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Mutations in the Consensus Helicase Domains of the Werner Syndrome Gene

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Summary

Werner syndrome (WS) is an autosomal recessive disease with a complex phenotype that is suggestive of accelerated aging. WS is caused by mutations in a gene, WRN, that encodes a predicted 1,432-amino-acid protein with homology to DNA and RNA helicases. Previous work identified four WS mutations in the 3′ end of the gene, which resulted in predicted truncated protein products of 1,060–1,247 amino acids but did not disrupt the helicase domain region (amino acids 569–859). Here, additional WS subjects were screened for mutations, and the intron-exon structure of the gene was determined. A total of 35 exons were defined, with the coding sequences beginning in the second exon. Five new WS mutations were identified: two nonsense mutations at codons 369 and 889; a mutation at a splice-junction site, resulting in a predicted truncated protein of 760 amino acids; a 1-bp deletion causing a frameshift; and a predicted truncated protein of 391 amino acids. Another deletion is >15 kb of genomic DNA, including exons 19–23; the predicted protein is 1,186 amino acids long. Four of these new mutations either partially disrupt the helicase domain region or result in predicted protein products completely missing the helicase region. These results confirm that mutations in the *WRN* gene are responsible for WS. Also, the location of the mutations indicates that the presence or absence of the helicase domain does not influence the WS phenotype and suggests that WS is the result of complete loss of function of the *WRN* gene product.

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Introduction

Werner syndrome (WS), a rare autosomal recessive disorder, is a potential model of accelerated human aging (Werner 1904; Thannhauser 1945; Epstein et al. 1966; Goto et al. 1978, 1981). WS subjects prematurely appear old, with graying and loss of hair typically beginning in the 2d decade of life. Scleroderma-like skin changes and voice alterations contribute to the general impression of accelerated aging. WS clinical features encompass a wide range of age-related pathology including arteriosclerosis, osteoporosis, diabetes mellitus, lenticular cataracts, and several types of benign and malignant neoplasms (Werner 1904; Thannhauser 1945; Reed et al. 1953; Riley et al. 1965; Epstein et al. 1966; Goto et al. 1978, 1981, 1996; Salk 1982; Tollefsbol and Cohen 1984). These diseases become prevalent in WS subjects in the 3d and 4th decades of life. The typical cause of death is either cancer or cardiovascular disease and occurs at a median age of 47 years (Epstein et al. 1966). Other WS features include short stature, hyperpigmentation, hyperkeratosis, tight skin, telangiectasia, a bird-like facies, subcutaneous atrophy, recalcitrant leg ulcers, blood-vessel calcification, hypogonadism, and reduced fertility in both sexes. Anecdotal evidence suggests that heterozygotes may also show some minor progeroid features such as early graying of hair (Epstein et al. 1966) and higher rates of malignancy (Goto et al. 1981, 1996). Laboratory findings include excess hyaluronic acid excretion in the urine, which is also found in progeria (Hutchinson-Gilford syndrome), a disease with a much earlier age at onset (Kieras et al. 1986).

Cellular studies also suggest accelerated aging in WS. Fibroblast cultures from young donors have a greater replicative potential than do cultures from elderly donors (Hayflick 1965; reviewed by Tollefsbol and Cohen 1984). Compared to cultures from age-matched controls and elderly subjects, WS cells grow slower, develop a senescent morphology earlier, and cease replication sooner, typically after only 10–20 population doublings (Martin, et al. 1970; Salk et al. 1981; Salk 1982; Tollefsbol and Cohen 1984). Thus, at least in culture, WS

fibroblasts behave more like cultures from normal elderly subjects than cultures from age-matched controls. The accelerated replicative senescence of WS fibroblasts could be related to the dermal atrophy observed in vivo.

The reduced replicative potential may be related to genomic instability, which is also part of the WS phenotype. Elevated rates of chromosomal rearrangements and deletions have been observed in vitro in fibroblast cultures (Hoehn et al. 1975; Salk et al. 1981; Salk 1982) and in vivo in fibroblasts and lymphocytes (Scappaticci et al. 1982; Salk et al. 1985). Another indication of genomic instability is the observed elevation of somatic mutation rates in WS cells (Fukuchi et al. 1985, 1989, 1990; Monnat et al. 1992). An elevation in spontaneous somatic mutation rates at the hypoxanthine phosphoribosyltransferase gene has been observed both in vitro by use of SV-40–transformed fibroblasts (Fukuchi et al. 1985, 1989) and in vivo in peripheral blood lymphocytes (Fukuchi et al. 1990). Deletions appear to be the dominant mutation type observed (Fukuchi et al. 1989; Monnat et al. 1992).

Some aspects of the WS phenotype are discordant with "normal" aging. For example, the ratio of epithelial to nonepithelial cancers is ~1:1 in WS versus 10:1 in the general population (Goto et al. 1996). The predominant cancers in WS are soft-tissue sarcoma, osteosarcoma, myeloid disorders, thyroid cancer, and benign meningiomas. Another discordant feature is the apparent lack of hypertension in WS subjects. Also, the central nervous system may not be affected. Autopsies of two WS subjects, 51 and 57 years old, who were not demented at the time of death, revealed no neuritic plaques, neurofibrillary tangles, amyloid deposits, or lipofuscinosis (Sumi 1985). However, a single case of WS with extensive cerebral atrophy (Kakigi et al. 1992) and another with spastic paraparesis (Umehara et al. 1993) have been reported. Additional studies of dementia in elderly WS subjects and additional autopsies are needed.

Recently, we reported the positional cloning of the WS gene (*WRN*) (Yu et al. 1996*a*). The predicted *WRN* protein consists of 1,432 amino acids with a central domain containing seven motifs, (I, Ia, II, III, IV, V, and VI), which are signatures of the DNA and RNA superfamily of helicases (Gorbalenya et al. 1989; fig. 1). The presence of the DEAH sequence and an ATP-binding motif (Gorbalenya 1989) further suggests that the *WRN* gene product is a functional helicase. The *WRN* helicase domains share significant homology with *Escherichia coli RecQ, Saccharomyces cerevisiae Sgs1,* human *RECQL* gene, and other helicases (Yu et al. 1996*a*). However, the N-terminal and C-terminal domains of the predicted protein show no similarity to other helicases or to any previously described protein. Since many helicases function as part of a multiprotein complex, the N-terminal and/or the C-terminal domain may contain interaction sites for these other proteins, while the central helicase domain functions in the actual enzymatic unwinding of DNA or RNA duplexes.

Initially, four different mutations in the C-terminal domain of *WRN* were identified. These mutations accounted for >83% of the Japanese WS patients in our collection. All four C-terminal domain mutations result in a predicted protein containing an intact helicase domain. We screened additional WS subjects to determine whether mutations that affected the helicase region of the gene could be identified. To facilitate identification of additional mutations, we determined the intron-exon structure of the *WRN* gene. The genomic structure information was then used to prepare PCR-primers for amplifying each exon so that mutational analysis of additional WS subjects could be performed. Five additional *WRN* mutations are described: two are located in the consensus helicase motifs, and another two are predicted to produce truncated proteins without the helicase domains. These mutations suggest that, in at least some WS subjects, the enzymatic helicase activity is destroyed and argues that WS is the result of complete loss-of-function of the *WRN* gene product.

Material and Methods

Cosmid Clones for WRN

Cosmid clones were isolated from a chromosome 8–specific cosmid library LA08NC01 (provided by Larry Deaven, Los Alamos National Laboratory; Wood et al. 1992). This library was arrayed for PCR screening as described by Amemiya et al. (1992). *WRN*-containing cosmids were identified using primer sets 5E6/5EY, 5ED/5E12, and CD-A/CD-B (table 1), which were derived from the *WRN* cDNA sequence (Yu et al. 1996*a;* GenBank accession no. L76937).

Genomic Structure of WRN

The genomic structure of the 3′ end of *WRN* (nt 2680–5208 of GenBank accession no. L76937) was originally defined from the complete sequence of P1 clone 2934 (Yu et al. 1996*a*). The remaining *WRN* genomic structure was determined by direct cycle sequencing of *WRN*-containing cosmids with a dye-terminator cycle-sequencing kit (Perkin Elmer) and an ABI373 DNA sequencer. Primers derived from the *WRN* cDNA sequence were used for the initial sequencing of cosmid clones. The resulting sequence was compared to the cDNA sequence to identify intron-exon boundaries. Sequencing primers were then designed from the intronic sequences to sequence in the reverse direction and to obtain the second boundary defining the intron-exon junction. This strategy was used to define the exons not in P1 clone 2934. Once intronic sequences were available from both ends of an exon, primers complementary to flanking intronic sequences could be designed to amplify each exon for mutation screening.

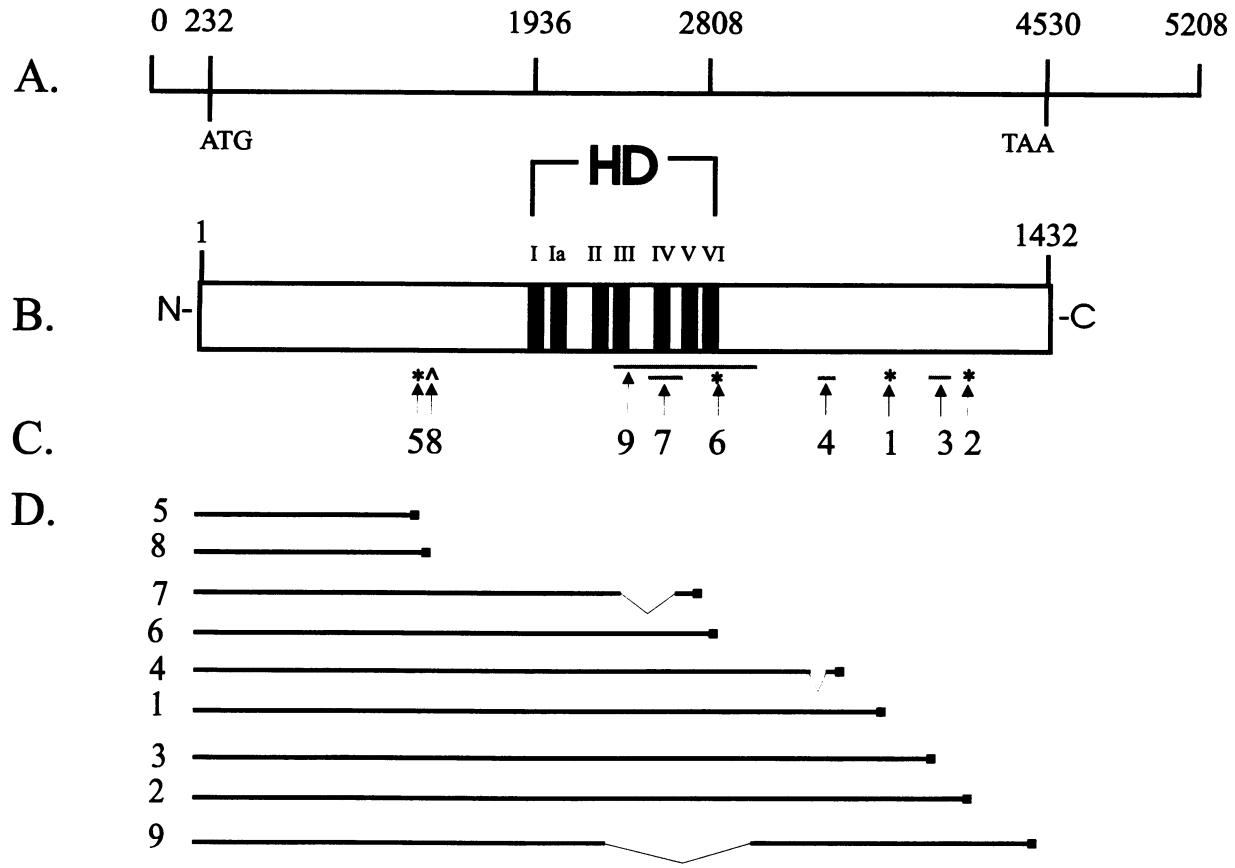

Figure 1 Schematic diagram of the *WRN* gene product with location of mutations. *A*, *WRN* cDNA. Numbering across the top refers to the cDNA sequence as numbered in GenBank L76937. *B*, Predicted *WRN* gene product. The helicase domain is designated as "HD," with motifs I–VI as indicated. *C*, Location of mutations. Numbering across the bottom refers to the mutations as described in table 3. An asterisk (*) denotes a nonsense mutation. A caret (^) denotes a frameshift mutation caused by a single base deletion. Gray lines indicate frameshift mutations causing deletion of exon(s). *D*, Predicted proteins. Lines represent the different predicted truncated proteins produced from mutations in the *WRN* gene.

Mutation Analysis

Mutations were detected by amplifying *WRN* exons from genomic DNA and directly cycle sequencing the PCR products by dye-terminator cycle sequencing (Perkin Elmer) and an ABI373 automated DNA sequencer. Prior to sequencing, the PCR-amplified exon fragments were purified using a QIAquick 8 PCR-purification kit (Qiagen). The resulting sequence results from WS and normal controls were aligned by FASTA analysis (GCG). Nucleotide differences between WS and controls were subsequently confirmed by sequencing the reverse strand.

Reverse transcriptase–PCR (RT-PCR)–based methods were initially used to identify some mutations (mutations 1–4 and 9; see below) and to confirm the predicted consequences of splice-junction mutations. RT-PCR products were synthesized from mRNA isolated from lymphoblastoid cell lines (Qiagen Oligotex, Qiagen). The large genomic deletion was detected in genomic DNA with long-range PCR (Expand Long Template PCR System; Boehringer Mannheim).

WS Subjects

WS patients were from an international registry of Werner syndrome subjects. The diagnostic criteria used has been described by Nakura et al. (1994). Family designations are as used elsewhere (Nakura et al. 1994; Goddard et al. 1996; Yu et al. 1996*a*).

Results

WRN *Gene Structure*

To facilitate mutational analysis of the *WRN* gene, the intron-exon structure was determined. The *WRN* gene was initially identified in the genomic sequence of P1 clone 2934 (Yu et al. 1996*a*). However, this clone contained only the 3′ end of the gene (exons 21–35). To obtain genomic clones for the 5′ end, a chromosome 8–specific cosmid library was screened for clones adjacent to P1 clone 2934. Four walking steps yielded cosmids 193B5, 114D2, 78D8, and 194C3, which contained the remaining exons (table 2). A total of 35 exons were identified, ranging in size from 68 bp (exon 14) to

768 bp (exon 35), with the coding region beginning in the second exon (table 2). As noted previously, there is a duplicated region in the *WRN* cDNA sequence, which is 27 amino acids in length. This duplication is also exactly conserved at the nucleotide level in the cDNA sequence. At the genomic level, the duplicated sequences were present as two exons (exons 10 and 11), each containing only the duplicated nucleotides. The intronic sequences adjacent to these two exons are also highly conserved, suggesting that a relatively recent duplication event is responsible for these repeated exons. Because the surrounding intronic sequences were conserved, it was not possible to identify primers that could specifically amplify exons 10 and 11.

The helicase region of the *WRN* gene, which shows homology to a variety of helicases, including *E. coli RecQ*, the *S. cerevisiae Sgs*1, *Caenorhabditis elegans* F18C5C, and human *RecQ* (Yu et al. 1996*a*), is contained in exons 14–21. This region, from codon 569 to 859, shows sequence homology to the seven signature helicase motifs. In addition, although the sequences between the motifs are not conserved, the spacing is very similar in genes from a wide range of species. For example the helicase motifs in the *E. coli RecQ* gene are found in a stretch of 288 amino acids compared to 291 amino acids for the *WRN* gene. Helicase motif 1 is split between exons 14 and 15, while the remaining motifs are each in an individual exon (table 2).

Mutations in WS Subjects

Initial screening of the *WRN* gene was based on a sequence from only the 3′ end of the gene (exons 23–35). Thus, the first four mutations (designated 1–4; table 3) were in the region 3′ to the helicase domains. Determination of the complete genomic structure permitted the rapid screening of the 5′ end of the gene with automated sequencing methods. For mutation screening, primers were designed so that exons 2–35 along with ~80 bp of flanking intronic sequence could be individually amplified (table 1). Initially, nine WS subjects (Caucasian subjects DJG, EKL, and FES and Japanese subjects IB, KO, OW, KUN, WKH, and WSF) were used to screen for mutations. These subjects were selected on the basis of haplotype analysis, which suggested that each subject could have different mutations (Yu et al. 1994; Goddard et al. 1996). As mutations were identified, a total of 30 Japanese and 36 Caucasian subjects were screened for each mutation by DNA sequence analysis of the appropriate exon.

Five new WS mutations were detected in the *WRN* gene (designated 5–9; table 3). Two of the mutations (5 and 6) were single-base substitutions creating nonsense codons. Mutation 5 results in a C→T transition, changing an Arg to a termination codon (table 3; fig. 1). The predicted protein is truncated at 368 amino acids, which would not include the helicase region that begins at codon 569. Three Japanese and three Caucasian subjects were homozygous, and one Japanese and four Caucasian subjects were heterozygous for this mutation (table 4). The presence of this mutation in some subjects (OW, KO, KUN, and SUG) has been confirmed by direct DNA sequence analysis of RT-PCR products (J. Oshima, unpublished data). Mutation 6 is also a C→T transition, changing an Arg to a nonsense codon. One Caucasian WS subject was homozygous for this mutation, and a second was a compound heterozygote. The predicted protein product is 888 amino acids. A third substitution mutation (mutation 7) was a G→T change at a splice-receptor site, generating a truncated mRNA devoid of exon 20 and a prematurely terminated *WRN* protein at amino acid 760. A single Japanese WS subject was homozygous for this mutation.

Two deletions were observed. One (mutation 8) is a 1-bp deletion at codon 389, resulting in a frameshift and a predicted truncated protein 391 amino acids long. This mutation was found in one Caucasian WS patient as a heterozygote. The second (mutation 9) was a much larger deletion. This deletion was first observed in RT-PCR experiments when two different RT-PCR products were obtained from RNA prepared from subject DJG. RT-PCR products produced by primers 5EE and B (table 1) yielded two different products, one with the expected size of 2,009 bp and a shorter product missing ~700 bp. The DNA sequence of the shorter RT-PCR product revealed that exons 19–23 were missing. To further establish the nature of this deletion, primers (exon 18A and exon 24A, table 1) derived from the exons flanking this potential gross deletion (exons 18 and 24) were used to amplify genomic DNA from subject DJG with a long-range PCR protocol. A single 5-kb fragment was observed corresponding to the shorter RT-PCR product. (The normal fragment, which is estimated to be >20 kb, was not observed.) This 5-kb fragment was completely sequenced and contained the expected 3′ and 5′ ends of exons 18 and 24, respectively. The exonic sequences were separated by intronic sequences adjacent to the 3′ and 5′ end of exons 18 and 24, respectively. No sequences from exons 19–23 were found in the 5-kb fragment. In other subjects and controls, the intronic sequence in the intron 3′ to exon 18 contained 531 bp of unique sequence followed by a 241-bp *Alu* repeat element. Likewise, for the region 5′ to exon 24, there is an *Alu* repeat element separated from exon 24 by 3,460 bp of unique sequence. The 4,938-bp fragment from subject DJG contained these unique exon-flanking intronic sequences separated by a single *Alu* element. Thus, this deletion presumably occurred by a recombination error at two highly homologous *Alu* elements within the *WRN* gene. A primer set, GD-A and GD-D (table 1), was then designed in order to specifically amplify a short fragment (426 bp) across this junction point and was used for screening our WS collection. A single additional

Table 1

Primer Sequence and PCR Conditions for *WRN* Analysis

PCR Fragment	Primer Sequence	Product Size (bp)	Mg^{+2} (mM)	pH
N-domain	5E6 5′-GATATTGTTTTGTATTTACCCATGAAGAC 5EY 5′-TCCGCTGCTGTGCAGTTGTTTCC	106	1.5	8.3
Center domain	5ED 5′-GATGGATTTGAAGATGGAGTAGAAG 5E12 5′-TCAGTAGATTTATAAGCAATATCAC	158	2.0	8.3
C-domain	CD-A 5′-CTGGCAAGGATCAAACAGAGAG CD-B 5′-CTTTATGAAGCCAATTTCTACCC	144	2.0	8.3
Exon 1	A 5′-AGGGCCTCCACGCATGACGC B 5′-AGTCTGTTTTTCCAGAATCTCCC	583	1.5	8.3
Exon 2	A 5′-CCTATGCTTGGACCTAGGTGTC B 5′-GAAGTTTACAAGTAACAACTGACTC	339	1.5	8.3
Exon 3	A 5′-ACTATAAATTGAATGCTTCAGTGAAC B 5′-GAACACACCTCACCTGTAAAACTC	316	1.5	8.3
Exon 4	E 5′-GGTAAACCACCATACCTGGCC F 5′-GTACATATCCTGGTCATTTAGCC	691	1.5	8.3
Exon 5	B 5′-ATTCAGATAGAAAGTACATTCTGTG E 5′-GTTAAGAAATACTCAAGGTCAATGTG	369	1.5	8.3
Exon 6	A 5′-GGTTGTATTTTGGTATAACATTTCC B 5′-ATATTTTGGTAGAGTTTCTGCCAC	374	1.5	8.3
Exon 7	A 5′-CTCTTCGATTTTTCTGAAGATGGG B 5′-CCCTAATAGTCAGGAGTGTTCAG	291	1.5	8.3
Exon 8	A 5′-GGAAAGAAAATGAAAATTTGATCCC B 5′-CAGCCTTAATGAATAGTATTCTTCAC	316	4.0	8.3
Exon 9	C 5′-ATTGATCTTTTAAGTGAAGGTCAGC D 5′-CTGCAACAGAGACTGTATGTCCC	668	1.5	8.3
Exon 12	A 5′-GCTTTCGACAAAATTGTAGGCCC B 5′-CCAAACCATCCAAAACTGGATCC	337	1.5	9.0
Exon 13	A 5′-TAACCCATGGTAGCTGTCACTG B 5′-CTGTTGCTGTTAAGCAGACAGG	285	1.5	8.3
Exon 14	C 5′-TTGAATGGGACATTGGTCAATTGG F 5′-GTAGTTGCATTTGTATTTTGAGAGT	348	1.5	8.3
Exon 15	C 5′-GTAAAAAGAAATGAAAGCATCAAAGG D 5′-TCACCCACAGAAGAAAAAAAGAGG	246	4.0	8.3
Exon 16	A 5′-CAAAAAAGAAAATTGCAAAGAACAGG B 5′-CAGCAACATGTAATTCACCCACG	282	4.0	8.3
Exon 17	E 5′-GAAGAGACTGGAATTGGGTTTGG F 5′-ATAGAGTATCATGGGATAAGATAGG	532	1.5	8.3
Exon 18	A 5′-TTCTCCTTTGGAGATGTAGATGAG B 5′-TCTTCAGCTTCTTTACCACTCCCCA	273	4.0	10
Exon 19	A 5′-CATGGTGTTTGACAACAGGATGG B 5′-GTTAAATATGCATTAGAAGGAAATCG	396	4.0	9.0
Exon 20	A 5′-ATAAAACCAAACGGGTCTGAAGC B 5′-AAAAGAAGTATTCAATAAAGATCTGG	342	4.0	8.3
Exon 21	A 5′-AATTCCACTTTGTGCCAGGGACT B 5′-ACTTGGGATACTGGAAATAGCCT	397	1.5	9.0
Exon 22	A 5′-TTTTTATCTTGATGGGGTGTGGG B 5′-AAATTCAGCACACATGTAACAGCA	356	1.5	9.0
Exon 23	A 5′-CTGAAGTCAAATAATGAAGTCCCA B 5′-GTTTGCTTTCTCATATCTAAACACA	360	4.0	8.3
Exon 24	A 5′-TCCTAGTCACCCATCTGAAGTC B 5′-CATGAAACTTGCTTCTAGGACAC	505	1.5	8.3
Exon 25	C 5′-GCTTGAAGGATGAGGCTCTGAG D 5′-TGTTCAGAATGAGCACGATGGG	461	1.5	8.3
Exon 26	A 5′-CTTGTGAGAGGCCTATAAACTGG B 5′-GGTAAACAGTGTAGGAGTCTGC	267	1.5	8.3
Exon 27	A 5′-GCCATTTTCTCTTTAATTGGAAAGG B 5′-ATCTTATTCATCTTTCTGAGAATGG	274	1.5	8.3
Exon 28	A 5′-TGAAATAGCCCAACATCTGACAG B 5′-GATTAATTTGACAGCTTGATTAGGC	291	1.5	8.3

(*continued*)

Table 1 (continued)

PCR Fragment	Primer Sequence	Product Size (bp)	Mg^{+2} (mM)	pH
Exon 29	A 5′-TGAAATATAAACTCAGACTCTTAGC B 5′-GTACTGATTTGGAAAGACATTCTC	303	1.5	8.3
Exon 30	A 5′-GATGTGACAGTGGAAGCTATGG B 5′-GGAAAAATGTGGTATCTGAAGCTC	307	1.5	8.3
Exon 31	A 5′-AAGTGAGCAAATGTTGCTTCTGG B 5′-TCATTAGGAAGCTGAACATCAGC	304	1.5	8.3
Exon 32	A 5′-GTTGGAGGAAATTGATCCCAAGTC B 5′-TGTTGCTTATGGGTTTAACTTGTG	351	1.5	8.3
Exon 33	A 5′-TAAAGGATTAATGCTGTTAACAGTG B 5′-TCACACTGAGCATTTACTACCTG	360	1.5	8.3
Exon 34	C 5′-GCAAAGGAAATGTAGCACATAGAG D 5′-AGGCTATAGGCATTTGAAAGAGG	491	1.5	8.3
Exon 35	A 5′-GTAGGCTCCCAGAAGACCCAG B 5′-GAAAGGATGGGTGTGTATTCAGG	406	1.5	8.3
Mutation 7	GD A 5′-ACAGGCCATAGTTTGCCAACCC GD D 5′-TGGTATTAGAATTTCCCTTTCTTCC	426	1.5	9.0
DJG RT-PCR	5EE 5′-TGAAAGAGAATATGGAAAGAGGCTTG B 5′-CTTTATGAAGCCAATTTCTACCC	2002	1.5	8.3
P2934AT1	A 5′-TCAAAATCAGTCGCCTCATCCC B 5′-CAATGTATCAGTCAGGGTTCACC	168	2.0	8.3

NOTE.—The annealing temperature was 60°C for all primer sets.

Caucasian WS patient, SUG, was also shown to contain this genomic deletion. Further PCR amplification of the exons within this deleted region demonstrated that both DJG and SUG are heterozygous for this mutation.

Origins of WRN *Mutations*

Since multiple subjects were identified with the same mutations, the possibility exists that at least some of the mutations originated in common founders. Prior to the identification of the *WRN* gene, haplotype analysis of 21 short-tandem-repeat polymorphisms (STRPs) spanning the 1.2-Mb *WRN* region (Yu et al. 1996*b*) had suggested that *WRN* in some Japanese subjects was the result of a common founder (Goddard et al. 1996). Cloning of the *WRN* gene and identifying the mutations in the same subjects used for haplotype analysis permitted this hypothesis to be tested. Also, during the process of analyzing the *WRN* gene, a new STRP within the *WRN* gene, D8S2300 (table 1), was identified, and a previously identified marker, D8S2162 (marker S in Goddard et al. 1996), was also found to be in the *WRN* gene and within 17.5 kb of D8S2300 (Yu et al. 1996*b*; for marker order and designation, see table 5). While D8S2162 is not particularly polymorphic (heterozygosity = 54% in Japanese and 70% in Caucasians) and is primarily a two-allele system (the 140- and 142-bp alleles, table 5), D8S2300 is highly polymorphic (heterozygosity = 78% in both Japanese and Caucasian populations).

Mutation 4 has been observed only in Japanese subjects. The following evidence suggests that most if not all carriers of this mutation originated from a common founder. For markers D8S2164 and D8S2300, all mutation 4 subjects had the same haplotype (140–148) except for JO2, who had haplotype 140–150, with the D8S2300 allele being a single repeat unit (2 bp) different from the 148-bp allele observed in other subjects with mutation 4. Mutations resulting in the addition or deletion of a single repeat unit are common at STRP loci (Weber and Wong 1993). Over a broader region, all mutation 4 families share essentially identical extended haplotypes for markers J–T, a region spanning ~800 kb which includes D8S2164, D8S2300, and the *WRN* gene; exceptions to the common haplotype in mutation 4 subjects consist of single discordant markers in a string of otherwise identical genotypes (Goddard et al. 1996; table 5), with a number of these discrepancies being alleles differing from the consensus haplotype by one repeat unit. The sharing of a common haplotype is consistent with the linkage disequilibrium previously reported in the same Japanese subjects (Yu et al. 1994; Goddard et al. 1996) and is consistent with a common genetic founder.

For mutation 2, the three Japanese subjects analyzed (HM, MH, and NN) all share the haplotype for markers D–U and D8S2300 (table 5; Goddard et al. 1996). The single discrepency is that subject NN has an allele at marker N that differs from the consensus haplotype by a single 2-bp repeat unit. In contrast, GAR, the Hispanic

Table 2

Intron-Exon Structure of the *WRN* Gene

Exon	cDNA Location	Exon Size (bp)	Intron-Exon Boundary Sequences	Exon Features
1	1–155	>155	... TTCTCGGGgtaaagtgtc	5′UTR
2	156–327	172	tacctctcagTTTTCTTT ... AAAGAAAGgtatgttgtt	5′UTR, ATG codon
3	328–440	113	taaactcaagGCATGTGT ... GATATTAGgtaagtgatt	
4	441–586	146	ctcactttagCATGAGTC ... CATGTCAGgttggtatct	
5	587–735	149	aatgttacagTTTTTCCC ... ATAAAAAGgtaaaagcaa	
6	736–885	150	tcatttctagCTGAAATG ... ATGCTTATgtacgtgctt	
7	886–955	70	ttttttatagGCTGGTTT ... AAATAAAGgtatgttaag	
8	956–1070	115	ttccccctagAGGAAGAA ... CCACGGAGgttaaatatt	
9	1071–1500	430	tttttttagGGTTTCTA ... CTACTGAGgtactaaaat	
10	1501–1581	81	ttttttaaagCATTTATC ... TGCTTAAGgtatgttta	Duplicated exon
11	1582–1662	81	ttttttaaagCATTTATC ... TGCTTAAGgtatgttta	Duplicated exon
12	1663–1807	145	aaactttcagTCTTTAGA ... TGATAAGGgtaagcactg	
13	1808–1883	76	ttatttccagACTTTTTG ... TTTAAACCgtgagtataa	
14	1884–1951	68	caccttcaagAGTTCAGT ... GGCAACTGgtaagttgta	Helicase motif I (5′ end)
15	1952–2060	109	tcatttcaagGATATGGA ... CAGCTTAAgtaagtcatg	Helicase motif I (3′ end) and Ia
16	2061–2129	69	cttcttatagAATGTCCA ... ATTAAATTgtgagtaatt	
17	2130–2212	83	gtttttacagAGGTAAAT ... TGATATTGgtaagtgata	
18	2213–2319	107	ttttttacagGTATCACG ... TGCCAATGgtaagctttg	Helicase motif II
19	2320–2504	185	catcattcagGTTCCAAT ... AAAACAAGgtaaggattt	Helicase motif III
20	2505–2679	175	ttttctttagTTCCCACT ... AAATTCAGgtatgaggat	Helicase motif IV
21	2680–2861	182	ttgttctcagTGTGTCAT ... TTAAATAGgtaaaaaaaa	Helicase motifs V and VI
22	2862–2963	102	taatcgacagGCACCTTC ... AGGAGACAgtatgtatta	
23	2964–3056	93	tcttgggtagAATCATCT ... AGGTCCAGgtaaagattt	
24	3057–3198	142	ttttatttagATTGGATC ... GAGGATCTgtaagtatat	
25	3199–3369	171	ctaatttcagAATTCTCA ... CGAAAAAGgtaaacagtg	
26	3370–3464	95	cttttaatagGGTAGAAA ... CTGCCTAGgttcattttt	
27	3465–3540	76	tatttttttagTTCGAAAA ... AGAAGAAGgtttgtttta	
28	3541–3614	74	ttaaatgcagTCTAACTT ... AAAAAAAGgtacagagtt	
29	3615–3690	76	aatattttagTATCATGG ... AGACTCAGgtaaggcttt	
30	3691–3803	113	ttttgttcagATTGTGTT ... AAAATGAGgtaaactatc	
31	3804–3918	115	ttaaacacagACCAACTA ... GTGTTCAGgtaaaatact	
32	3919–4050	132	aattctgtagACAGACCT ... TGCCTTTGgtaagtgtga	
33	4051–4213	163	ctttctctagAAGAGCAT ... CAACTCAGgtgagaggca	
34	4214–4422	209	tcgtttacagATATGAGT ... ATACTGAGgtattaatta	
35	4423–5190	768	tttcctacagACTTCATC ...	TAA codon, 3′UTR

NOTE.—Exons are in uppercase and intron sequences are in lowercase letters.

subject homozygous for mutation 2, differs from the Japanese mutation 2 subjects at 6 of the 12 markers from J–T (discordant markers are J, K, M, O, D8S2300, and T; table 5 and data not shown) that bracket the *WRN* gene. Thus, while the Japanese subjects may be descendants from a common founder, mutation 2 in GAR is probably the result of an independent mutation.

For mutation 5, the two Japanese families KO and OW are identical for markers J–S, including D8S2300 (table 5; Goddard et al. 1996). In contrast, the Caucasian families differ from the Japanese families at 4–5 (depending on the family) of the 11 markers from J–S. In addition, even in the Caucasians studied, mutation 5 may have multiple origins. AG00780 differs from EKL at both R and D8S2300, which are close adjacent markers. CP3, KUN, and DJG also differ from AG00780 at D8S2300 at both alleles. CP3 also differs from the consensus haplotype for this group at markers O and T, although marker O may have a high mutation rate (Goddard et al. 1996). Thus, in the different Caucasian subjects, mutation 5 may have occurred by independent mutational events. It should be noted that absence of evidence for a common founder does not necessarily exclude the possibility of a single originating mutational event. Intragenic recombination and/or mutations creating new alleles at the two STRP loci could, over an extended period of time, obscure the origins of the different *WRN* mutations.

Discussion

The *WRN* gene described here was identified by positional cloning methods as one of the transcribed sequences in a 1.2-Mb region known to contain the *WRN*

Table 3

Summary of *WRN* Mutations

Mutation	Codon	Exon	Type of Mutation	Nucleotide Sequence	Comment	Predicted Protein Length (aa)
None	...	...	...	...	...	1432
1	1165	30	Substitution	CAG (Gln) to TAG (terminator)	Nonsense	1164
2	1305	33	Substitution	CGA (Arg) to TGA (terminator)	Nonsense	1304
3	1230	32	4-bp deletion	gtag-ACAG to gt-AG	4-bp deletion at splice-donor site	1247
4	1047–1078	26	Substitution	tag-GGT to tac-GGT	Substitution at splice-donor site	1060
5	369	9	Substitution	CGA (Arg) to TGA (terminator)	Nonsense	368
6	889	22	Substitution	CGA (Arg) to TGA (terminator)	Nonsense	888
7	759–816	20	Substitution	GAG-gta to CAG-tta	Substitution at splice-receptor site	760
8	389	9	1-bp deletion	AGAG (Arg) to GAG (Glu)	Frame-shift	391
9	697–942	19–23	Deletion (>15 kb)	...	Genomic deletion	1186

gene (Yu et al. 1996*a*). Initial evidence that this gene is responsible for WS was that four mutations were identified in the homozygous state in WS subjects. In the work presented here, five additional mutations in the *WRN* gene were identified. Evidence that the helicase gene is the locus responsible for WS is as follows: first, the gene is located in the minimal region defined by recombinant events, linkage disequilibrium studies, and haplotype analysis of ancestral recombinant events (Yu et al. 1996*a*, 1996*b*; Goddard et al. 1996); second, the mutations observed are not found in the homozygous state in unaffected subjects; and third, all nine mutations severely alter the predicted protein and are either nonsense mutations or mutations that result in a frameshift and a predicted truncated protein product. Thus, there is little doubt that mutations in the helicase gene are responsible for WS.

The *WRN* gene can be divided into three regions. The 5′ end, from codons 1–539, shows no homology to other genes, including other helicases. This end of the protein is acidic, with 109 aspartate or glutamate residues, including a stretch of 14 acidic residues in a 19–

Table 4

Mutation Status of WS Subjects

	Japanese WS Subjects		Caucasian WS Subjects	
Mutation	Homozygous	Heterozygous	Homozygous	Heterozygous
1	SY[a]			
2	HH[a], HM[a], MH[b], NN[a]		GAR[a]	
3			SYR[c]	
4	FJ[a], FUW[a], HA[c], HW[a], IU[a], JO1[a], JO2[a], KAKU[d], KY[a], MCI[a], MIE2[c], SK[a], ST[a], TH[c], TK[b], TO[a], ZM[a]			
5	KO[a], OW[d]	KUN[c]	EKL[a], AG0780[c], AG4103[b]	DJG[d], CP3[c], NF[b]
6			CTA[a]	SUG1[d]
7	WKH[a]			
8				FES[c]
9				DJG[d], SUG1[d]

NOTE.—The countries of origin (ethnic group) of non-Japanese subjects are AG00780, United States (Caucasian); AG04103; United States (Caucasian); CTA, England (Indian, east African, Asian); CP3, France (Caucasian); DJG, Germany (German); EKL, Switzerland (German); FES, Germany (German); NF, France (Caucasian); SUG, United States (Caucasian); SYR, Syria (Syrian). AG04103 and AG00780 were obtained as cell lines from the Aging Cell Repository (Camden, NJ).

[a] Definite.

[b] Possible.

[c] Insufficient data.

[d] Probable.

Table 5

STRP Genotypes at the *WRN* Gene

Subject	Ethnic Group	Mutation	D8S2162	D8S2300
FJ, FUW, HA, HW, JO1, KAKU, KY, MIE2, TO	Japanese	4	140/140	148/148
JO2	Japanese	4	140/140	150/150
HM, MH, NN	Japanese	2	140/140	144/144
GAR	Hispanic	2	140/140	156/156
OW, KO	Japanese	5	140/140	148/148
AG00780	Caucasian	5	142/142	136/136
EKL, AG04103	Caucasian	5	142/142	128/128
CP3	Caucasian	5/?	142/150	128/142
KUN	Japanese	5/?	140/142	128/148
DJG	Caucasian	5/9	140/142	128/del[a]

NOTE.—Genotype data for HH, SK, ST, TH, TK, and ZM were not available. For D8S2162, alleles in bp (frequency for Caucasians, frequency for Japanese) were as follows: 136 (.030, .025); 138 (.020, .010); 140 (.460, .576); 142 (.337, .359); 144 (.084, .010); 146 (0, .010); 148 (.009, .010); 150 (.059, 0). For D8S2300, alleles in bp (Caucasian frequency, Japanese frequency) were as follows: 114 (.006, 0); 122 (0, .009); 124 (.011, 0); 128 (.253, .079), 130 (0, .018); 132 (.006, .009); 134 (.046, .096); 136 (.086, .009); 138 (.011, 0); 140 (.034, 0); 142 (.052, .035); 144 (.023, .061); 146 (.023, .053); 148 (.034, .132); 150 (.034, .105); 152 (.057, .123); 154 (.063, .088); 156 (.086, .070); 158 (.098, .070); 160 (.046, .018); 162 (.029, .009); 166 (0, .009); 168 (0, .009). Markers used for haplotype analysis are in the following order, listed from 8ptel to 8cen with the alphabetical designations from Goddard et al. (1996): A = D8S137; B = D8S131; C = D8S2194; D = D8S2192; E = D8S2196; F = D8S2198; G = D8S339; H = D8S2204; I = D8S2202; J = D8S2206; K = D8S2134; L = D8S2144; M = D8S2156; N = D8S2138; O = D8S2168; P = D8S2174; Q = D8S2150; R = D8S2180; D8S2162; D8S2300; WRN; S = D8S2162; T = D8S2186; U = D8S278. Markers C–T span ~1.2 Mb (Yu et al. 1996*b*). Markers A and B are ~6.7 cM telomeric to the WRN region, and marker U is ~1.6 centromeric of the WRN cluster. The order of marker pairs C/D and L/M cannot be presently resolved. D8S2162 and D8S2300 are within the WRN gene.

[a] del = D8S2300 locus is deleted on one chromosome.

amino acid sequence (codons 507–526). Similar stretches of acidic residues are found in the xeroderma pigmentosum (XP) complementation group B helicase (XPB; Weeda et al. 1990), the Bloom syndrome helicase (Ellis et al. 1995), and the X chromosome–linked α-thalassemia mental retardation syndrome helicase (Stayton et al. 1994; Ion et al. 1996). In the *WRN* gene, this region also contains a tandem duplication of 27 amino acids, with each copy encoded for by a single exon that contains only the duplicated codons. Because this duplication is exact at the nucleotide level, and because flanking intronic sequences for the two exons that encode the duplication are also highly similar, this duplication is presumed to be the result of a relatively recent event. The duplicated regions are also highly acidic, with 8 glutamate or aspartate residues of 27 amino acids with only 2 basic amino acids (a histidine and a lysine residue).

The middle of the *WRN* gene, spanning codons 540–963, is highly homologous to other helicases from a wide range of organisms, including the *ReqQ* gene from *E. coli,* the SGS1 gene from *S. cerevisiae,* a predicted helicase (F18C5C) from *C. elegans,* and several human helicases (Yu et al. 1996*a*). Thus, the *WRN* gene is a member of a superfamily of DExH-box DNA and RNA helicases. The principle conserved sequences consist of seven motifs found in other helicases. These motifs include a predicted nucleotide-binding site (motif I) and a Mg^{2+} binding site (sequence DEAH, motif II), and helix unwinding presumably occurs with the concomitant hydrolysis of ATP. Some or all of the seven motifs are presumed to form the enzymatic active site for DNA/RNA unwinding. In other human helicases, which include XPB, XPD, and CSB, motif III contains a p53 binding site (Wang et al. 1995). When bound, p53 inhibits the helicase activity of these proteins.

The 3′ end of the *WRN* gene, codons 964–1432, has limited homology to other genes. The only homology identified is a loose similarity to the *E. coli ReqQ* gene and the putative *C. elegans* gene *F18C5.2.* The sequence of this region gives essentially no clue as to its function.

The initial four helicase mutations identified were in the 3′ end of the gene (Yu et al. 1996*a*). The bias in identifying mutations in this region occurred because this was the first portion of the gene screened. All four mutations (1–4, table 2) altered only the C-terminal portion of the gene, leaving the seven helicase motifs intact (fig. 1). Thus, the possibility existed that the predicted truncated proteins could have a functional helicase activity but still give the WS phenotype. The five mutations identified here demonstrate that WS mutations are not restricted to the 3′ end of the gene but are also found in other regions of *WRN.* In addition, mutations 5 and 7–9 each disrupt either part or all of the helicase region. In particular, mutation 5, which is a nonsense mutation and results in a predicted protein of 368 amino acids, is observed in the homozygous state in both Japanese and Caucasian subjects. Thus, the WS

subjects carrying this mutation will completely lack the *WRN* helicase domains as well as the 3′ end of the protein. Although the possibility exists that the truncated 368–amino acid protein has some partial remaining function, mutation 5 probably results in complete loss of all activity of the *WRN* protein. The WS phenotype in these subjects is not appreciably distinct from the WS phenotype generated by the other mutations described here. Thus, it is possible that all mutations in the WS gene are complete loss-of-function mutations. This could occur if mutations throughout the gene sufficiently disrupt protein folding to abolish function completely or to induce rapid turnover of the altered protein. The presence of nonsense mutations may also result in reduced mRNA levels (Aoufouchi et al. 1996), which, in the homozygous state, could severely reduce protein production and also result in loss of function. Additional experiments are needed to determine whether stable truncated proteins are observed in WS cells.

The function of the *WRN* gene product and how the loss of this function generates the WS phenotype remains to be determined. The close homology between the *WRN* gene and other helicases suggests that the *WRN* protein will be a DNA-unwinding enzyme. However, the actual unwinding function of the helicase needs to be demonstrated. By analogy with other helicases, the *WRN* protein may be part of a multiprotein complex. For example, the helicase XPD (ERCC-2), identified as defective in XP-complementation group D (Flejter et al. 1992; Frederick et al. 1994; Broughton et al. 1995; Takayama et al. 1995) and trichothiodystrophy (Broughton et al. 1994; Takayama et al. 1996), and helicase XPB (ERCC-3), which is mutated in XP group B (Weeda et al. 1990), are both part of the human RNA polymerase II basal transcription factor TFIIH (Schaeffer et al. 1993). Likewise, the helicase CSB (ERCC-6), which is defective in Cockayne syndrome (CS) group B (Troelstra et al. 1992), may also be part of, or interact with, the TFIIH complex. CSB binds to CSA, the product of the gene found mutated in CS group A subjects (Henning et al. 1995). In turn, CSA interacts with TFIIH. Sequences in the 3′ and/or the 5′ region of the *WRN* protein presumably will contain the interaction sites needed for forming a complex with other proteins.

The WS phenotype provides limited clues as to the function of the *WRN* protein. WS cells appear to have a mutator phenotype (Fukuchi et al. 1985, 1989, 1990; Monnat et al. 1992) and an elevated rate of chromosomal rearrangement (Hoehn et al. 1975; Salk et al. 1981; Salk 1982). Thus, genomic instability appears to be part of the WS phenotype. Such DNA damage could be the result of a repair defect. CS (Troelstra et al. 1992) and some forms of XP are caused by mutations in genes encoding helicases. Both disorders exhibit defective DNA repair, being either defective in nucleotide-excision repair or strand-specific transcription-coupled repair. However, defective DNA repair has not been reproducibly observed in WS. Post-UV irradiation cell survival (Saito and Moses 1991) and unscheduled DNA synthesis (Fujiwara et al. 1977; Higashikawa and Fujiwara 1978; Stefanini et al. 1989) are both normal in WS cells. Likewise, WS cells show normal sensitivity to a wide variety of DNA-damaging agents, including bleomycin, methyl methansulfonate, *cis*-dichlorodiamine platinum, isonicotinic acid hydrazide, and diepoxybutane (Arlett and Harcourt 1980; Gebhart et al. 1985, 1988; Saito and Moses 1991; Stefanini et al. 1989). In one study, elevated rates of chromosomal breakage in WS cells was observed with 4-nitroquinoline-1-oxide (Gebhart et al. 1988). Sister chromatid exchange rates are normal and are not abnormally altered after treatment with clastogenic agents (Gawkrodger et al. 1985; Gebhart et al. 1985). The possibility exists that a cryptic DNA-repair defect remains to be identified in WS. However, a more likely alternative is that loss of the *WRN* protein function causes DNA damage that is not repaired, or at least is incompletely repaired.

In addition to genomic instability, several other alterations in DNA metabolism have been reported in WS cells. Elevated rates of homologous recombination have been observed in fibroblasts from some but not all WS cells (Cheng et al. 1990). In studies of the ligation of linearized plasmid vectors by WS lymphoblast cell lines, DNA ligation efficiency is not altered in WS cells, but the fidelity of ligation events is reduced (Runger et al. 1993, 1994). DNA replication may be altered in WS cells; both reduced DNA chain–elongation rates (Fujiwara et al. 1977) and increased distances between DNA synthesis initiation sites (Takeuchi et al. 1982*a*; Hanaoka et al. 1983) have been observed in WS cells relative to normal control cultures. Consistent with altered DNA synthesis, the S-phase in lymphoblastoid cell lines from WS subjects is prolonged (Takeuchi et al. 1982*b*; Poot et al. 1992). To determine whether any of these alterations in DNA metabolism is directly related to the defective WS protein or is simply an epiphenomenon will require additional work.

By analogy with the function of other helicases, the *WRN* protein could function in DNA repair, regulation of transcription, regulation of progression through the cell cycle (possibly via P53), or any number of other potential functions involving DNA or RNA unwinding. Identification of the proteins that complex with the *WRN* protein will be critical in identifying the DNA metabolism events in which the *WRN* protein participates. Understanding the molecular mechanism by which the WS defect contributes to accumulation of DNA damage will, it is hoped, add to our understanding of human aging.

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