Probes. These molecules have low photobleaching rates (10^{-6} to 10^{-8} per excitation), near-unity quantum yield in a semirigid environment, and an absorption cross section of 1.35×10^{-16} cm^2 at 532 nm, estimated by us on the basis of the measured absorption in methanol.
15. A quartz cover slip was first spin-coated with one drop (0.2 ml) of 0.1% by weight PMMA in chlorobenzene and then spin-coated with one drop of a 1-nM solution of $DiI_{12}C(12)$ in toluene. The nanomolar dye solution was freshly prepared from a micromolar dye solution with each sample.
16. Although the calculated saturation intensity is 1 MW cm^{-2}, in a separate study we found that the saturation of the fluorescent transition was determined by transitions from the excited singlet state to a metastable state, probably the triplet state, with an intersystem crossing rate of about 0.15% and a triplet lifetime of 0.4 ms.
17. The maximum fluorescence rate is $R_0 = \eta \Phi_F(\sigma I/h\nu)$, where σ is the absorption cross section at the laser frequency ν, Φ_F is the fluorescence quantum yield, I is the laser intensity, and η is the collection efficiency. In general, both η and Φ_F can depend on the emission dipole orientation. However, the 1.25-NA oil-immersion objective used here collected a calculated 65% of the total light emitted by a molecule at the PMMA-air interface [E. H. Helen and D. Axelrod, *J. Opt. Soc. Am. B* **4**, 337 (1987)], nearly independent of the emission-dipole orientation. The total collection or detection efficiency was 15%.
18. A focused laser beam has a very small longitudinal field component along the z axis, on the order of E/kw, where E is the tangential laser field, k is $2\pi/\lambda$, and w is the focused spot size [M. Lax, W. H. Louisell, W. B. McKnight, *Phys. Rev. A* **11**, 1365 (1975)].
19. The emission dipole, which lies along the long axis of the molecule that connects the two indole rings, is oriented 28° from the absorption dipole [D. Axelrod, *Biophys. J.* **26**, 557 (1979)].
20. The radiative lifetime for a broad molecular spectrum is proportional to the spectrally averaged inverse cube of the emission frequency [S. J. Strickler and R. A. Berg, *J. Chem. Phys.* **37**, 814 (1962)] or, to a small approximation, proportional to the cube of the peak fluorescence wavelength.
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22. For a dipole on the air side of the interface, $L_{\parallel}/L_{\infty}$ is 0.92, $L_{\perp}/L_{\infty}$ is 1.6, and the radiative lifetime is decreased for a perpendicular emission dipole.
23. Aliphatic hydrocarbon immersion oil, type FF; Cargille Laboratories, Cedar Grove, NJ.
24. We deposited several micrometers of PMMA by spin-coating several drops of a solution of 5% by weight PMMA in toluene and heating the sample to dry.
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Positional Cloning of the Werner's Syndrome Gene

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Werner's syndrome (WS) is an inherited disease with clinical symptoms resembling premature aging. Early susceptibility to a number of major age-related diseases is a key feature of this disorder. The gene responsible for WS (known as *WRN*) was identified by positional cloning. The predicted protein is 1432 amino acids in length and shows significant similarity to DNA helicases. Four mutations in WS patients were identified. Two of the mutations are splice-junction mutations, with the predicted result being the exclusion of exons from the final messenger RNA. One of these mutations, which results in a frameshift and a predicted truncated protein, was found in the homozygous state in 60 percent of Japanese WS patients examined. The other two mutations are nonsense mutations. The identification of a mutated putative helicase as the gene product of the WS gene suggests that defective DNA metabolism is involved in the complex process of aging in WS patients.

Werner's syndrome is a rare autosomal recessive disorder that is considered a partial model of human aging (*1*–*3*). WS patients prematurely develop a variety of the major age-related diseases, including several forms of arteriosclerosis, malignant neoplasms, type II diabetes mellitus, osteoporosis, and ocular cataracts; these individuals also manifest early graying and loss of hair, skin atrophy, and a generally aged appearance. Growth retardation occurs, typically around the time of puberty, but medical problems are rare during childhood. Cell culture studies also suggest a parallel between WS and aging; the replicative life-span of fibroblasts from WS patients is reduced compared with age-matched controls and is similar to the life-span of fibroblasts taken from more elderly individuals (*4*). However, some prevalent geriatric disorders such as Alzheimer's disease and hypertension are not observed in WS. Moreover, there are subtle discordances between WS and normal aging, such as a disproportionately severe osteoporosis of the limbs relative to the trunk and the high prevalence of nonepithelial neoplasms in WS. Finally, there are unusual clinical features unrelated to aging, including ulcerations around the ankles and soft tissue calcification (*1*, *2*).

The WS locus (*WRN*) was initially localized to 8p12 (*5*) by linkage analysis and the genetic position refined by both meiotic and homozygosity mapping (*5*–*7*). Initial mapping (*6*–*8*) placed *WRN* in an 8.3-centimorgan (cM) interval flanked by markers D8S137 and D8S87 (Fig. 1); D8S339, located within this interval, was the closest marker. Subsequently, short tandem repeat polymorphism (STRP) markers at the glutathione reductase (*GSR*) gene and D8S339 were shown to be in linkage disequilibrium with WS in Japanese WS patients (*9*, *10*), indicating that these markers are most likely close to *WRN*.

To clone the WS gene, we generated a map from yeast artificial chromosomes (YACs), P1 clones, and cosmid contigs (Fig. 1), starting at *GSR* and extended by walking methods to cover approximately 3 Mb (*11*). Eighteen STRP markers (Fig. 1B) were identified in the contig; probable recombinants were detected at D8S2194 (which excluded the region telomeric to this marker) and at D8S2186 [which excluded the region centromeric to this marker (*12*)], making the 1.2 to 1.4 Mb interval

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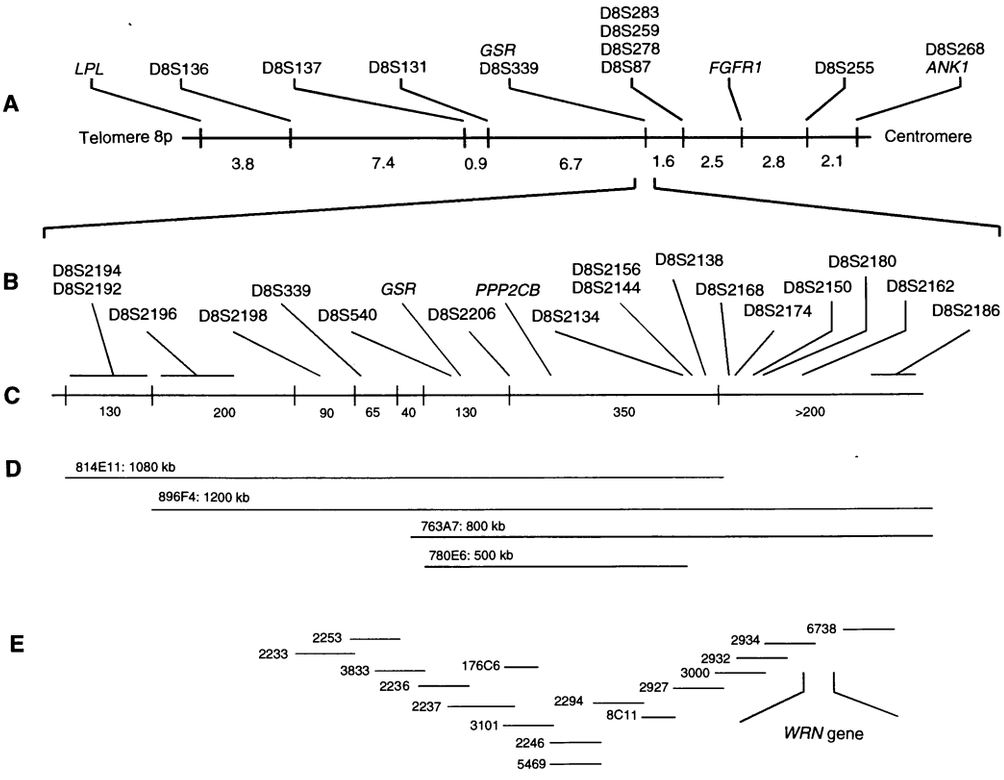

Fig. 1. Genetic and physical maps of the *WRN* region. (**A**) The genetic map units are in centimorgans, assuming sex-equal recombination rates (*8*). (**B**) The polymorphic loci are STRP markers except for *PPP2CB* and D8S2180 which are bi-allelic insertion-deletion polymorphisms (*11*). The STRP loci at *GSR* and *PPP2CB* are D8S2202 and D8S2208, respectively. (**C**) The physical map has approximate distances in kilobases determined from sizes of overlapping nonchimeric YACs, and from genomic DNA sequence from overlapping P1 clones 2233, 2253, 3833, 2236, and 3101. Marker and clone order was determined from the sequence-tagged site (STS) content of YACs, P1 clones, and cosmid clones and from genomic DNA sequence. (**D**) The YACs represent the minimal number of YACs to cover the *WRN* region and are the YACs used for cDNA selection experiments. The small insert clones shown in (**E**) are the minimum number needed for overlapping coverage of the *WRN* region and are P1 clones except for 8C11 and 176C6, which are cosmids. A total of 20 P1 clones and 109 cosmid clones were identified from the D8S2194 to D8S2162 region. The complete genomic sequence (*30*) was determined for eight P1 clones (2233, 2253, 3833, 2236, 2237, 2932, 2934, and 6738) and one cosmid clone(176C6). Partial sequences were obtained for three P1 clones (2234, 2927, and 3000), and one mouse P1 clone (5469) was completely sequenced.

from D8S2194 to D8S2186 (Fig. 1C) the minimal *WRN* region. Significant evidence for linkage disequilibrium ($P < 0.03$) between single markers and WS in a Japanese patient population was obtained for 10 [D8S2198, D8S2196, D8S339, D8S540 (GSR2), D8S2202 (GSR1), D8S2206, D8S2134, D8S2162, D8S2174, and D8S2180] of the 18 STRP loci in the interval (*12*).

Potential expressed sequences in the interval between D8S2192 to D8S2186 were identified by exon trapping, hybridization of complementary DNA (cDNA) libraries to immobilized YACs, comparison of the genomic sequences to DNA sequence databases by means of BLAST, and analyzing genomic sequence with the exon-finding computer program GRAIL (*13*). BLAST searches of the Eukaryotic Promotor Database (*14*) were performed to identify potential regulatory elements. Each gene detection method identifies short segments of expressed sequences, which can then be used to screen an arrayed fibroblast cDNA library (*15*) to identify longer cDNA clones. Genes identified by this process were screened for *WRN* mutations by sequencing reverse transcriptase–polymerase chain reaction (RT-PCR) products (*16*). Before identification of the *WRN* gene, GSR, protein phosphatase 2 catalytic subunit β (*PPP2CB*), general transcription factor IIEβ (*GTF2E2*), a β-tubulin pseudogene 1 (*TUBBP1*), and six genes of unknown function were screened for mutations and none were detected. WS patients were also screened for mutations in 14 GRAIL-predicted exons and three putative promotor elements by sequencing PCR amplification fragments generated from the genomic DNA from WS and normal individuals.

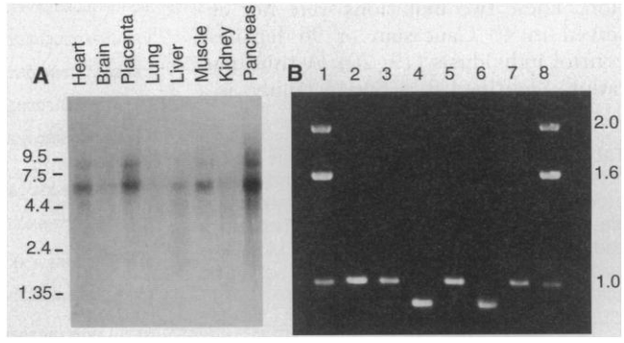

Fig. 2. Expression and DNA sequence of the *WRN* gene. (**A**) A Northern blot (Clontech) of multiple tissues, prepared with 2 μg of polyadenylated RNA per lane, was hybridized with a 1970-bp probe generated from the WRN helicase cDNA clone by using primers K (5′-AGTGCAGTGGTGTCATCATAGC-3′) and L (5′-CCTATTTAATGGCACCCAAAATGC-3′). The filter was hybridized at 42°C in 50% formamide for 16 hours and washed twice in 1× standard saline citrate (SSC) and 0.1% SDS at room temperature, followed by two washes in 0.1× SSC and 0.1% SDS at 65°C (30 min each wash). (**B**) RT-PCR products (*16*) were produced with primers K and N (5′-ACCGCTTGGGATAAGTGCATGC-3′) and the products resolved by agarose gel electrophoresis. Lanes 1 and 8, 1-kb ladder size standard (Life Technologies); lane 2, Japanese normal control; lane 3, SY family, Japanese WS, mutation 2; lane 4, ZM family, Japanese WS, mutation 4; lane 5, Japanese normal control; lane 6, MCI family, Japanese WS, mutation 4; lane 7, Caucasian WS. RNA for RT-PCR was from lymphoblastoid cell lines (lanes 2 to 4) or from fibroblast cultures (lanes 5 to 7). Molecular sizes are indicated to the sides of (A) and (B) in kilobases.

The candidate *WRN* gene was detected by means of the genomic sequence of P1 clone 2934, which was used to search the expressed sequence tag (EST) database. A 245–base pair (bp) EST (R58879) was identified that was identical to three regions of genomic sequence of 145, 94 (a complete exon), and 6 bp in length, separated in the genomic sequence by two introns (81 and 851 bp in length). An

exon of the same gene was also identified by exon trapping. The sequence from R58879 was used to identify longer cDNA clones (*17*). Northern (RNA) blot analysis showed that R58879 is expressed; transcripts of ~5.8 and 8.1 kb were detected in all tissues tested (Fig. 2A). The predominant smaller transcript was present at highest quantities in pancreas, followed by placenta, muscle, and heart. Transcripts were also detected in RT-PCR products from fibroblast and lymphoblastoid cell line RNA (Fig. 2B). The completed sequence was assembled from 12 additional overlapping cDNA clones and by 5′-RACE experiments (*17*) to yield a 5.2-kb final sequence (GenBank accession number L76937).

Four mutations in WS patients were detected in the gene corresponding to R58879 (Table 1). Two mutations were nonsense mutations creating premature stop codons (mutations 1 and 2). Four Japanese WS patients, the offspring of first cousin marriages, and one Caucasian, from a second cousin marriage (*18*), were homozygous for the Arg1305TGA mutation (mutation 1) (where the arginine codon at position 1305 is mutated to stop codon TGA). The Gln1165TAG mutation (mutation 2) was found in one Japanese patient who is the offspring of a first cousin marriage and homozygous for the mutation. These two mutations were not observed in 48 Caucasian or 96 Japanese control individuals (*19*, *20*). A third mutation, identified in a Syrian family, is a 4-bp deletion spanning a splice junction (mutation 3). In this kindred, three sibs with WS are homozygous for this mutation. A fourth sib, aged 21, is homozygous for the same mutation but too young for a definitive diagnosis of WS (*1*, *2*). Although these individuals are not from a consanguineous marriage, they do share the same haplotype across the WS region (*12*). This mutation was not observed in 96 Caucasian or 48 Japanese control individuals (*19*, *20*).

A fourth mutation was first detected as a RT-PCR product that was 95 bp shorter than products from other WS and control individuals (Fig. 2B). Comparison of the RT-PCR product sequence and the genomic sequence from P1 clone 2934 revealed that the missing 95 bp corresponded to a single exon. The missing exon and flanking genomic segments were sequenced from the WS patient and control individuals, and a single base change of G→C was detected that changes a splice donor sequence from ApG to ApC (mutation 4) and results in a frame shift of codons 1078 to 1092. This WS patient is the offspring of a first cousin marriage and is homozygous for this mutation. For 30 Japanese kindreds examined, affected individuals from 18 of the kindreds are homozygous for mutation 4. In three of these families two affected sibs were sampled, and in each case both are homozygous. Among mutation carriers, 12 of 16 have the 141-bp allele at the GSR2 STRP, which is overrepresented in WS cases (frequency = 0.40) and relatively rare in Japanese control individuals (frequency = 0.07) (*10*). The association of this allele with WS was responsible for the initial detection of linkage disequilibrium in this region (*9*). This mutation was not observed in 48 Caucasian WS patients. In 187 Japanese control individuals, one heterozygote was observed for an estimated gene frequency of 0.003 (*19*, *20*), which is comparable with gene frequency estimates (0.001 to 0.005) based on WS prevalence rates and consanguinity estimates (*2*, *21*).

The protein predicted from the cDNA sequence is 1432 residues in length (Fig. 3) and is highly similar to DNA helicases from a wide range of organisms (Fig. 4). All seven helicase consensus domains are present, including the nucleotide binding

Table 1. WS mutations. Splice junctions are denoted by a double-headed arrow (↔) and deleted bases with a hyphen (-). Mutated or deleted bases are in bold. Intronic sequence is in small letters and exonic sequence in capital letters. Mut., mutation; Ind., individual; Norm., normal; aa, amino acids.

Mut.	Ind.	Nucleotide sequence	Predicted protein length (aa)
1		LeuGluArgAla	1304
	Norm.	TTGGAGCGAGCA	
		↓	
	WS	TGA	
2		AlaArgGlnLys	1164
	Norm.	GCTAGGCAGAAA	
		↓	
	WS	TAG	
3		ThrAspLeuPhe	1392
	Norm.	ctgtag↔ACAGACCTCTTT	
		↓	
	WS	ctgt--↔--AGACCTCTTT	
4		GlyArgAsn	1060
	Norm.	ttttaatag↔GGTAGAAAT	
		↓	
	WS	**c**	

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MSEKKLETTAQQRKCPEWMNVQNKRCAVEERKACVRKSVFEDDLPFLEFTGSIVYSYDAS   60
DCSFLSEDISMSLSDGDVVGFDMEWPPLYNRGKLGKVALIQLCVSESKCYLFHVSSMSVF  120
PQGLKMLLENKAVKKAGVGIEGDQWKLLRDFDIKLKNFVELTDVANKKLKCTETWSLNSL  180
VKHLLGKQLLKDKSIRCSNWSKFPLTEDQKLYAATDAYAGFIIYRNLEILDDTVQRFAIN  240
KEEEILLSDMNKQLTSISEEVMDLAKHLPHAFSKLENPRRVSILLKDISENLYSLRRMII  300
GSTNIETELRPSNNLNLLSFEDSTTGGVQQKQIREHEVLIHVEDETWDPTLDHLAKHDGE  360
DVLGNKVERKEDGFEDGVEDNKLKENMERACLMSLDITEHELQILEQQSQEEYLSDIAYK  420
   | Repeat 1                  | Repeat 2
STEHLSPNDNENDTSYVIESDEDLEMEMLKHLSPNDNENDTSYVIESDEDLEMEMLKSLE  480
NLNSGTVEPTHSKCLKMERNLGLPTKEEEEDDENEANEGEEDDDKDFLWPAPNEEQVTCL  540
                                I                          Ia
KMYFGHSSFKPVQWKVIHSVLEERRDNVAVMATGYGKSLCFQYPPVYVGKIGLVISPLIS  600
LMEDQVLQLKMSNIPACFLGSAQSENVLTDIKLGKYRIVYVTPEYCSGNMGLLQQLEADI  660
    II                                 III
GITLIAVDEAHCISEWGHDFRDSFRKLGSLKTALPMVPIVALTATASSSIREDIVRCLNL  720
                                                IV
RNPQITCTGFDRPNLYLEVRRKTGNILQDLQPFLVKTSSHWEFEGPTIIYCPSRKMTQQV  780
                                    V
TGELRKLNLSCGTYHAGMSFSTRKDIHHRFVRDEIQCVIATIAFGMGINKADIRQVIHYG  840
      VI
APKDMESYYQEIGRAGRDGLQSSCHVLWAPADINLNRHLLTEIRNEKFRLYKLKMMAKME  900
KYLHSSRCRRQIILSHFEDKQVQKASLGIMGTEKCCDNCRSRLDHCYSMDDSEDTSWDFG  960
PQAFKLLSAVDILGEKFGIGLPILFLRGSNSQRLADQYRRHSLFGTGKDQTESWWKAFSR 1020
                        Missing exon, Mutation 4
QLITEGFLVEVSRYNKFMKICALTKKGRNWLHKANTESQSLILQANEELCPKKFLLPSSK 1080
          * ← Location of the premature stop codon for mutation 4
TVSSGTKEHCYNQVPVELSTEKKSNLEKLYSYKPCDKISSGSNISKKSIMVQSPEKAYSS 1140
                    * ← Mutation 2 (stop codon)
SQPVISAQEQETQIVLYGKLVEARQKHANKMDVPPAILATNKILVDMAKMRPTTVENVKR 1200
                              Missing exon, Mutation 3
IDGVSEGKAAMLAPLLEVIKHFCQTNSVQTDLFSSTKPQEEQKTSLVAKNKICTLSQSMA 1260
__________ Mutation 1 (stop codon) → *
ITYSLFQEKKMPLKSIAESRILPLMTIGMHLSQAVKAGCPLDLERAGLTPEVQKIIADVI 1320
RNPPVNSDMSKISLIRMLVPENIDTYLIHMAIEILKHGPDSGLQPSCDVNKRRCFPGSEE 1380
ICSSSKRSKEEVGINTETSSAERKRRLPVWFAKGSDTSKKLMDKTKRGGLFS  1432
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Fig. 3. Predicted protein sequence of the *WRN* gene product (*22*). Helicase domains I through VI are in bold type, and their location was adapted from previously described helicase domain alignments (*31*). The amino acids corresponding to the exon missing from RT-PCR products from mutation 4 individuals are overlined. The locations of mutations 1 and 2 are noted with asterisks. The exon presumed to be missing as a result of the mutation 3 splice junction deletion is also overlined. Exon boundaries are from the genomic sequence of P1 clone 2934.

motif in domain I and the DExH sequence (*22*) (where X is any amino acid) in domain II. Across the seven helicase domains, the WRN predicted protein is 62 to 64% identical to the human *RECQL* and *Escherichia coli recQ* gene products and putative helicases from *Caenorhabditis elegans* and yeast (Sgs1p). In addition to the seven-domain region, at the 3′ end of the gene there are regions of limited similarity between the *WRN* gene product and the *C. elegans* putative helicase F18C5.2 and the *E. coli* RecQ protein (*23*). The WRN protein also contains a 98–amino acid acidic region that includes 13 aspartates or glutamates in a stretch of 17 amino acids (Fig. 3); the same region contains a 27–amino acid repeat that is a perfect duplication at the nucleotide level.

As a putative helicase, the WRN protein could be involved in DNA replication, recombination, chromosome segregation, DNA repair, transcription, or other functions requiring DNA unwinding. Indicators of defective DNA metabolism in WS include chromosomal instability, an elevated mutation rate at specific genes, elevated rates of nonhomologous recombination, decreased accuracy of ligation of disrupted plasmids, decreased repair rate of telomeres, rapid decrease in telomere length, and possibly altered DNA replication (*24*). In contrast, WS cells do not show an increased susceptibility to ultraviolet exposure or to other DNA damaging agents, do not appear to be defective in nucleotide excision repair, and do not show elevated sister chromatid exchange (*25*). These characteristics set WS apart from other disorders (xeroderma pigmentosum, Cockayne's syndrome, and Bloom's syndrome) for which inherited defects have been identified in helicases potentially involved in DNA repair or chromosome exchange events (or both) (*26*). However, a cryptic DNA repair defect in WS cannot be ruled out, possibly because the appropriate DNA-damaging conditions have not been tested, or because other partially redundant repair systems may exist. For example, *E. coli recQ* mutants only show recombination defects and DNA damage sensitivity when other helicases (such as RecBC) are absent (*27*). An alternate hypothesis to a DNA repair deficiency is that the *WRN* defect leads directly to DNA damage and mutations. For example, the *Saccharomyces cerevisiae* Sgs1p helicase (Fig. 4), as part of a topoisomerase complex, functions to decatenate intertwined chromosomes; mutations in the gene *SGS1* lead to hyperrecombination between repeated sequences with the deletion of intervening DNA, chromosome breakage, and nondisjunction (*28*).

The consequence of the WS defect in DNA metabolism may be the accumulation of DNA mutations, leading to the age-related diseases observed in WS. Disease susceptibility in WS could be the result of mutations at specific genes. For example, the increased incidence of neoplasia in WS may be caused by somatic mutations at oncogenes and tumor suppressor genes. Mutations in *WRN* may also play a role in tumorigenesis in non-WS individuals because this gene is located in one of two chromosome 8p regions where loss-of-heterozygosity has been observed in tumors (*29*). A more general mechanism of disease susceptibility in WS may be accumulated DNA damage that leads to premature replicative senescence and subsequent pathology. Whatever the specific mechanisms involved in the WS phenotype, identification of the WS gene now

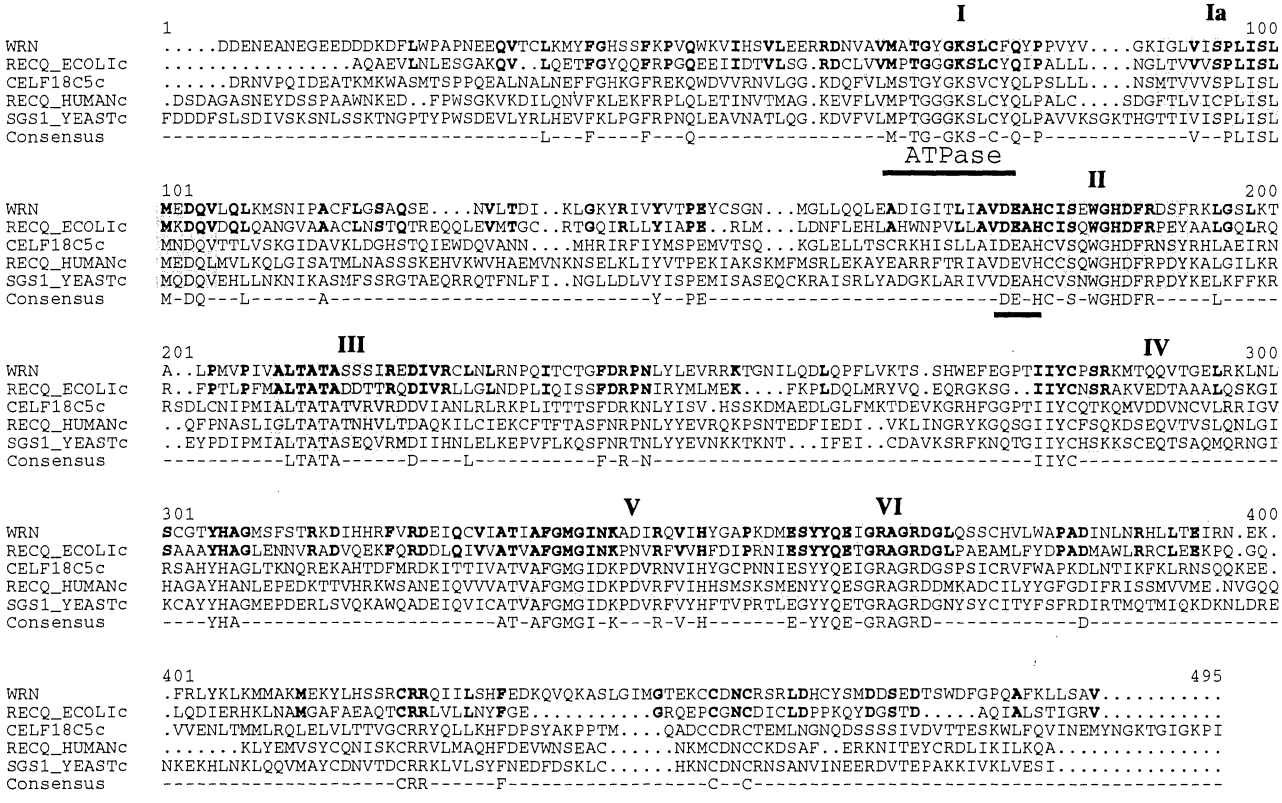

Fig. 4. Protein alignment of genes showing homology to predicted protein WRN (*22*). Helicase domains are marked as shaded regions (*31*). F18C5.2 (GenBank entry CELF18C5) is a predicted gene from a *C. elegans* cosmid that has been sequenced (*32*). When the predicted WRN protein was aligned to F18C5.2, one region, highly conserved in other helicases, was missing. The missing region was identified by aligning the *WRN* gene to the genomic sequence between exons 6 and 7. Manual inspection of the alignment led to the identification of a missing exon (nucleotides 10240 through 10427), which was included in the F18C5.2 sequence shown. Sequences for human RECQL and *E. coli* RecQ proteins and yeast Sgs1p are from GenBank. Numerous other helicases (not shown here) also showed significant homology.

provides evidence that at least some components of "normal" aging and disease susceptibility in late life may be related to aberrations in DNA metabolism.

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16. RT-PCR products synthesized from RNA (Qiagen Oligotex, Qiagen, Chatsworth, CA) prepared from affected WS and control individuals were amplified with a variety of primers and the PCR products cycle-sequenced with the dye terminator cycle sequencing kit (Perkin-Elmer). In other experiments, RT-PCR products were gel purified, reamplified with the same primers, and sequenced with a U.S. Biochemical Sequenase PCR Product sequencing kit.
17. *WRN* clones were from a normal fibroblast cell line cDNA library that was primed with polyadenylate and cloned in Lambda ZAP pBK-CMV (Stratagene). The library was arrayed for PCR screening (*15*) and could be screened either by PCR amplification or by conventional plaque hybridization. The initial 2.1-kb cDNA clone containing the EST R58879 and corresponding to the 3′ end of the gene was obtained by PCR screening with primers A (5′-ACTGGCAAGGATCAAACAGAGAG-3′) and B (5′-CTTTATGAAGCCAATTTCTACCC-3′), which were designed from the DNA sequence of R58879 and produce a 145-bp fragment from *WRN* cDNA clones. To obtain longer clones, we designed primers 5EA (5′-GAACTTTGAAGTCCATCACGACC-3′) and 5EB (5′-GCATTAATAAAGCTGACATTCGCC-3′) from a GRAIL-predicted exon located 5′ in the P1 clone 2934 genomic sequence to exons containing the initial 2.1-kb clone. Primers 5EA and 5EB produce a 110-bp fragment from genomic or cDNA clones, and six additional clones were obtained. An additional eight clones were obtained by plaque hybridization of the same library. The longest clone produced by these methods was 4.0 kb. Additional sequence was obtained by 5′ rapid amplification of cDNA ends (5′-RACE) experiments. We used primer 5EA to synthesize first-strand cDNA by reverse transcriptase (SuperScript II RT, Life Technologies). RNA was removed by ribonuclease H, and single-strand DNA was purified with a GlassMAX spin cartridge (Life Technologies). A deoxycytidine tail was added to the 3′ end of the DNA by using terminal deoxynucleotide transferase. After heat inactivation of the terminal transferase (70°C, 10 min), the resulting DNA (5 μl) was amplified with a nested primer 5EM (5′-TCGATCAAAACCAGTACAGGTG-3′) and the 5′-RACE anchor primer (5′-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′, Life Technologies). A 2.5-kb product was obtained that contained an additional 1.4 kb of unique 5′ sequence.
18. The WS patients were from an International Registry of Werner's Syndrome (G.M.M.). The diagnostic criteria and the ethnic origins of the individuals are as previously described (*7, 12*). Contributors to the registry are S. S. Agarwal, F. Amato, P. Amblard, J. Anders, R. Anschuetz, M. Y. Apak, S. Balci, M. Biancalana, T. D. Bird, J. Bonar, A. Braganza, I. Bournerias, J. Chevrant Breton, W. T. Brown, G. Burg, D. P. Cavalcanti, C. Danesino, V. Dumas, J. Ellis, C. J. Epstein, W. Fischer, M. Fraccaro, K. Fukuchi, Y. Fujiwara, P. Garnier, S. Gilgenkranz, E. Hachulla, T. J. Harrison, H. Hoehn, Y. Hosokawa, A. F. Hurlimann, J. Jabkowski, K. Kamino, H. Kayserili, S. Kiso, G. Klein, J. Lamit, D. Lewis, H. Little, M. C. Martin, P. Martinet, K. Marumo, M. I. Melagrano, W. Mills, A. Mohan, A. Motulsky, M. Mumenthaler, S. Murano, N. Marakami, J. Matthews, P. Modiano, Tsukasa Murakami, O. Nikaido, G. Natchiar, T. Ogihara, S. Ouais, A. Partalcl, F. Pasquali, N. Philip, M. Poot, C. Puissan, J. Revuz, M. W. Rizzo, C. Rubin, T. Saida, K. R. Sathish, S. Scappaticci, J. Schmidtke, K. Singh, R. Singh, M. W. Steele, V. P. Sybert, C. Tannock, J. Taziri, B. Uyeno, A. Verloes, A. Wakayama, and M. Yuksel.
19. Control individuals were screened for mutations 1, 2, and 4 (Table 1) by DNA sequence analysis of PCR products amplified from genomic DNA. For mutation 1, primers E8A (5′-GATGTGACAGTGGAAGCTATGG-3′) and E8B (5′-GGAAAAATGTGGTATCTGAAGCTC-3′) were used to produce a 267-bp product. Both strands were sequenced by using the same primers. The same procedure was used for mutation 2 [primers E11A (5′-TAAAGGATTAATGCTGTTAACAGTG-3′) and E11B (5′-TCACACTGAGCATTTACTACCTG-3′)] and mutation 4 [primers E4A (5′-CTTGTGAGAGGCCTATAAACTGG-3′) and E4B (5′-GGTAAACAGTGTAGGAGTCTGC-3′)], which produced 360- and 267-bp fragments, respectively. Some control individuals were also tested for mutations by a PCR mismatch amplification assay. Exons were PCR-amplified by exon primers in 20-μl reaction. The entire mixture was then diluted into 200 μl of distilled water. From the dilution mixture 2 μl were used for mismatch PCR. For each point mutation, two mismatched primers were designed in which the 3′ end of the primer corresponds to either wild-type (WT) or mutant (MT) nucleotide. Mismatch PCR is prepared in two separate tubes with the combination of one mismatched primer and one exon primer. PCR was then carried out under high-stringency conditions for 30 cycles. The mismatched primer sequences and PCR conditions were as follows: Exon 4 WT primer E4D (5′-CTTTATGAAGCCAATTTCTACCCT) together with primer E4B amplify a 106-bp fragment under conditions of annealing temperature (T_A) = 63°C, 1.0 mM Mg and pH 9.5. Exon 4 MT primer E4C (5′-TAAAAGATCCTTTTTGCTTTTAATAC) together with primer E4A amplify a 212-bp fragment under conditions of T_A = 63°C, 1.5 mM Mg, and pH 10.0.
20. An upper confidence bound on the allele frequency, P, when no mutations are found, can be calculated by means of the equation $(1 - P)^N = \alpha$, where N is the number of chromosomes tested, and α is the desired significance level. For mutations 1 to 3, 95% upper confidence limits are 0.03 and 0.015, based on zero observed mutations in 48 and 96 independent controls, respectively. For mutation 4, based on one heterozygote in 178 controls, and assuming a Poisson distribution, the standard deviation is equivalent to the estimated allele frequency (0.0027), giving a 95% upper confidence limit of 0.008.
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30. P1 DNA for PCR analysis was prepared by the standard alkaline lysis method followed by phenol-chloroform extraction and ethanol precipitation [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning, a Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989), pp. 1.25–1.28] and then by CsCl density gradient centrifugation in the presence of ethidium bromide. For sequencing, P1 clones were randomly fragmented and subcloned into an M13 phage–derived vector. Single-stranded template DNA from recombinant M13 clones was prepared by a standard miniprep procedure. DNA quality and yields were determined by agarose gel electrophoresis and by measuring absorbance at 260 nm. Dideoxynucleotide sequencing was performed with *Taq* DNA polymerase and fluorescently labeled dye primers. DNA sequences were determined with a fluorescent DNA sequencer (ABI 373A).
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