DNA and double-stranded DNA with blunt ends are poor substrates for both enzymes (Plchova et al., in press and Kamath-Loeb, 1998; Huang et al, 2000; Li and Comai, 2000). 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 (Huang et al, 2000; Li and Comai, 2001) 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 (Kamath-Loeb et al, 1998), no 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 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 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 (Shen and Loeb, 2000).

As for hWRN-p, cholesterol adduct completely blocks AtWRNexo-p and uracil, hypoxanthine and ethenoadenine allow AtWRNexo-p to digest through the regions containing these modifications (Machwe et al., 2000). On the contrary 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 at high concentrations of the exonuclease such damaged nucleotides are removed. This is not the case for hWRN-p (Machwe et al., 2000).

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 but DNA containing two mismatched nucleotides on the 3'-recessed strand is poorly degraded (Kamath-Loeb et al., 1998) 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 connected to the absence of the helicase domain in AtWRNexo-p, because another report 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 tested only the N-terminal part of hWRN-p (Huang et al., 2000).

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. As hWRN-p (Kamath-Loeb et al., 1998), AtWRNexo-p is able to hydrolyze both kinds of substrates efficiently.

Both AtWRNexo-p and hWRN-p (Huang et al., 2000) degrade the DNA/RNA hybrids. The hydrolysis of single-stranded RNA catalyzed by AtWRNexo-p is similarly weak as the degradation of single-stranded DNA.

The summary of AtWRNexo-p and hWRN-p exonucleolytic activities is shown in Figure 33.

## 6.2. What could be the reason(s) for the different activities of AtWRNexo-p and hWRN-p?

In general, the experiments show that certain DNA structures are better substrates for AtWRNexo-p 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 either due to experimental limitations of this approach or might indeed reflect a meaningful biological difference. Although the formed explanation cannot be totally excluded, the latter explanation is more favourable.

One has to stress that a 27 aa N-terminal fusion of the AtWRNexo ORF to the calmodulin-binding peptide (CBP) required for protein purification was used in these experiments. The attempts to eliminate this fusion by peptidase digest were always coupled with the precipitation of the enzyme and therefore to perform experiments with a protein without the respective tag was not possible. 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 (Kamath-Loeb et al., 1998; Machwe et al., 2000). In contrast to the studies with hWRN-p the characterization of the exonuclease activity of AtWRNexo-p was performed at 22°C. This is the optimal temperature

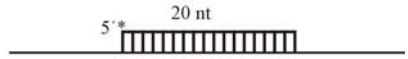
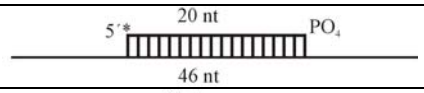
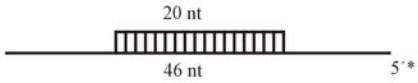
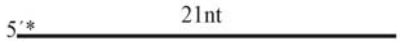
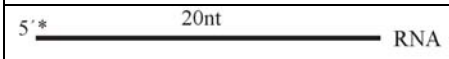
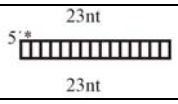
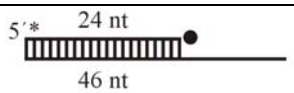
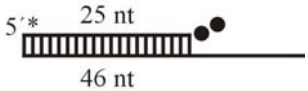
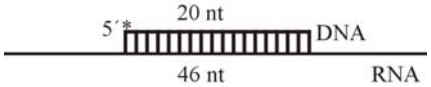
| Substrate | | AtWRNexo-p | hWRN-p |
|---|---|---|---|
|  | ATP<br>ATPγS<br>$MgCl_2$<br>$MnCl_2$<br>$ZnCl_2$<br>$CaCl_2$ | -<br>-<br>+++<br>++<br>+<br>- | - (a)<br>- (a)<br>+++<br>++<br>-<br>- |
|  | | + | + |
|  | | + | - |
|  | | Weak activity | - |
|  | | Weak activity | Not tested |
|  | | - | - |
|  | Undamaged<br>8-oxoG<br>8-oxoA<br>Uracil<br>Abasic site<br>Hypoxanthine<br>Ethenoadenine<br>Cholesterol | +<br>Inhibited/+<br>Inhibited/+<br>+<br>Inhibited<br>+<br>+<br>Blocked | +<br>Blocked/Blocked<br>Blocked/Blocked<br>+<br>Blocked<br>+<br>+<br>Blocked |
|  | | = Match | Match < |
|  | | + | Weak activity |
|  | | + | + |

**Fig. 33.** Comparison of AtWRNexo-p and hWRN-p exonuclease activities. In the second column the conditions, at which the substrates were tested, or peculiarities of the substrates are described. "(a)" indicates, that although the WRN exonuclease doesn't need ATP or ATPγS ("-"), is stimulated by these compounds. The activity in the presence of different ions is depicted as different number of "+" (the highest activity is marked by "+++"). The description of activities on substrate carrying the damages on the left side means the activity

at low protein concentration, on the right side with higher protein concentration. The fields concerning one mismatch show the activity relative to the fully matched nucleotide.

for growth of *Arabidopsis thaliana*.

This small protein may interact with other factors of the repair and recombination machinery in plants and in course of these interactions also 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. The interaction of AtWRNexo protein with AtRecQl2 and AtRecQl5 may indicate that activities that reside in a single protein chain in mammals might in plants be complemented *in trans***.** Via EST search, homologues of AtWRNexo in *Medicago sativa*, *Oryza sativa*, *Lotus japonicus* and *Glycine max* were found. Thus, this gene is common at least for higher plants. One is therefore tempted to speculate that during eukaryotic genome evolution both genes were fused together to generate a single ORF. As such a gene is present in *Xenopus laevis*, *Mus musculus* and *Homo sapiens*, the fusion remained stable during the animal evolution (Figure 34).

The interaction of AtWRNexo protein with itself is in accordance with a finding that N-terminal fragment of human WRN exonuclease forms both trimer or hexamer (Huang et al., 2000; Xue et al., 2002). The hWRN-p interacts with Ku70/80 (Li and Comai, 2000, 2001; Karmakar et al., 2002b; see also section 1.5.3.3.). The observation that AtWRNexo protein interacts with AtKu80 indicates that AtWRNexo may function together with AtKu80 in the same pathway as hWRN-p does. Additional observation that AtWRNexo interacts with Ubc9 is again in agreement with that what was already reported for hWRN-p (see section 1.5.4.2).

An interaction between hWRN-p and MLH1, a protein required in mismatch repair, has not been reported. However, AtWRNexo strongly interacts with AtMLH1, suggesting a possible role in the mismatch repair pathway.

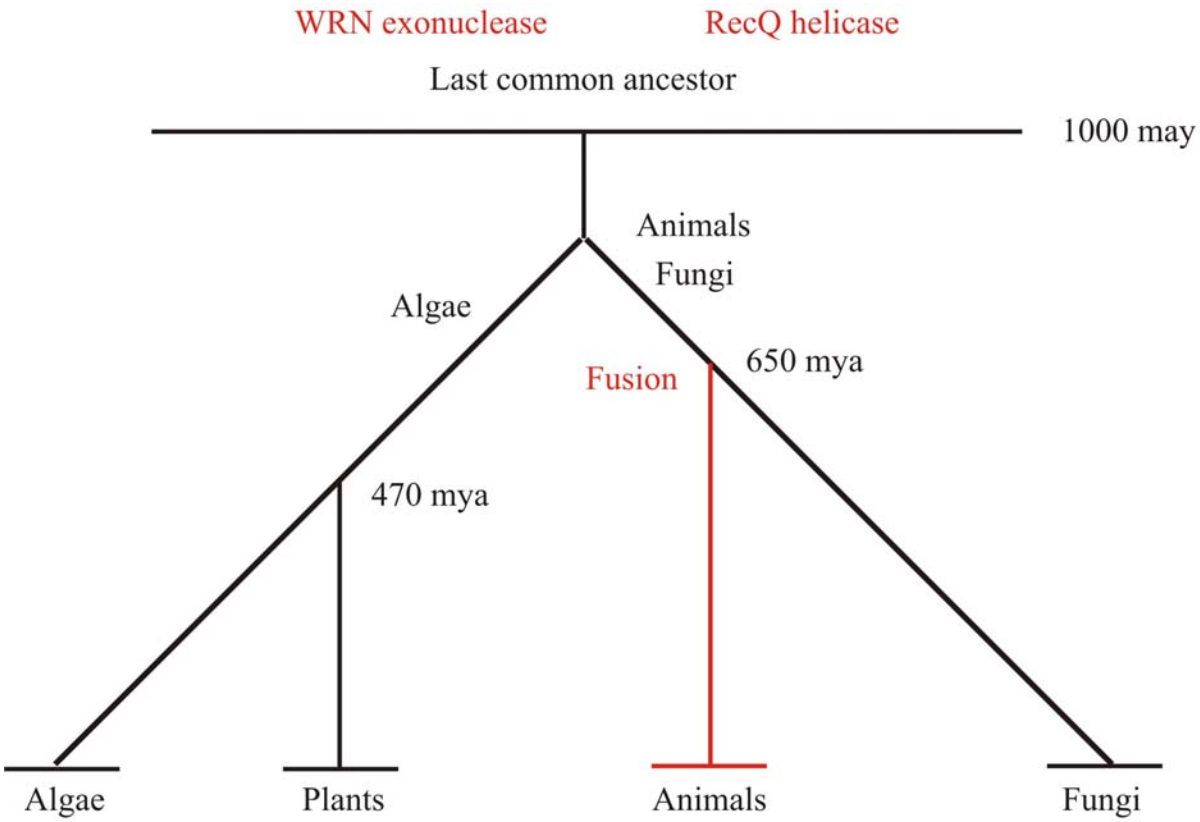



**Fig. 34.** Putative fusion event of WRN exonuclease and RecQ helicase during the animal evolution. The fusion (in red) most probably happened 650 million years ago (mya), when animals and fungi splitted from each other.

## 6.3. Possible roles of hWRN-p in nucleic acid 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.

### 6.3.1. Role in DNA replication

hWRN-p is able to unwind intermediates in DNA replication and repair processes (Brosh et al., 2002a), interacts with the human 5' flap endonuclease/5'→3' exonuclease (FEN-1) and stimulates the cleavage activity of the latter (Brosh et al., 2001c, Brosh et al., 2002b). This interaction implicates hWRN-p in the processing of Okazaki fragments during lagging strand

synthesis. 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 (polδ), proliferating cell nuclear antigen (PCNA) or replication protein A (RPA) (Kamath-Loeb et al., 2001; Kamath-Loeb et al., 2000; Szekely et al., 2000; Lebel et al., 1999; Brosh et al., 1999). The interaction of WRN helicase with RPA allows the helicase to unwind long DNA duplexes up to nearly 1000 bp. *Xenopus laevis* FFA-1 protein which is 66% homologous to hWRN-p, is involved together with RPA in replication foci formation (Chen et al., 2001). The hWRN-p exonuclease is also active on the DNA substrates having on the 3'-recessed strand mismatched nucleotides (Kamath-Loeb et al., 1998; Huang et al., 2000). It can be envisaged that misincorporated nucleotides can be removed in manner of a 3' → 5' proofreading exonuclease (Shevelev and Hübscher, 2002). DNA secondary structures, such as hairpins or G-quadruplexes, might impede a progression of a replication fork. WRN helicase activity enables polδ to overcome these secondary structures of the $d(CGG)_n$ trinucleotide repeat sequence (Kamath-Loeb et al., 2001). A schematic model of such structures is shown in Figure 35.

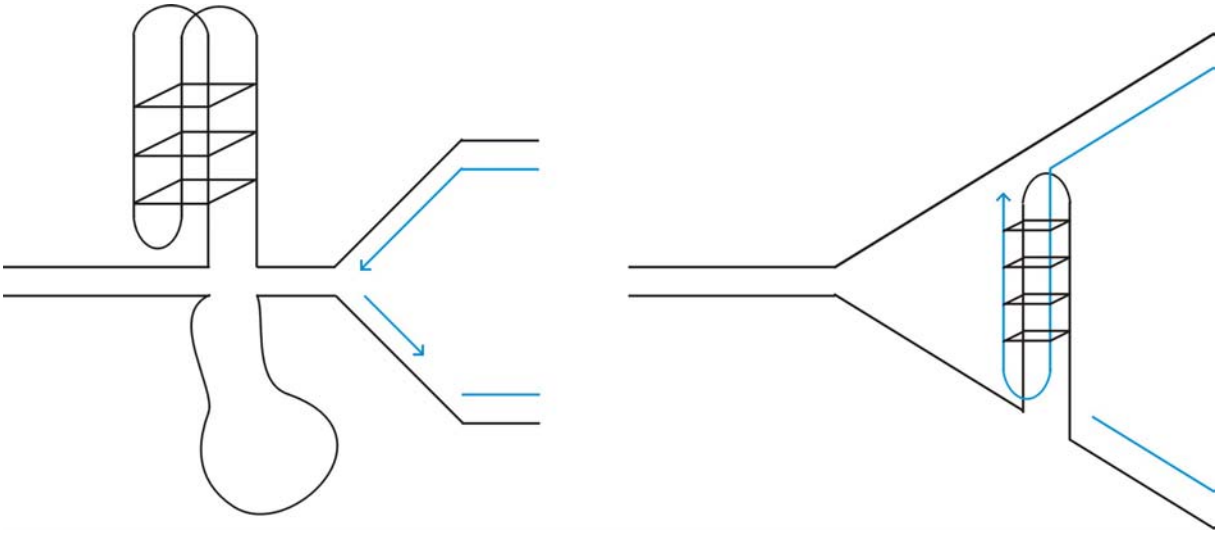


**Fig. 35.** Examples of DNA replication blocks. G4-DNA structure in the leading-strand template (*left*) and G4-DNA-stabilized junction (*right*).

The WRN helicase unwinds G4-DNA structure (Fry and Loeb, 1999; Mohagheg et al., 2001). Potential role of this helicase in defending genome integrity during DNA replication is shown in the Figure 36.

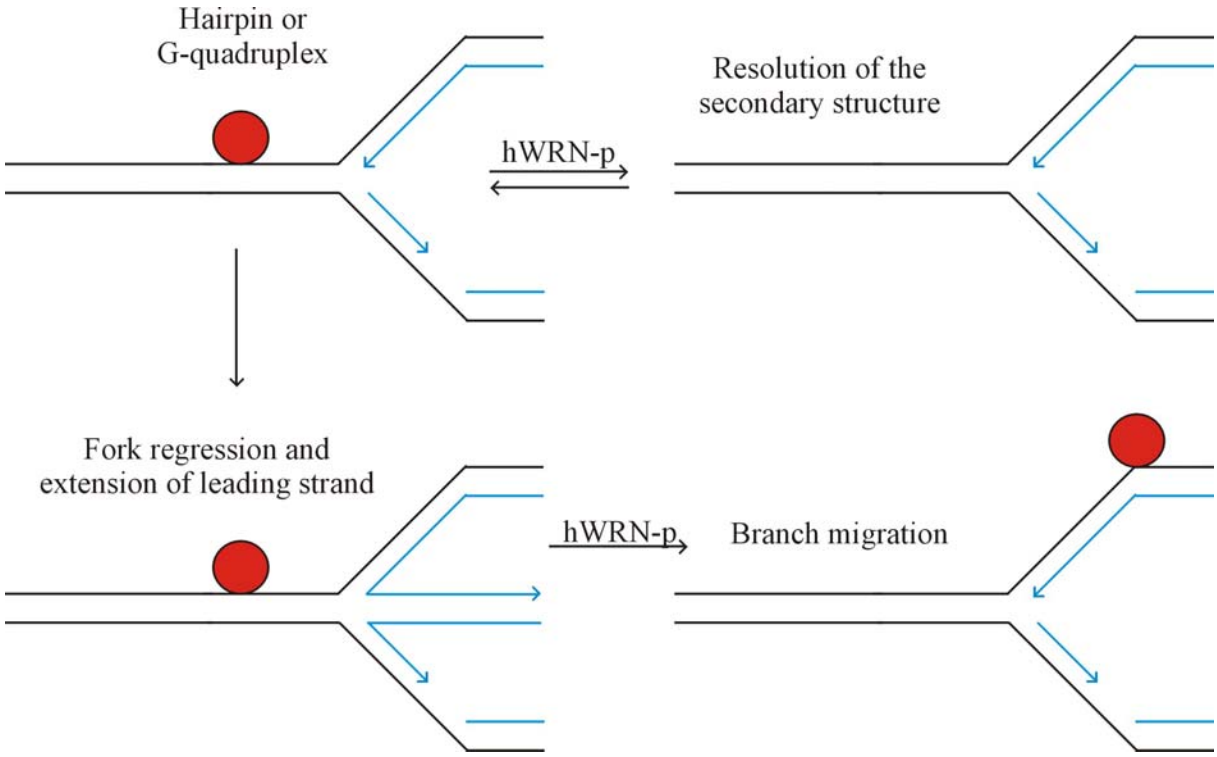



**Fig. 36.** Possible role for the Werner syndrome protein in DNA replication. During DNA replication structures may arise that impede fork progression (red circle). Such structures might be directly processed by hWRN-p (upper part of the figure). If a fork encounters already the blocking lesion, the new synthesized strands might anneal to form a four-way junction. The leading strand could be extended using the longer lagging strand as a template. Branch migration catalyzed by hWRN-p might then extend the leading strand beyond the lesion to restore the replication fork (lower part of the figure).

## 6.3.2. Role in DNA repair

hWRN-p may also function in the repair of double-strand breaks by non-homologous end joining (NHEJ), because it was shown to interact with Ku70/Ku80 (Cooper et al, 2000, Karmakar et al, 2002b). Moreover, recent studies revealed an interaction with DNA-$PK_{cs}$ which forms together with Ku70/Ku80 a complex involved in DSB repair. Displacement of DNA-$PK_{cs}$ from DNA was achieved by addition of hWRN-p to a Ku:DNA-PKcs:DNA complex, so that the DNA-$PK_{cs}$ could no longer protect DNA ends from the exonucleolytic

action of hWRN-p (Li and Comai, 2002). Ku70/Ku80 has been found to stimulate the exonucleolytic activity of hWRN-p and recruit hWRN-p to DNA (Li and Comai, 2001, Karmakar et al., 2002b). 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 (Vogel et al, 1999).

A role for the hWRN-p helicase in mitotic recombination has been suggested. The WS fibroblast cell lines showed a reduction in cell proliferation following mitotic recombination. The spontaneous mitotic recombination events in human somatic cells were measured using two different chromosomally integrated mitotic recombination reporter plasmids. The initiation of mitotic recombination is comparable in both wild type and WS cells, but WS cells offen fails to resolve recombinant products (Prince et al., 2001). The defective recombination resolution is also connected to the Rad51-dependent homologous recombination pathway. Rad51 plays a central role in recombinational repair and together with RPA coats and stabilizes single-strand DNA for a strand exchange activity. Rad51 promotes the formation of recombination products, such as Holliday junctions, which could be substrates for hWRN-p. A failure to resolve these products may lead to mitotic arrest, cell death or genetic instability. The suppression of mitotic recombination using a dominant-negative Rad51 protein led to a higher rate of surviving WS cells following DNA damage (Saintigny et al., 2002).

### 6.3.3. Role in telomere maintenance

There is also some evidence, that the hWRN protein might participate in telomere maintenance. The hWRN protein was shown to unwind large telomere repeat DNA complex (TRDC) in the presence of replication protein A (Ohsugi et al., 2000). Another report describes unwinding of tetrahelical structures of guanine-rich sequences by Werner syndrome

helicase (Fry and Loeb, 1999). Helicase/exonuclease activities disrupt and degrade D-loops *in vitro* (Orren et al., 2002). D-loop structures occur in telomeric regions (Greider, 1999; Griffith et al., 1999; Murti and Prescott, 1999) and are also 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. Although WS fibroblasts demonstrate a faster rate of telomere shortening, the senescent WS cells reveal longer telomeres compared with those of the senescent controls (Schulz et al., 1996). Recent reports revealed that the replicative senescence is most probably connected to an alteration in telomere structure rather than to a telomere length (Karlseder et al., 2002). It was also shown that the yeast WRN homologue Sgs1 is involved in telomere lengthening by a telomerase – independent pathway (Huang et al., 2001; Johnson et al., 2001; Cohen and Sinclair, 2001). Recent studies described the stimulation of Werner helicase activities by the telomere-binding protein TRF2 (Opresko et al., 2002). The Werner syndrome protein interactions with several proteins that have been found to localize to telomeres suggest that hWRN-p may function in a maintenance of telomeres. Ku interacts with the telomere binding proteins TRF1 and TRF2 (Song et al., 2000; Hsu et al., 2000). Ku86 also prevents the fusion of telomeric ends (Samper et al., 2000). Furthermore, DNA-$PK_{cs}$ and Mre11-Rad50-Nbs1 complex required for double-strand break repair localize to telomeres too (Gilley et al., 2001; Zhu et al., 2000). The schematic structure, how hWRN-p may be involved in a telomere maintenance is shown in Figure 37.

### 6.3.4. Role in base excision repair (BER)

The Werner syndrome protein may also participate in long-patch base excision repair (LP-BER). This pathway remove damaged bases with a help of different proteins (Wilson and Thompson, 1997). The repair pathway initiates a DNA glycosylase that recognizes and removes a damage base. An abasic intermediate is subsequently cleaved by an apurinic/apyrimidinic (AP) endonuclease to generate a nick with a 3'-OH and 5'-deoxyribose

phosphate (dRP). In short patch pathway, the polymerase β incorporates a single nucleotide and a nick is sealed by DNA ligase I. When dRP is modified, polymerase replaces approximately 7 nucleotides. The displaced strand is then removed by FEN-1, which is stimulated by PCNA. A nick is again sealed by DNA ligase I. In this pathway, hWRN-p stimulates the displacement activity of polymerase β in a long-patch BER (Harrigan et al., 2003). Moreover, hWRN-p interacts with FEN-1, PCNA, polymerase δ and RPA. It is therefore very likely, that the Werner syndrome protein participates in LP-BER (Figure 38).

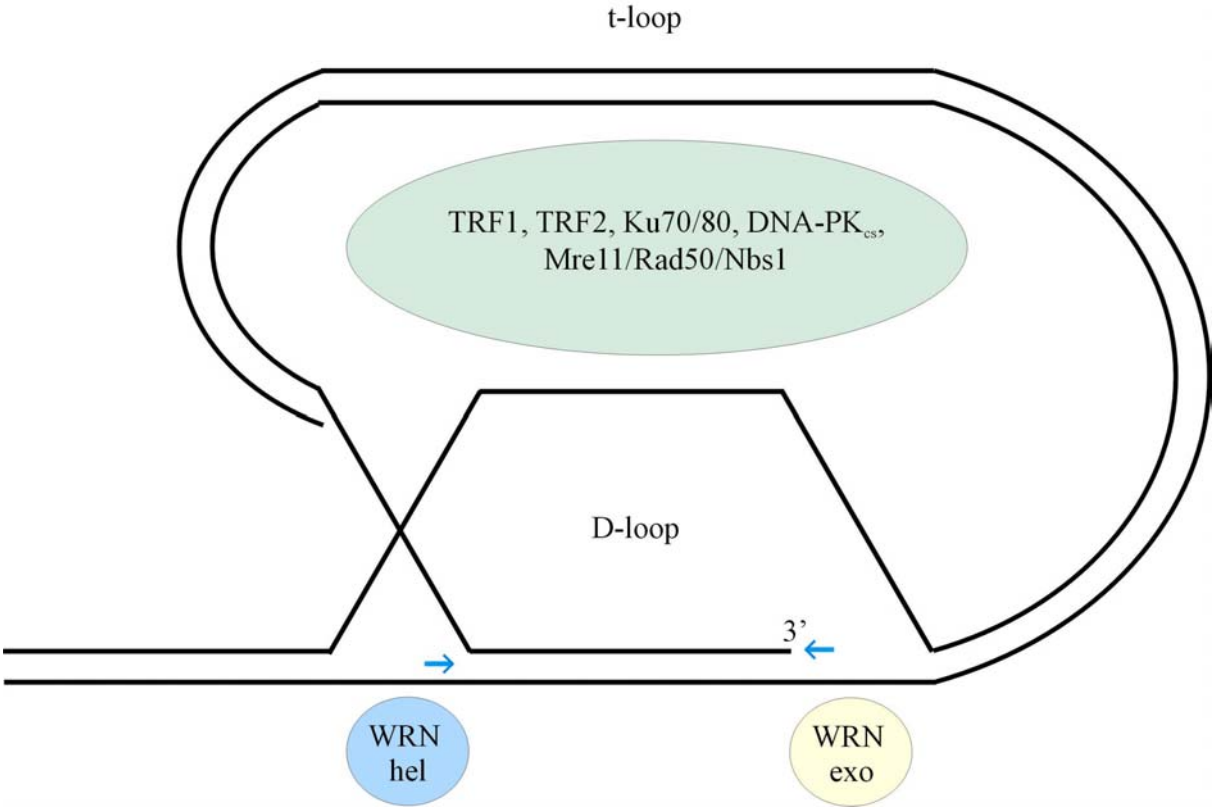



**Fig. 37.** The structure of telomeric ends. hWRN-p may process the invading strand either by the helicase or the exonuclease activity. The direction of unwinding and exonucleolytic degradation is marked by blue arrows. Other proteins that associate with telomeric sequences and are necessary for formation and stabilization of t-loops are presented in the upper part of the figure.

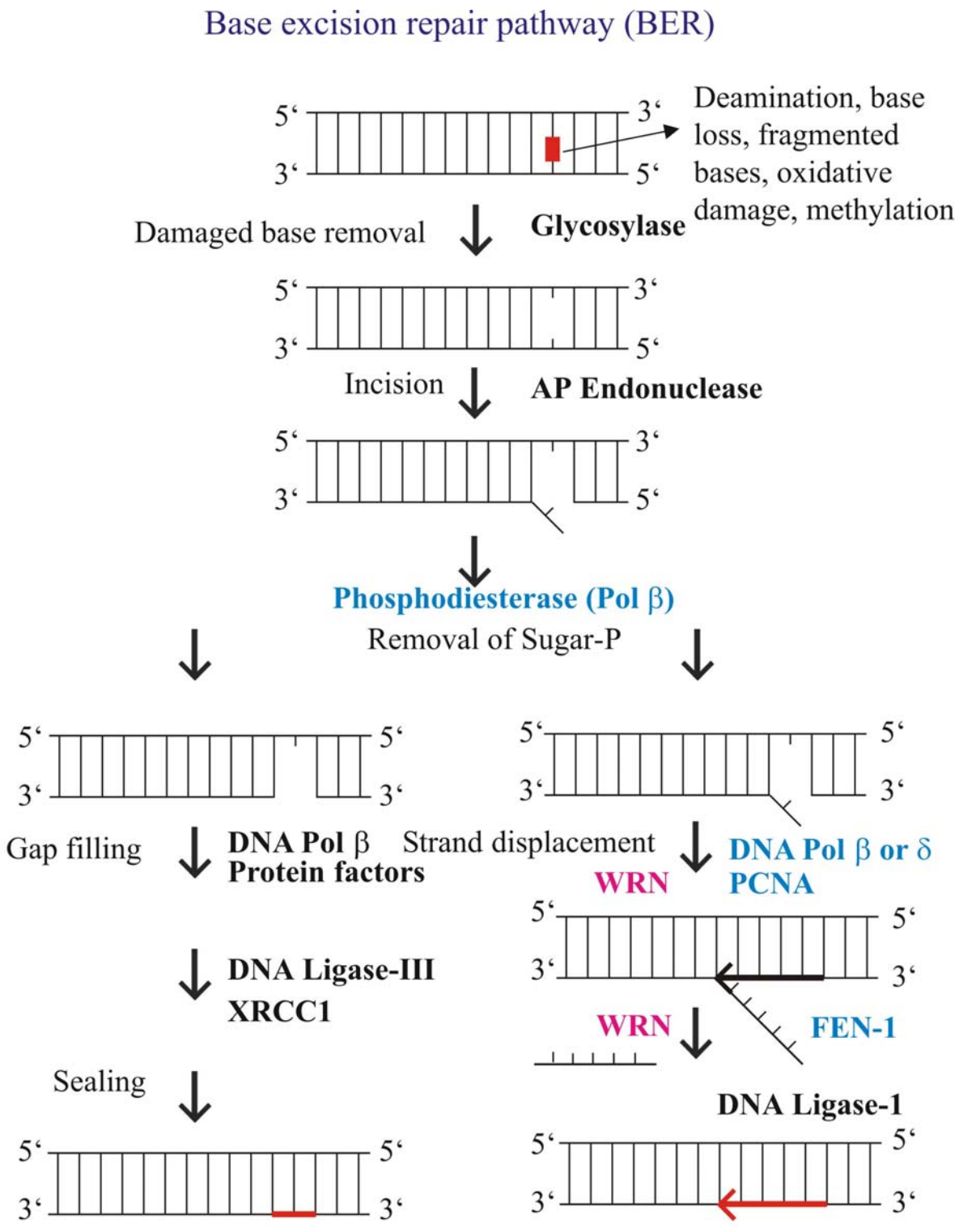



**Fig. 38.** The base excision repair pathway. In the blue colour are shown the proteins, which interact with hWRN-p either physically and/or functionally. The steps, in which may the human Werner syndrome protein participate are marked with pink coloured “WRN”.

### 6.3.5. Role in posttranscriptional gene silencing (PTGS)

There are three genes belonging to the RecQ family of helicases in *Neurospora crassa* required for a post-transcriptional gene silencing (Cogoni and Macino, 1999; Catalanotto et al., 2002). The phenomenon PTGS is a process, in which a double-stranded RNA triggers degradation of homologous mRNA in the cytoplasm and thus promotes silencing of the gene, which mRNA is transcribed. This phenomenon is known also for plants, where PTGS may participate in plant defense against viruses. Recently, the homologue of the Werner syndrome exonuclease from *Arabidopsis thaliana* AtWRNexo protein, newly named WEX, has been shown to be involved in PTGS (Glazov et al., 2003). The generated mutant *wex-1* revealed an impaired PTGS. An ectopic expression of WEX again restored the PTGS to the level of a wild type. It was speculated that the exonuclease may be required for the final hydrolysis of fragments of target RNAs.

## 6.4. Possible roles of AtWRNexo-p in nucleic acid metabolism

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 support the hypothesis that the Werner-like exonuclease activities are a common feature of DNA metabolism in animals and plants. The ability of AtWRNexo-p to degrade also the mismatched nucleotides from the 3'-recessed end of the partial duplexes suggests the role for AtWRNexo-p as a 3' → 5' proofreading exonuclease in DNA replication. AtWRNexo-p is able to degrade the DNA/RNA hybrid and only weakly single-stranded RNA. It is actually not surprising, because the AtWRNexo-p possesses an exonucleolytic domain similar to a domain of ribonuclease D (RNaseD). The RNaseD domain reveals similarities to the

proofreading domain of DNA polymerases (Moser, 1997). Thus, AtWRNexo-p may be able to degrade both DNA and RNA and therefore might also be involved in some aspect of mRNA degradation during posttranscriptional gene silencing. Indeed, pilot experiments showed that partially duplex RNA molecules could be digested by this enzyme (Plchova, unpublished). AtWRNexo-p is similarly to the WRN exonuclease inhibited or blocked by certain type of DNA lesions. It is therefore possible that AtWRNexo-p may potentially be involved in sensing such damaged nucleotides and may need the participation of other protein partners for their removal. Interactions of the AtWRNexo-p and several members of the AtRecQl helicase family in the two hybrid assay indicate that the exonuclease function is involved in processes that require RecQl-helicase activities. Since this exonuclease is not physically linked to a helicase, one is tempted to speculate that it might be involved also in processes, which do not require helicase activities. AtWRNexo protein interacts with AtMLH1. This interaction is very strong and may suggest, that AtWRNexo-p function is required for mismatch repair in plants. The interaction of AtWRNexo protein with AtKu80 may be important for non-homologous end joining pathway or telomere maintenance. Furthermore, the activity of AtWRNexo-p might be modulated by AtKu80 as it is shown for the WRN exonuclease (Cooper et al., 2000). AtWRNexo-p might also be posttranslationally modified by AtUbc9, another protein that interacts with AtWRNexo-p. This modification could be probably important for the regulation of the degradation of the AtWRNexo protein.

## 6.5. Outlook

In this study, the Werner-like exonuclease from *Arabidopsis thaliana* (AtWRNexo-p) was biochemically characterized. The characterization revealed similarities but also significant differences to the human Werner syndrome exonuclease. AtWRNexo-p is able to exonucleolytically degrade DNA/RNA hybrids and weakly single-stranded RNA. A recent study showed that this protein is involved in posttranscriptional gene silencing. It will be

therefore interesting to investigate, whether this exonuclease can degrade also substrates postulated to occur during the PTGS process, and RNAs of pathogens like viroids.

The two-hybrid assays revealed the interactions between AtWRNexo protein and other proteins required for different aspect of genome maintenance. The purification and biochemical characterization of AtRecQ-like and AtRecQsim proteins will provide an insight in the possible roles of these enzymes *in vivo*. Furthermore, the isolation of AtRecQl2 and AtRecQl5 proteins, found to interact with AtWRNexo protein *in vivo*, will allow to confirm these interactions on the protein level. Some secondary DNA structures, such as bubbles, are not possible to degrade by only an N-terminal part of the Werner syndrome protein and require the helicase part of WRN protein for a degradation (Machwe et al., 2002). It will be therefore interesting to determine, if AtWRNexo-p together with one of the other AtRecQ proteins will hydrolyze such structures.

The heterodimer Ku stimulates the exonucleolytic activity of hWRN-p (Cooper et al., 2000). The expression and purification of AtKu heterodimer will allow to test such effect on AtWRNexo-p.

Many proteins were identified to interact with hWRN-p. Thus, it will be interesting to identify other putative interacting partners from *Arabidopsis thaliana*. This will give us a new insight in which pathways the AtWRNexo protein may participate.

Besides defining partners of the exonuclease by two-hybrid screens, the analysis of T-DNA insertion lines will help to elucidate the function of AtWRNexo-p *in vivo*. It will be important to test the telomere length of such mutant plants, as it has been postulated for human, that the Werner syndrome protein may participate in telomere maintenance. Moreover, the AtWRNexo mutant should be tested for deficiences in DNA repair.

## 7. Summary

The aim of this study was the characterization of the Werner syndrome exonuclease homologue (AtWRNexo-p) from *Arabidopsis thaliana*.

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 a small ORF (AtWRNexo) with homology to the exonuclease domain of hWRN-p was identified in *Arabidopsis* genome. To identify a function and determine the substrate specificity of AtWRNexo protein (AtWRNexo-p), the ORF was cloned into an expression vector and expressed in *E. coli* as a 27 aa calmoduline-binding peptide fusion-protein. Purified AtWRNexo-p was incubated with different DNA substrates. The analysis revealed that AtWRNexo-p is indeed an exonuclease with activities that are similar but also significantly different from activities of the human WRN exonuclease. The protein digests recessed strands of DNA duplexes in 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. DNA/RNA hybrids are also hydrolyzed by AtWRNexo-p, but the single-stranded RNA only weakly. A mutation of a conserved residue within the exonuclease domain (E135A) completely abolished the exonucleolytic activity.

To identify possible interaction partners of AtWRNexo protein, its ORF was cloned also into the vectors used in GAL4 and LexA two-hybrid systems. Additionally, the ORFs of putative interaction partner were also cloned into these vectors. An interaction between AtWRNexo

protein and AtMLH1, AtKu80, AtRecQl2, AtRecQl5, AtUbc9 proteins could be detected. Furthermore, two-hybrid analysis revealed, that AtWRNexo protein interacts with itself, probably, it persists as multimer in solution.

The results indicate, that the WRN-like exonuclease activity seems to be an important feature of nucleic acid metabolism in animals and plants.

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