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Advances in Brief

WRN or Telomerase Constructs Reverse 4-Nitroquinoline 1-Oxide Sensitivity in Transformed Werner Syndrome Fibroblasts[1]

Fuki M. Hisama, Yao-Hui Chen, M. Stephen Meyn, Junko Oshima, and Sherman M. Weissman[2]

Boyer Center for Molecular Medicine [F. M. H., Y-H. C., S. M. W.] and Departments of Neurology [F. M. H.] and Genetics [Y-H. C., S. M. W.], Yale University School of Medicine, New Haven, Connecticut 06520; Department of Medical and Molecular Genetics, Hospital for Sick Children, University of Toronto, Toronto, Ontario, M5G 1X8 Canada [M. S. M.]; and Department of Pathology, University of Washington, Seattle, Washington 98195 [J. O.]

Abstract

WRN encodes a RecQ helicase, which is mutated in Werner syndrome. Werner syndrome is a genetic condition of young adults characterized by premature aging, limited replicative capacity of cells *in vitro*, and increased cancer risk. Telomerase is a reverse transcriptase that extends the G-rich strand of telomeric DNA. Primary cells *in vitro* typically lack telomerase activity and undergo senescence, whereas telomerase is reactivated in many, but not all, tumors. The roles of the two genes are not known to be related. Here we report the development of an effective colony-forming assay in which a SV40-transformed Werner fibroblast cell line is 6–18-fold more sensitive to 4-nitroquinoline 1-oxide than SV40-transformed normal cell lines. The sensitivity can be partially reversed by transfecting a normal *WRN* gene but not a mutated *WRN* gene into the cells. Curiously, the sensitivity can be reversed equally well by transfecting a telomerase gene (*TERT*) into the cells. These data indicate the possibility of an interdependent function of these two genes.

Introduction

WS[3] is a rare, autosomal recessive condition that results in the premature appearance in young adults of features of old age, including gray hair, cataracts, type 2 diabetes, and mesenchymal cancers (1, 2). Cells from Werner subjects demonstrate limited replicative capacity *in vitro* compared with age-matched controls and show an increase in somatic mutations, chromosome loss or deletions, and a prolonged S phase. Werner lymphoblastoid cell lines are hypersensitive to the DNA-damaging effects of 4NQO (3). The cause of this disease is truncation of the gene product (WRN), a 1432-amino acid RecQ helicase (4), which is a member of the DexH-containing superfamily of helicases that includes the xeroderma pigmentosum genes *XPB* and *XPD* as well as the Bloom syndrome gene *BLM* and the gene for Rothmund-Thompson syndrome (5). ATP-dependent helicase activity and 3′→5′ exonuclease activity have been verified for the baculovirus-produced WRN protein (6, 7). Some but not all mutations in *WRN* result in impaired nuclear localization of the protein (8). However, the precise cellular functions of WRN remain to be elucidated. The lack of a convenient *in vitro* model for correlating the molecular structure and biological effects of WRN has hampered functional studies, particularly because the published knockout mouse model does not recapitulate many of the expected features of WS (9).

Telomeres, which form a protective cap on the ends of eukaryotic chromosomes, are composed of short, G-rich DNA repeats complexed with proteins. Telomeres prevent inappropriate joining of chromosome ends and permit full replication of the chromosome during each cycle of DNA replication. They are replicated by the specialized reverse transcriptase telomerase, using an intrinsic RNA template (10). Primary cells in culture typically lack telomerase activity and undergo progressive shortening of the telomeres, which has been proposed as a "cellular clock" leading to *in vitro* senescence (11). Many but not all immortalized cell lines show a reappearance of telomerase activity. Transfection of the telomerase gene causes some primary cell lines to fail to senesce in culture without producing a transformed phenotype (12, 13). Although both *TERT* and *WRN* have been identified separately as having roles in carcinogenesis, the roles of the two genes are not known to be related. Here we describe a colony-forming assay in which a SV40-transformed Werner fibroblast cell line is 6–18-fold more sensitive to 4NQO than SV40-transformed normal cell lines. The sensitivity, as predicted, can be partially reversed by transfecting a normal *WRN* gene but not any of several control constructs into the cells. Unexpectedly, the 4NQO sensitivity of the *WRN(−/−)* SV40-transformed cells can also be reversed by transfecting a construct containing the human *TERT* gene. Thus, these results raise the possibility of potentially interdependent functions of these two genes.

Materials and Methods

Chemicals. A stock solution of 4NQO (Sigma Chemicals, St. Louis, MO) was prepared by dissolving it in absolute ethanol at a 2 mM concentration and stored at −20°C. The same stock solutions were used throughout the experiments.

cDNA Expression Constructs. pEGFP-WRN (Fig. 1*B*), a *Sal*I-*Eco*RV fragment from pBK-NWRN containing the coding region of the *WRN* gene including the 3′-untranslated region and nine nts of the 5′-untranslated region, was cloned into pBluescript to generate pBSK-WRN. A *Xho*I-*Sac*II fragment containing WRN from nt 223-4747 was cloned into pEGFP-C (Clontech) containing the neomycin resistance marker.

PCINeo-hEST2-HA expressing the telomerase catalytic subunit with a HA epitope tag at the COOH terminus was obtained from Dr. Robert Weinberg (Massachusetts Institute of Technology, Cambridge, MA).

pcDNA3.1-His/Myc-Mut 5, an *Eco*RV and *Hin*dIII fragment containing a nonsense point mutation (C→T) at nt 1336 (GenBank L76937), was amplified from pBSK-WRN plasmid using oligonucleotide W5F (5′-GTAGGAATT-GAAGGAGATCAGTGG-3′; nt 643–666) as the sense primer and oligonucleotide Mut5R (3′-CCTTTATTTCACCTTACTTTTCGAATA-5′; nt 1321–1342) with an additional five nts to create a *Hin*dIII site as the antisense primer. The PCR product was confirmed by sequencing, and was subsequently cloned into pcDNA3.1-His/myc. A *Xho*I-*Eco*RV fragment containing the 5′ region of the *WRN* gene was subsequently ligated into the construct. The final construct contains a premature nonsense mutation at nt 1336.

pEGFP-WRN-Mut5, a *Sal*I-*Hin*dIII fragment from the pcDNA3.1-His/Myc-Mut 5 construct, was ligated into the *Xho*I-*Hin*dIII sites of pEGFP-C (Clontech) to generate the truncated form of WRN fused to a GFP tag at the 5′ end.

POLD2 (GenBank NM006230) including 78 bases upstream of the ATG

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[2] To whom requests for reprints should be addressed, at Sterling Professor of Medicine and Genetics, Yale University School of Medicine, BCMM 336, 295 Congress Avenue, New Haven, CT 06520. Phone: (203) 737-2282; Fax: (203) 737-2286; E-mail: sherman.weissman@yale.edu.

[3] The abbreviations used are: WS, Werner syndrome; 4NQO, 4-nitroquinoline 1-oxide; HA, hemagglutin; nt, nucleotide; GFP, green fluorescence protein.

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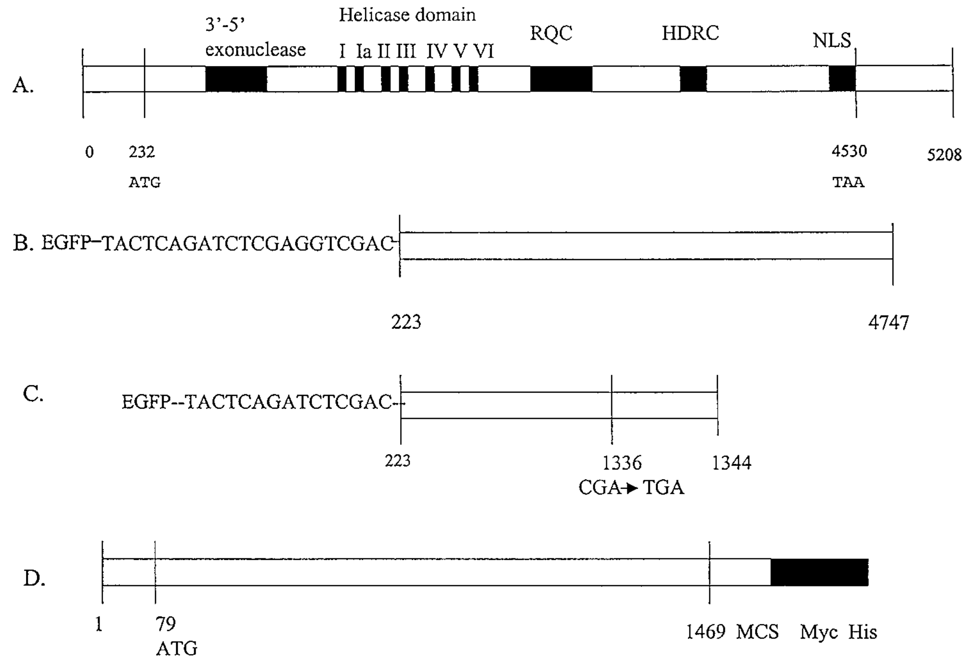

Fig. 1. WRN constructs. *A*, *WRN* gene product. Numbering refers to the cDNA sequence in GenBank L76937. The helicase domain contains motifs I–VI. *RQC*, a region of unknown function conserved in RecQ helicases. *HDRC*, helicase RNase D, a COOH-terminal conserved region found in helicases and RNase proteins. *NLS*, nuclear localization signal. *B*, pEGFP-WRN. *C*, pEGFP-WRN-Mut5 contains a premature stop mutation at nt 1336, resulting in a truncated protein associated with symptoms of WS. *D*, p50 is the δ small subunit of p50 DNA polymerase (GenBank NM006230) lacking the terminal 19 bases of the coding region fused to a c-myc epitope and a His tag at the COOH terminus. The construct includes 78 bases upstream of the ATG start codon and a 5′ *Sal*I site. *MCS*, multiple cloning site. *Myc*, c-myc epitope. *His*, histidine tag. The TERT construct used has been reported previously (Ref. 16; GenBank AF043739).

start codon and lacking the terminal 19 bases of the coding region was ligated into the pcDNA3.1 His/Myc vector to form the construct pcDNA-p50 DNA polymerase δ small subunit.

Primers and Sequencing. Sequencing of the *WRN* gene to confirm mutations was performed using previously published primer sequences and PCR conditions to amplify the *WRN* gene from genomic DNA prepared by standard methods. (14). A vector-specific and gene-specific primer pair was used to amplify the 5′ and 3′ ends of the WRN and TERT constructs. For WRN, the primers used were W5F (5′-GTGGAGAGGGTGAAGGTGATGC-3′) and WFR (5′-CATCCATTCAGGACATTTCCGC-3′), with a product size of 688 bp, and W3F (5′-GACACGTACCTTATCCACATG-3′) and a standard SK primer (5′-CCGCTCTAGAACTAGTGGATC-3′), with a product of 526 bp. For TERT, the primers were based on the GenBank construct sequence (AF043739) and consisted of TERT5F (5′-AGAGAAGACTCTTGCGTT-TCTG-3′) and TERT5R (5′-GTGACACCACACAGAAACCAC-3′), which produced a 1018-bp product. Amplification with TERT3F (5′-TCTGC-TACTCCATCCTGAAAGC-3′) and TERT3R (5′-GTCATAGGGATAGC-CCGCATAG-3′) yielded a 313-bp product. Primers designed to amplify part of the neomycin resistance casette from pEGFP-C (GenBank U19278) were also used [NeoF (5′-ATGATTGAACAAGATGGATTGC-3′) and NeoR (5′-CCAAGCTCTTCAGCAATATCAC-3′), with a predicted PCR size of 698 bp].

Cell Lines and Culture. The SV40-transformed WS fibroblast cell line (AG11395/WS780), a second SV40-transformed Werner fibroblast cell line (AG07066B/PSV811), and SV40-transformed control fibroblast cell lines WI-38 (AG07217A) and MRC-5 (AG 11076) were obtained from the Aging Cell Culture Repository, Coriell Institute for Medical Research (Camden, NJ) and grown in the recommended medium. Cells were grown in high-glucose DMEM (Werner fibroblasts) or MEM with 2× concentration of vitamins and essential and nonessential amino acids (control fibroblasts) supplemented with 20% FCS (Life Technologies, Inc., Rockville, MD) at 37°C in a 5% CO_2 atmosphere. Each cell line was tested for *Mycoplasma* by PCR assay (Stratagene).

Stable Expression Cell Lines. Stably transfected cell lines were obtained by incubating either the SV40-transformed Werner cell line or the SV40-transformed WI-38 cell line (10^6 cells/100-mm tissue culture dish) with the cDNA constructs in the presence of LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's instructions. Two days later, the cells were placed in neomycin-containing medium (500 mg/ml) for 2 weeks, and then individual colonies (100–200 colonies/vector) were pooled to form a population for additional studies.

Colony-forming Assay. Fibroblasts were harvested in exponential growth phase and seeded in triplicate at the appropriate densities in Corning 60-mm tissue culture dishes to produce fewer than 300 discrete colonies. The appropriate densities were determined from: (*a*) the plating efficiency for each cell line (1000, 2000, or 4000 cells/dish) in the absence of 4NQO; and (*b*) preliminary 4NQO sensitivity studies, in which cells were plated (10^5 to 10^6 cells/dish for each cell line) and treated with 2.0 μM 4NQO for 1 h as described below. Because of differences in growth response to different lots of serum, a single lot number was used for the entire set of experiments. After incubation overnight to permit cell attachment, the cells were rinsed with 1× PBS and incubated in serum-free medium supplemented with concentrations of 4NQO of 0, 0.5, 1.2, 2.0, or 2.8 μM for 1 h. After chemical treatment, the cultures were rinsed with 1× PBS and incubated in growth medium until visible colonies appeared (11–14 days), at which time the cells were rinsed, fixed, and stained in a crystal violet solution (0.25% crystal violet dissolved in 72% methanol, 10% formaldehyde, and 18% deionized water). The number of survivors (defined as colonies containing ≥50 cells) was scored. Survival curves were plotted as colony-forming ability (expressed as a percentage of the sham-treated control) on a logarithmic scale as a function of chemical concentration on a linear scale.

Western Blot Analysis. COS-7 cells were transfected with pEGFP-WRN containing the full-length cDNA, pEFGP-WRN-mut5 encoding the *WRN* gene with a stop codon at residue 1336, or pCINeo-hEST2-HA containing the telomerase catalytic subunit with a HA tag at the COOH terminus. Cells were harvested after 60 h and solubilized in radioimmunoprecipitation assay buffer. The protein concentration of each sample was quantified, and equal amounts of protein (20 μg) were loaded on a 10% SDS-polyacrylamide gel. For WRN, immunoprecipitation was performed with anti-WRN and anti-GFP antibodies before loading on the gel. Immunoblotting was performed using anti-GFP or anti-HA antibody at a 1:1000 dilution.

Telomere Length and Telomerase Activity. The TeloQuant kit was used according to the manufacturer's (PharMingen, San Diego, CA) protocol. Telomere lengths were analyzed by Southern blotting of terminal restriction fragments. Briefly, genomic DNA samples were prepared using the Qiagen, Inc. (Chatworth, CA) genomic protocol for cultured cells using the following six SV40-transformed cell lines: (*a*) WI-38; (*b*) MRC-5; (*c*) *WRN(−/−)*/AG11395; (*d*) *WRN(−/−)*/AG11395 stably transfected with vector alone; (*e*) *WRN(−/−)*/AG11395 stably transfected with the WRN construct; and (*f*) *WRN(−/−)*/AG11395 stably transfected with the TERT construct. For each sample, 5.0 μg of genomic DNA were digested with *Rsa*I/*Hin*fI overnight, separated on a 0.6% agarose gel, transferred to a nylon membrane (Amersham Hybond-N^+), and hybridized with a biotinylated telomere probe at 5 ng/ml, and telomere length was compared with known standards by chemiluminescence detection. To assay telomerase activity, 1×10^6 cells were washed with PBS, lysed in 1× 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfo-

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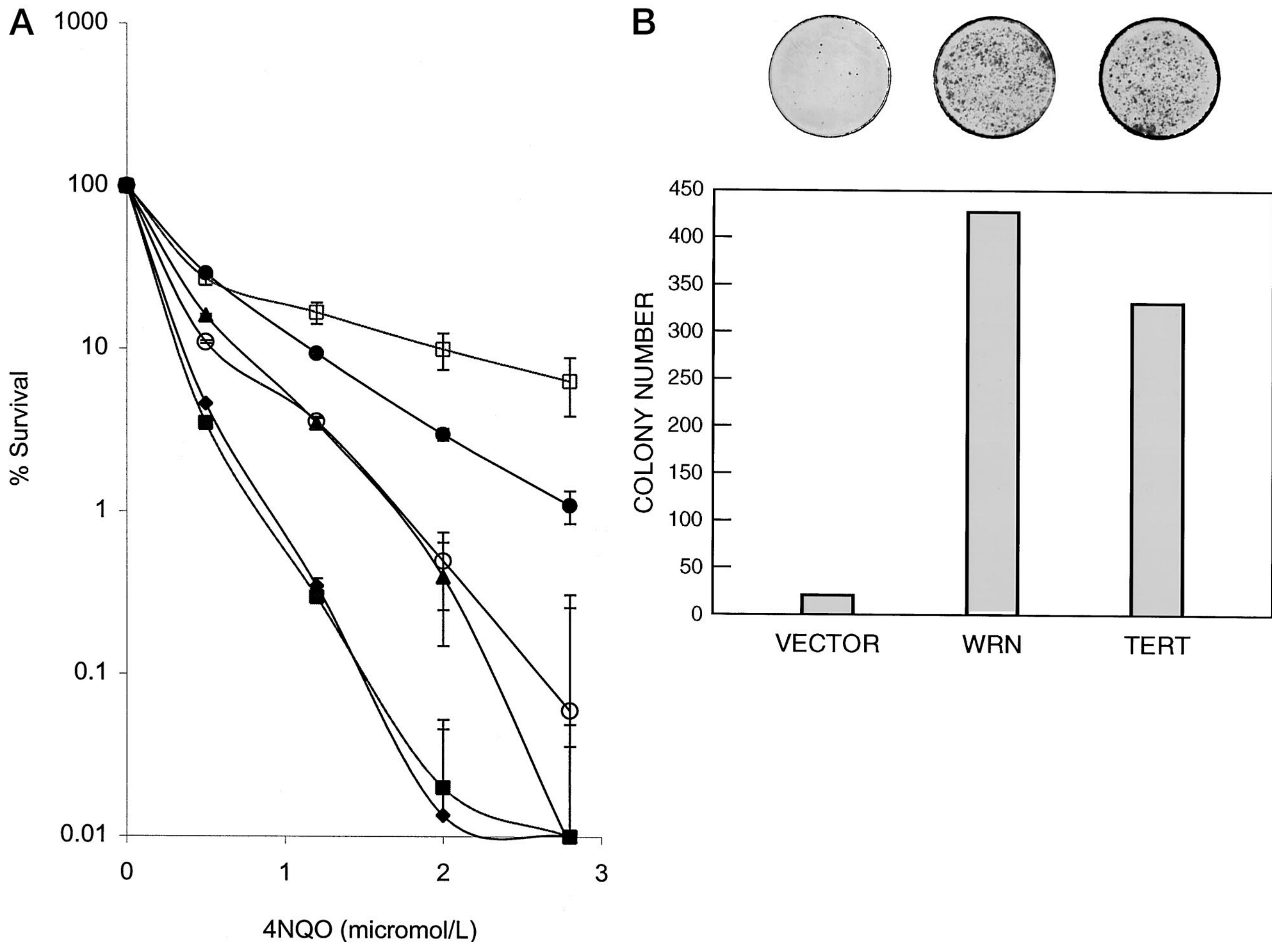

Fig. 2. *A*, graphs representing the clonogenic survival of SV40-tranformed WRN(−/−) fibroblasts (♦), normal cell lines WI-38 (□) and MRC-5 (●), or *WRN(−/−)* cells transfected with pEGFP vector alone (■), with pEGFP-WRN expressing the full-length Werner protein (▲), or with pCINeo-hEST2-HA expressing the telomerase catalytic subunit with a COOH terminus HA epitope (○) after treatment with increasing concentrations of 4NQO. Each curve represents the mean of triplicate platings. *Bars*, 1 SD. *B*, WRN or TERT constructs reverse 4NQO sensitivity in SV40-transformed WRN(−/−) fibroblasts. Plates from a representative experiment demonstrate that compared with transfection with vector alone, transfection of a construct containing either the *WRN* gene or a *TERT* gene and treatment with 0.5 μM 4NQO for 1 h as described in the text increased the number of surviving colonies.

nic acid buffer, incubated on ice for 30 min, and centrifuged at 12,000 × *g* for 30 min. The protein concentration was determined by Coomassie Protein Assay Reagent (Bio-Rad), and telomerase activity was determined for 1 μg of protein by its ability to synthesize telomeric repeats onto an oligonucleotide substrate in the presence of deoxynucleotide triphosphates and the supplied primers. The resulting extended products were PCR-amplified using a radiolabeled primer and visualized by autoradiography.

Results

4NQO Sensitivity in SV40-transformed Werner Fibroblasts. Similar to previously reported hypersensitivity of T and B lymphocytes (3) and fibroblasts (15) in Werner subjects, we found a SV40-transformed fibroblast cell line from a Werner subject was hypersensitive to the cytotoxic effects of 4NQO. In the initial studies, we used the *WRN(−/−)* SV40-transformed fibroblast cell line AG11395 with a nonsense mutation (C→T at codon 369, as confirmed by sequencing genomic DNA), resulting in a WRN protein truncated before the helicase domains. The mean colony-forming efficiency was determined for each SV40-transformed cell line as well as for the stably transfected lines and was 0.5% for AG11395 [*WRN(−/−)*], 1.2% for *WRN(−/−)* + TERT, 2% for *WRN(−/−)* + WRN, 4% for *WRN(−/−)* + vector, 1.2% for WI-38 + mut5, 1% for MRC-5, and 11% for WI-38. Although we counted only colonies containing >50 cells to exclude cells with limited proliferative capacity, this number is not critical. Counting colonies with >10 or 20 cells resulted in a proportionate increase in the plating efficiency for each cell line.

Treatment with increasing concentrations of 4NQO (Fig. 2*A*) resulted in a marked reduction in colony-forming ability of the AG11395/WS780 cell line compared with control SV40-transformed cell lines. As controls for the 4NQO treatment, we tested two SV40-transformed normal cell lines: (*a*) WI-38; and (*b*) MRC-5.

Transfection of WRN or TERT Constructs Rescues 4NQO Sensitivity. To test the effects on rescuing 4NQO sensitivity, we prepared constructs containing the full-length *WRN* gene. For controls, we prepared constructs containing a vector alone; WRN containing a C→T mutation at nt 1336, resulting in a the introduction of a premature stop at codon 369; a vector expressing the p50 polymerase δ small subunit; or *TERT* with a HA epitope tag at the COOH-terminus. As expected, control transfections with vector alone, a vector expressing the p50 subunit of δ DNA polymerase, or WRN containing a nonsense mutation and subsequent selection with neomycin had no effect on colony-forming ability. Stable transfection with the WRN construct fused with a GFP tag at the 5′ end strongly increased the ability of 4NQO-treated *WRN(−/−)* fibroblasts to form colonies. Unexpectedly, transfection with the TERT constructs rescued the 4NQO sensitivity of SV40-transformed *WRN(−/−)* cells to an equal degree as transfection with WRN. (Fig. 2, *A* and *B*). The effects of rescue by transfection with either WRN or TERT were repeated a minimum of six times using the original stably transfected cell lines as well as reproduced at least three times in a second set of stable transfectants. Stable transfection of SV40-transformed WI-38

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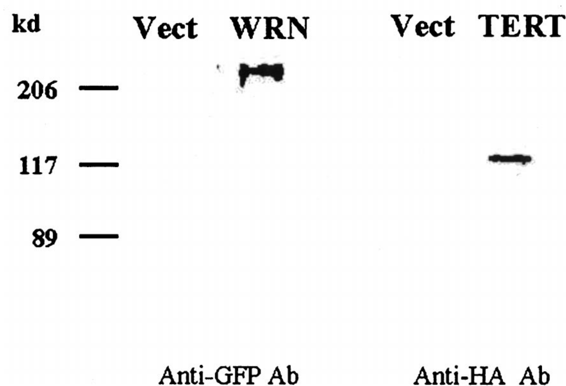

Fig. 3. Transient transfection of COS-7 cells with the constructs confirmed expression of the WRN or TERT proteins.

fibroblasts with the same WRN construct had no effect on the baseline resistance to 4NQO (data not shown).

To confirm the presence of the constructs in the stably transfected cell lines, we used a gene-specific and vector-specific primer pair in each case to PCR-amplify the 5′ and 3′ ends of the WRN and TERT constructs from genomic DNA extracted from the cell lines. As another control, we amplified the neomycin resistance gene. To confirm that the constructs were able to express WRN or TERT full-length proteins, we used the same constructs in transient transfection of COS-7 cells using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's protocol. After 60 h, the cells were solubilized, and immunoblotting was performed (Fig. 3). We also assayed telomerase activity, but we detected no significant differences between transfection with vector alone or transfection with the WRN construct or the TERT construct (Fig. 4). In addition, we measured telomere length in two separate sets of stably transfected cell lines by Southern blotting. These results showed an equal increase in telomere length after transfection with vector, WRN, or TERT.

Discussion

The Werner gene and the telomerase gene have both been implicated in very different models of carcinogenesis as well as aging. Both have been cloned in the last 4 years, allowing for more detailed studies of their molecular mechanisms. Here we report the use of a colony-forming assay as an *in vitro* test of the biological function of WRN. The fibroblast cell survival assay reported here appears to meet several of the requirements desired for a model system for functional studies of WRN: it is convenient, inexpensive, and reproducible. By testing a series of deletion and substitution mutants in this assay, it will be possible to map regions of the WRN protein essential for this biological effect and to determine in what manner TERT has a similar effect.

The roles of *WRN* and *TERT* are not known to be related. The report presented here demonstrates a potential overlap in the field of effects of WRN and TERT, the catalytic subunit of telomerase, because either WRN or TERT substantially reversed the sensitivity of transformed Werner fibroblasts to the DNA-damaging agent 4NQO. We found that this effect is most marked at higher concentrations ($\geq$2.0 μM). It is worth noting that Counter *et al.* (16) found a dissociation between TERT activity *in vitro* and the biological effect of TERT and TERT-HA constructs in human cells. Both constructs had robust TERT activity, but after transduction, only TERT (not TERT-HA) conferred the ability to bypass growth arrest and crisis in postsenescent cells.

In our assay, stable transfection of any construct, including empty vector, resulted in cells with increased TERT activity. This result is interesting and, at present, is unexplained. Perhaps the process of transfection itself and selection for neomycin resistance generated a subset of cells with higher telomerase activity. However, there was a dissociation between increased telomerase activity and 4NQO resistance measured by our assay. Transfection with vector or a mutant form of WRN produced no change or even increased the sensitivity of the cells to 4NQO toxicity. It would be useful to reproduce the effect in a second SV40-transformed Werner cell line or in primary Werner cell lines; nevertheless, the present assay provides a valuable measure of biological function. Two other putative Werner transformed fibroblast cell lines have been reported. One of them, AG07066/PSV811, was derived from a patient who had a normal *WRN* gene despite his clinical phenotype. This is so because sequencing of the *WRN* gene and its promoter region in this cell line by our group as well as others has identified a single common polymorphism (F1074L) but no functional mutation and because of the known expression of normal amounts of full-length WRN protein in this cell line (15, 17). Furthermore, in our assay, this cell line was no more sensitive to 4NQO than normal cell lines. In the case of the third Werner fibroblast line, WV (18), we were not able to obtain a *Mycoplasma*-free culture of this stable cell line or to produce retrovirally transfected stable cell lines from primary Werner fibroblasts, presumably due to their limited replicative capacity.

The observed effect may reflect not the WRN mutation but some second mutation acquired by the cell line. Nevertheless, these data, together with the previous study of Ogburn *et al.* (3) demonstrating 4NQO hypersensitivity of lymphoblastoid cell lines from 17 subjects either homozygous or heterozygous for WRN mutations, support the hypothesis that mutations in WRN render the cell preferentially sensitive to the effects of 4NQO, a chemical whose carcinogenic effects are mediated through an acetylated metabolite of 4NQO to produce bulky base damage, similar to the effects of UV radiation, but also

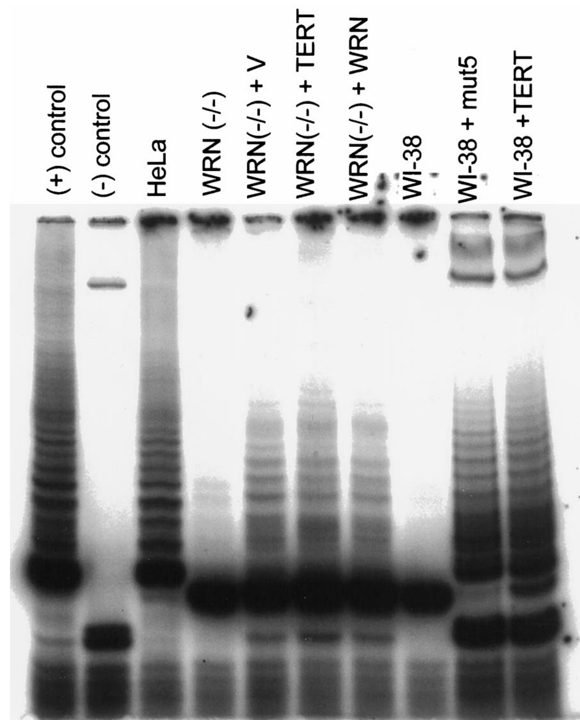

Fig. 4. Telomerase assay. The untransfected SV40 cell lines WRN(−/−) and WI-38 showed low levels of endogenous telomerase activity. Stable transfection with one of the constructs (vector, TERT, WRN, or WRN-mut5) resulted in equal increases of telomerase activity, which showed no correlation with 4NQO sensitivity.

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result in the formation of 8-hydroxyguanine, similar to oxidation-mediated DNA damage (19). As to why partial rather than full correction of the 4NQO sensitivity phenotype was observed, it may be that WRN, like its *Saccharomyces cerevisiae* homologue SGS1, normally acts in concert with a topoisomerase partner (20, 21) and that an imbalance in the ratio of WRN and topoisomerase may not function as well as in the native state. In studies of another RecQ homologue, the Bloom syndrome gene, correction of the pathognomonic elevated sister chromatid exchange rate was dependent on the level of expression of the ectopic gene, with only partial correction after two cell divisions, but stable introduction of the BLM cDNA yielded full correction of sister chromatid exchange frequency, poor growth, and S-phase defects (22).

There are several possible explanations for the observed effect of ectopic TERT in cells with endogenous telomerase. Hypothetically, forced expressed of TERT could select for 4NQO-resistant cells. Alternatively, there could be a critical threshold of TERT protein necessary for 4NQO resistance. However, a more plausible explanation is based on the observations of Burger *et al.* (23), who showed that treatment with the DNA-damaging apoptotic agent cisplatin resulted in a decrease in telomerase activity in human testicular cells. These results were extended when telomerase was shown to suppress apoptotic signaling in cultured pheochromocytoma cells (24). If endogenous TERT were down-regulated by 4NQO, then in our assay, the continued expression of ectopic telomerase could potentially overcome the apoptotic signal triggered by NQO treatment.

Recent evidence has suggested that telomerase has an unknown role in addition to its known function to maintain telomere length. In fibroblasts expressing ectopic human TERT, telomeres continue to shorten even as they proliferate well beyond the expected crisis point, suggesting that TERT allows cell proliferation by some mechanism other than net lengthening of telomeres (25). The results reported here suggest the possibility of a functional interaction not previously recognized that may provide a unique insight into some recent studies. For example, telomeric DNA ends by looping back on itself, its single-stranded terminus tucked back into the double-stranded DNA (26). Perhaps this unique chromatin structure acts as a sensor of DNA damage. It has been proposed that oxidative stress results in accelerated telomere shortening and inhibits proliferation of human fibroblasts (27). The mechanism of NQO damage at least partially resembles oxidative damage. One explanation of the failure to form colonies may be that oxidative damage or, by analogy, 4NQO-induced damage occurs preferentially at telomeres (28) and that WRN may play a role in protecting or maintaining telomeres. Alternatively, given that at least a portion of TERT RNA is in the nucleolus and the possible (yet controversial) nucleolar localization of WRN (9), it may be that TERT or WRN is recruited into the nucleolus in response to DNA damage. In conclusion, these results provide an assay system for a biological function of WRN and raise the possibility of relating two very divergent models for aging and carcinogenesis.

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Note Added in Proof

After submission of this paper, Wyllie *et al.* (29) reported that forced expression of telomerase in WS primary fibroblasts extends cellular life span.

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