

## Functional Interaction between Ku and the Werner Syndrome Protein in DNA End Processing\*

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Werner syndrome (WS) is an autosomal recessive disease characterized by premature aging. The gene responsible for the syndrome was recently cloned and shown to encode a protein with strong homology to DNA/RNA helicases. In addition, the Werner syndrome protein (WRN) possesses an exonuclease activity. Based on the homology to helicases it has been proposed that WRN functions in some aspects of DNA replication, recombination, or repair. However, there is currently no evidence of a role of WRN in any of these processes; therefore, its biological function remains unknown. Using a biochemical approach, we have identified two polypeptides that bind to the WRN protein. Peptide sequence analysis indicates that the two proteins are identical to Ku70 and Ku80, a heterodimer involved in double strand DNA break repair by non-homologous DNA end joining. Protein-protein interaction studies reveal that WRN binds directly to Ku80 and that this interaction is mediated by the amino terminus of WRN. In addition, we show that the binding of Ku alters the specificity of the WRN exonuclease. These results suggest a potential involvement of WRN in the repair of double strand DNA breaks.

Werner syndrome (WS)<sup>1</sup> is a recessive autosomal disorder leading to premature aging (1). Individuals with WS develop accelerated atherosclerosis, osteoporosis, and a higher incidence of several types of tumors (2–4). Somatic cells of WS patients display a shortened replicative life span and elevated rates of chromosome translocation, rearrangements, and deletions (5, 6). The gene defective in WS has been identified and encodes a protein that possesses both exonuclease and helicase activities (7–13). A nuclear localization signal has also been identified at its carboxyl-terminal end. All the WS mutations that have been identified result in a nonsense mutation or

frameshift, leading to a predicted truncated protein. Thus, it is thought that the truncated protein fails to enter the nucleus and is subsequently degraded. Immunocytochemical studies have localized the Werner syndrome protein (WRN) in the nucleoli of actively growing human cells, whereas serum deprivation causes a relocalization to the nucleus (14, 15). Despite all the available information, the cellular function of WRN remains unknown (16). To understand the molecular basis of WS, we searched for nuclear factors that associate with the WRN protein. Here we report that WRN interacts with the Ku70/80 heterodimer (Ku), a factor involved in the repair of DNA double strand breaks (DSBs) (17–20). More importantly, our results show that the exonuclease activity of WRN on 3'-recessed DNA ends is strongly stimulated by Ku. Moreover, in the presence of Ku, WRN can efficiently process both blunt-ended DNA and the 3'-protruding strand of a partial duplex DNA. These results suggest that WRN, through direct physical interaction with the Ku70/80 heterodimer, may be involved in processing of DSBs, providing a link between DNA repair and aging.

### EXPERIMENTAL PROCEDURES

**Protein Binding Assay and Peptide Sequencing**—SF9 cells were infected with recombinant baculoviruses encoding Flag-tagged WRN. The cells were harvested three days postinfection, and whole cell lysates were prepared in RIPA buffer (50 mM Tris (pH 7.9), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride). Flag-tagged WRN or Flag-tagged HCV polymerase were immobilized on anti-Flag beads and then incubated with nuclear extracts from HeLa cells at 4 °C for 1 h. Bound proteins were then eluted from the beads with BCO buffer (1 M KCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5% glycerol, 1 mM DTT, and a mixture of protease inhibitors). The eluted proteins were precipitated with trichloroacetic acid, washed with acetone, and then resolved by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Bound proteins were visualized by silver staining. For protein sequencing, the preparation of the associated proteins was accomplished starting from approximately 10 mg of HeLa nuclear extracts. The proteins were separated by SDS-PAGE and stained with Coomassie Blue. After extensive destaining, the protein bands were excised from the gel and sent to the Biopolymers Facilities (Dr. John Rush, Howard Hughes Medical Institute, Harvard Medical School) for peptide sequencing. Sixteen peptides derived from the 65-kDa polypeptide (DSLIFLVDASK, DLLAVFYGTEK, NIYVLQELD-NPGAK, ILELDQFK, IMLFTNEDNPHGNDSAK, DTGIFDLMLHLK, KPGGFDISLFYR, DIISAEDEDLR, VFHEESSK, LEDLLR, TFFNTST-GGLLPSDTK, SQIYGSR, SDFENPVLQGHFR, ELVYPPDYNPEGK, VEYSEELK, KQELLEALTK) and ten peptides derived from the 90-kDa polypeptide (HIEIFTDLSSR, SQLDIIHSLK, LGGHGSPFPLK, LTIGSNLSIR, YGSDIVPFSK, FFMGNQVLK, ANPQGVAFPHIK, TDTLEDLFPTTK, TLFPLIEAK, AFREEAIK) were sequenced.

**Protein Purification**—Recombinant Flag-WRN proteins were expressed with a baculovirus system. Whole cell lysates were prepared with lysis buffer (0.5% Nonidet P-40, 1.5 mM MgCl<sub>2</sub>, 10 mM Hepes (pH 7.5), 100 mM NaCl). Flag-WRN protein was purified through the DEAE-cellulose column and further purified by affinity chromatography on anti-Flag resin. Recombinant Ku70 bearing an amino-terminal polyhistidine (His) epitope tag and Ku80 baculoviruses were used to co-infect SF9 cells, and 48 h after infection, cell lysates were prepared using lysis buffer. Ku70/80 complex was purified by metal affinity (Talon, CLONTECH) and DNA cellulose chromatography. For the purification of Ku80, lysates from insect cells expressing recombinant Ku80 were incubated with immobilized Flag-WRN beads. After extensive washes, bound Ku80 was eluted with BCO buffer. Purified proteins were dialyzed in dialysis buffer (10 mM Tris (pH 7.9), 80 mM NaCl, 4 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10% glycerol).

**Immunoprecipitation and Western Blot Analysis**—Nuclear extracts from HeLa cells were prepared as described in Ref. 21. Immunoprecipi-

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<sup>1</sup> The abbreviations used are: WS, Werner syndrome; WRN, Werner Syndrome protein; HR, homologous recombination; NHEJ, non-homologous end joining; DSB, double strand break; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HCV, hepatitis C virus.

tation was carried out with anti-WRN antibodies (Santa Cruz Biotechnology Inc.). Immunoprecipitation products were resolved by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using anti-WRN and anti-Ku80 antibodies (Santa Cruz Biotechnology Inc.).

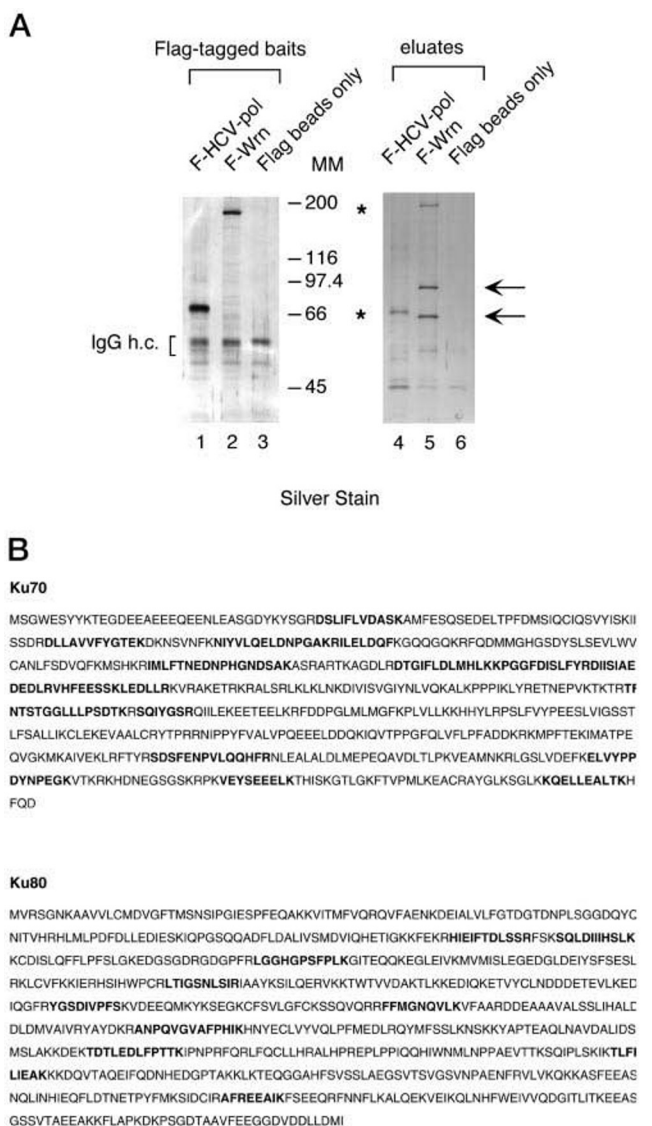
**DNA Exonuclease Assay**—DNA exonuclease activity was measured with the following DNA substrates: 20-oligomer A1 (CGCTAGCAATAT-TCTGCAGC), 20-oligomer A2 (GCTGCAGCAATATTGCTAGCG) complementary to A1, and 46-oligomer A3 (GCGCGGAAGCTTGGCTGCA-GAATATTGCTAGCGGGAATCGGCGC) partially complementary to A1. Oligonucleotides were labeled at the 5' end with [ $^{32}$ P]ATP. The appropriate oligonucleotides were annealed by boiling and slow cooling to room temperature. Reaction mixtures contained 40 mM Tris-HCl (pH 7.5), 4 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM ATP, 0.1 mg/ml bovine serum albumin, DNA substrates (~30 fmol, 100,000 cpm), and 5 ng of WRN protein, 5 ng of Ku70, 5 ng of Ku80, or 5 ng of Ku70/80 in a final volume of 10  $\mu$ l. The reaction mixtures were incubated at room temperature for 10 min, and then the reactions were terminated by the addition of 2  $\mu$ l of a formamide-dye solution (95% formamide, 50 mM EDTA, 0.5% bromophenol blue, 0.5% xylene cyanol). After incubation at 95 °C for 3 min, DNA products were resolved by either 12 or 16% polyacrylamide-urea gel electrophoresis and visualized by autoradiography.

## RESULTS

To identify nuclear proteins that interact with WRN, HeLa nuclear extracts were incubated with Flag-tagged WRN protein immobilized on anti-Flag resin. In parallel, HeLa nuclear extracts were also incubated with Flag-tagged HCV polymerase and anti-Flag resin only. Analysis by SDS-PAGE and silver staining revealed that two polypeptides of approximately 65 and 90 kDa (Fig. 1A, lane 2) respectively, were eluted specifically from the Flag-WRN resin. Neither the 65- nor the 90-kDa protein was present in the eluates from the control resins (Fig. 1A, lanes 1 and 3). Therefore, they are likely to represent specific cellular partners of the WRN protein. To ensure that DNA did not mediate the interaction between WRN and the two polypeptides, the experiment was also carried out with nuclear extracts treated with DNase I, yielding identical results. To identify the two polypeptides, bands were excised from the gel and digested with protease, and the resulting peptides were subjected to electrospray ionization/ion trap mass spectrometry. The results of the peptide sequencing indicated that the 65- and the 90-kDa proteins were identical to human Ku70 and Ku80, respectively (Fig. 1B). Ku70/80 is a heterodimer with high affinity for DNA ends, and it has been shown to be involved in DNA double strand break repair by non-homologous DNA end joining (NHEJ) and V(D)J recombination (17–19).

To determine which subunit of the Ku heterodimer interacts with the WRN protein, we performed co-infection in SF9 cells using recombinant baculoviruses expressing Flag-tagged WRN and either Ku70, Ku80, Ku70/Ku80, or a wild-type baculovirus. After 48 h, cells were lysed and lysates were incubated with anti-Flag resin. After extensive washing, the bound proteins were eluted with BCO buffer and analyzed by SDS-PAGE and silver staining. Ku70/80 heterodimer interacted with the WRN protein, as determined by the presence of the 70- and 80-kDa polypeptides in the eluate from the anti-Flag immunoprecipitation (Fig. 2, lane 2). Moreover, Ku80 alone also co-immunoprecipitated with WRN (Fig. 2, lane 3). In contrast, Ku70 did not associate with WRN because it was absent in the immunoprecipitation with anti-Flag resin (Fig. 2, lane 4). Thus, Ku80 mediates the molecular interaction between WRN and the Ku heterodimer.

To provide further evidence that the WRN protein interacts with Ku *in vivo*, we immunoprecipitated endogenous WRN from HeLa nuclear extracts using antibodies against the WRN protein. As a control,  $\beta$ -actin antibodies were also used in parallel experiments. Western blot analysis indicated that Ku80 is present in the immunoprecipitation reaction with anti-

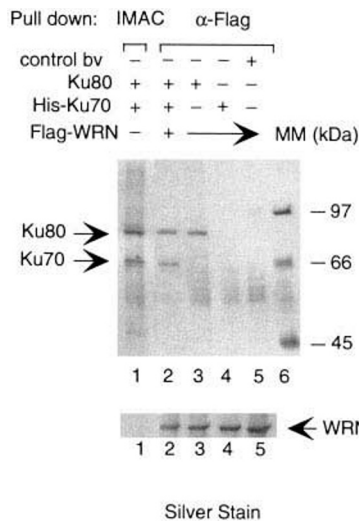


**FIG. 1. Purification of WRN-binding proteins from HeLa nuclear extracts.** A, Flag-tagged WRN (lane 2) and Flag-tagged HCV polymerase (HCV-pol, lane 1) were immobilized to anti-Flag beads and incubated with nuclear extracts from HeLa cells. After extensive washes, the bound proteins were eluted from the beads with BCO buffer. The eluted proteins were separated by 8% SDS-PAGE and analyzed by silver staining. Flag-tagged HCV polymerase and anti-Flag beads were used as negative controls. IgG h.c., immunoglobulin heavy chains; MM, molecular mass markers (kDa). Asterisks indicate Flag epitope-tagged HCV polymerase and WRN proteins partially eluted from the beads. Arrows indicate 65-kDa and 90-kDa associated proteins. B, Ku70 and Ku80 protein sequences. Peptide sequences obtained by LC/MS mass spectrometry are in bold characters.

WRN antibodies but absent from the control immunoprecipitation with  $\beta$ -actin antibodies. This result indicates that Ku80 is associated with the WRN protein *in vivo*.

To define the domain of the WRN protein that interacted with Ku80, recombinant Ku80 was incubated with a series of Flag-tagged WRN deletion mutants. After extensive washing, bound proteins were resolved by SDS-PAGE and analyzed by Western blotting with antibodies against Ku80. The results of this experiment indicated that a specific region of WRN encompassing the amino-terminal amino acid sequence (amino acid residues 1 to 388) was sufficient for binding to Ku80 (Fig. 3). In addition, this region of WRN can efficiently interact with Ku heterodimer (data not shown). In a recent study the carboxyl-terminal region of WRN (amino acids 940 to 1432) was used to





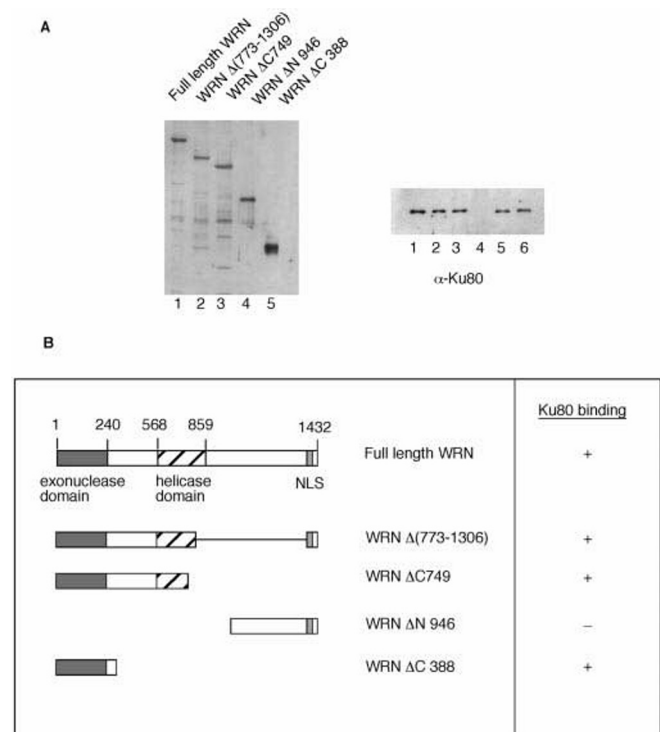
**FIG. 2. Interaction between WRN and Ku70/80.** Flag-tagged WRN was coinfecting in Sf9 cells with either Ku80, Ku70/80, Ku70, or wild type baculovirus (*control bv*) as indicated. Equal amounts of cell extracts from each infection were prepared and incubated with anti-Flag beads. Bound proteins were then eluted from the beads and analyzed by SDS-PAGE and silver staining. The amount of immunoprecipitated Flag-WRN, which under this elution condition remains bound to the beads, was determined by SDS-PAGE and silver staining (*lower panel*).

isolate Ku from HeLa nuclear extracts, leading to the interpretation that this region of WRN interacts directly with Ku (22). Because WRN can form dimers or higher order multimers (19), it is possible that the reported interaction between the carboxyl-terminal region of WRN and Ku may be mediated by full-length WRN or other uncharacterized cellular proteins.

The WRN protein possesses 3' to 5' exonuclease activity, and this activity has been shown to specifically hydrolyze 3'-recessed strands in a partial DNA duplex (8, 9). The finding that the WRN protein interacts with Ku70/80 prompted us to ask whether Ku70/80 affects WRN exonuclease activity. To test this hypothesis, we performed DNA exonuclease assays with recombinant WRN protein in the presence of Ku70, Ku80, or Ku70/80 heterodimer. As previously shown, WRN can weakly hydrolyze 3'-recessed DNA ends (Fig. 4A, *lane 4*). However, as also shown in a recent report (22), in the presence of Ku70/80 heterodimer, the WRN exonuclease activity was strongly stimulated (Fig. 4A, *lane 7*). This dramatic activation was strictly dependent on a functional Ku heterodimer, because in the presence of either Ku70 or Ku80 alone, the stimulation was abolished (Fig. 4A, *lanes 5 and 6*). We then examined whether the Ku70/80 heterodimer could also activate the WRN exonuclease activity on blunt-end and 3'-protruding DNA substrates. As reported by others, the WRN protein does not digest either 3'-protruding (Fig. 4B, *lane 4*) or blunt-end DNA substrates (Fig. 4C, *lane 4*). However, in the presence of a Ku70/80 heterodimer, WRN exonuclease activity was active on both 3'-protruding ends (Fig. 4B, *lane 7*) and blunt-end DNA molecules (Fig. 4C, *lane 7*). Therefore, these results indicate that direct interaction between the WRN protein and Ku70/80 strongly stimulates the 3'-exonuclease activity on 3'-recessed DNA substrates. In addition, the presence of Ku70/80 induces rapid processing of blunt end and 3'-protruding end DNA by the WRN exonuclease.

#### DISCUSSION

The functional interaction between Ku70/80 and WRN protein provides a biochemical basis for the phenotypes of WRN<sup>-/-</sup> fibroblasts. WRN<sup>-/-</sup> cells exhibit large DNA deletions and chromosome rearrangements and are characterized



**FIG. 3. The amino-terminal region of WRN is sufficient for binding to Ku80.** *A, left panel*, silver-stained gel of purified Flag-tagged WRN proteins. *Right panel*, Ku80 was incubated with purified Flag-tagged wild type WRN (*lane 1*), WRNΔ773-1306 mutant (*lane 2*), WRNΔC749 (*lane 3*), WRNΔN946 (*lane 4*), and WRNΔC388 (*lane 5*). *Lane 6* shows input Ku80. *B*, schematic representation of WRN wild type and mutant proteins used in this study. Functional domains are as indicated. NLS, nuclear localization signal.

by a shortened lifespan. Mouse cells deficient for Ku80 show elevated frequencies of chromosomal aberrations such as breakage, translocation, and aneuploidy (23, 24). In addition, Ku80 knock out mice display an early onset of age-specific changes that are reminiscent of Werner syndrome patients (25). Taken together, these genetic analyses and our biochemical studies strongly support the idea that there is a functional interaction between Ku70/80 and the WRN protein.

The finding that WRN binds to Ku70/80 suggests potential cellular functions for this protein. One possible scenario is that WRN is involved in some aspects of DNA DSB repair. In eukaryotic cells, DSBs can be caused by a variety of exogenous and endogenous agents. DSBs pose a major threat to the integrity of the genome, and if left unrepaired, they can cause cell death or neoplasia. DSBs are repaired either by using an intact copy of the broken region as a template (homologous recombination (HR)) or by direct rejoining of the broken ends (NHEJ). Both mechanisms operate in eukaryotic cells; however, it is thought that NHEJ is the prevalent pathway in higher eukaryotes. Many of the proteins involved in HR have been identified. These include Rad50, an ATP-dependent DNA-binding protein, Mre11, a double-stranded DNA 3'-exonuclease and single-stranded endonuclease, and NBS1, a protein that is specifically mutated in patients with Nijmegen breakage syndrome (19, 20). Mre11 is thought to function in the resection of DSB ends in HR, and the Rad50-Mre11-NBS1 complex has been proposed to serve a crucial function in the recognition of double strand DNA breaks (26).

In contrast to HR, NHEJ does not require homology with a second DNA duplex. Biochemical analyses have established that Ku binds to the ends of broken DNA and plays a direct role in NHEJ (18-20). Upon binding of Ku to the DNA end, the

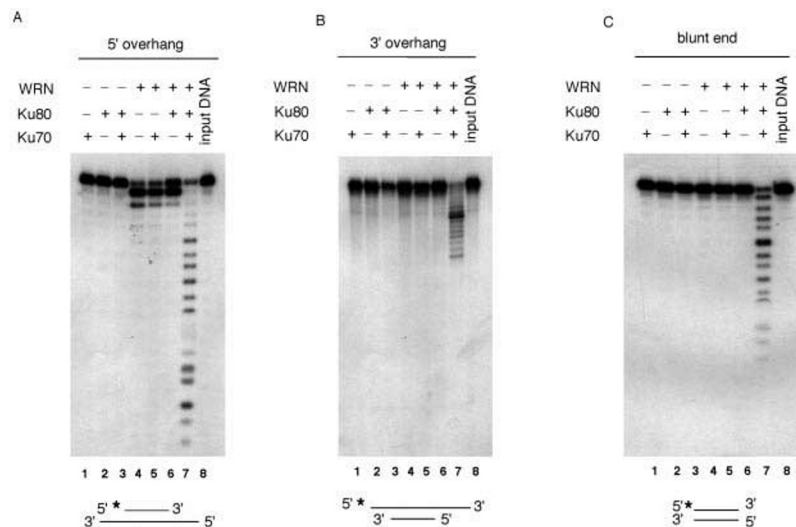


FIG. 4. **Stimulation of WRN 3' to 5' exonuclease activity by Ku70/80.** A, purified WRN, WRN-Ku70, WRN-Ku80, WRN-Ku70/80 were incubated with 3'-recessed,  $^{32}$ P-labeled 5'-20-mer (A1)/46-mer (A3) DNA substrate at room temperature for 10 min. Products were analyzed by 16% polyacrylamide-urea denaturing gel and autoradiography (lane 1, Ku70; lane 2, Ku80; lane 3, Ku70/80; lane 4, WRN; lane 5, WRN-Ku70; lane 6, WRN-Ku80; lane 7, WRN-Ku70/Ku80; lane 8, DNA probe only). B, purified WRN, WRN-Ku70, WRN-Ku80, WRN-Ku70/80 were incubated with 3'-protruding,  $^{32}$ P-labeled 5'-46-mer (A3)/20-mer (A1) DNA substrate at room temperature for 10 min. Products were analyzed by 12% polyacrylamide-urea denaturing gel and autoradiography (lane 1, Ku70; lane 2, Ku80; lane 3, Ku70/80; lane 4, WRN; lane 5, WRN-Ku70; lane 6, WRN-Ku80; lane 7, WRN-Ku70/Ku80; lane 8, DNA probe only). C, purified WRN, WRN-Ku70, WRN-Ku80, WRN-Ku70/80 were incubated with blunt-ended,  $^{32}$ P-labeled 5'-20-mer (A1)/20-mer (A2) DNA substrate at room temperature for 10 min. Products were analyzed by 16% polyacrylamide urea denaturing gel and autoradiography (lane 1, Ku70; lane 2, Ku80; lane 3, Ku70/80; lane 4, WRN; lane 5, WRN-Ku70; lane 6, WRN-Ku80; lane 7, WRN-Ku70/Ku80; lane 8, DNA probe only).

DNA-dependent protein kinase is recruited to the DNA-Ku complex. DNA-dependent protein kinase can phosphorylate itself and Ku; however the functional significance of these modifications remains to be determined. Most double strand DNA breaks are not blunt ends but have single-stranded overhangs; therefore the DNA ends must be trimmed by exonucleases and/or endonucleases before they can be rejoined (20). Mre11 has been proposed to play a role in NHEJ in yeast; however it remains to be demonstrated whether it also functions in NHEJ in higher eukaryotes. The WRN protein contains a 3' to 5' exonuclease activity and interacts with Ku *in vivo* and *in vitro*; therefore, WRN may be involved in nucleolytic processing of double strand DNA ends.

An alternative hypothesis is derived from the observation that the yeast Ku 70/80 heterodimer (yKu) is found associated with the chromosome ends, and it has been proposed that yKu helps cap the telomeres and/or regulating 3' end processing enzymes (27). More recently, the Ku70/80 complex has also been proposed to be required for capping the ends of mammalian chromosome (28, 29). Therefore, it is possible that the association between WRN and Ku70/80 may be involved in some aspects of telomere maintenance.

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