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Preferential Expression of an Intact *WRN* Gene in Werner Syndrome Cell Lines in Which a Normal Chromosome 8 Has Been Introduced

Genro Kashino,* Seiji Kodama,* Keiji Suzuki,* Mitsuo Oshimura,† and Masami Watanabe*,[1]

**Laboratory of Radiation and Life Science, Department of Health Sciences, School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan; and †Department of Molecular and Cell Genetics, School of Life Science, Faculty of Medicine, Tottori University, Yonago 638-8503, Japan*

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Werner syndrome (WS) is a premature aging syndrome caused by mutations in the *WRN* gene. All mutations of the *WRN* gene reported thus far are predicted to produce the truncated WRN proteins. The mRNAs that contain chain-termination mutations are supposed to be unstable due to degradation by nonsense-mediated mRNA decay (NMD). In the present study, we investigated the expressions of intact and nonsense-mutated *WRN* genes in Werner syndrome cell lines in which a normal chromosome 8 had been introduced by microcell fusion. We demonstrate here that the expression of the mutated *WRN* gene that produces nonsense mRNAs remains at low levels, resulting in the preferential expression of the intact *WRN* gene in the WS microcell hybrids. This result supports the idea that imperfect messages containing premature termination codons are eliminated by the RNA surveillance system, suggesting the significance of the NMD mechanism in the etiology of Werner syndrome.

Key Words: **Werner syndrome; WRN protein; RNA surveillance; nonsense-mediated decay; gene expression.**

Werner syndrome (WS) is a rare autosomal recessive disease. Affected individuals typically have multiple progeroid features such as premature graying of the hair, cataracts, osteoporosis, diabetes, and accelerated atherosclerosis, accompanied by a high incidence of cancers (1, 2). The study of cultured cells has demonstrated that WS cells show abnormal phenotypes including accelerated senescence, a high proportion of deletion mutation, and hypersensitivity to 4-nitroquinoline-1-oxide (3–8). The gene responsible for WS (*WRN*) was identified in 1996 by positional cloning, and turned out to encode a 1432 amino acid protein (9). The WRN protein possesses $3' \rightarrow 5'$ helicase and exonuclease activities, and is one of five human RecQ DNA helicases (10–12). Among those, three helicases including WRN are associated with heritable human diseases. The other two helicases are BLM and RTS, the activities of which are defective in Bloom syndrome and Rothmund–Thomson syndrome patients, respectively (13, 14). All of the *WRN* mutations identified thus far in WS patients result in the truncated WRN protein with loss of a C-terminal nuclear localization signal (15). As a result, mutant WRN proteins show impaired nuclear transportation in WS cells, and this defect might be crucial for the pathogenesis of WS (16).

The expression of the truncated *WRN* gene was reported to be diminished in WS cells. Yamabe *et al.* (17) investigated the expression of the *WRN* gene in fibroblasts and EBV-transformed B-lymphoblastoid cells of WS patients by Northern blot analysis, and demonstrated that the levels of mutant WRN mRNA from patient cells were lower than those of intact mRNA from the cells of normal individuals by an average of 70%. Moser *et al.* (18) showed that the expression and activity of WRN helicase was negligible in WS patient cell lines. These results are well explained by the RNA surveillance mechanism, which prevents the production of truncated proteins (19, 20). This RNA surveillance system, referred to as nonsense-mediated mRNA decay (NMD), eliminates imperfect messages containing premature translation termination codons, and the result is that most of the mRNAs that cannot be translated along their full-length are rapidly degraded.

As reported in the previous study (5), we established WS cell lines expressing a normal WRN protein by introduction of a normal human chromosome 8 via microcell fusion. In the present study, we investigated the expressions of intact and mutated *WRN* genes by direct sequencing of a fragment of *WRN* cDNA prepared from the mRNAs of the WS microcell hybrids.

[1] To whom correspondence should be addressed. Fax: +81-95-844-5504. E-mail: nabe@net.nagasaki-u.ac.jp.

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We show here that the expression of truncated WRN mRNA is diminished compared to the normal counterpart derived from an externally introduced chromosome 8 in the WS microcell hybrids. The result indicates that the NMD mechanism that discriminates normal transcripts from abnormal ones is active in the WS microcell hybrids, supporting the idea that NMD plays a significant role in the etiology of WS.

MATERIALS AND METHODS

Cells and cell culture. The WS cell lines used were SV40-transformed WS780 cells and their microcell hybrids in which human chromosome 8 or chromosome 9 has been introduced (5). WS780 cells have a point mutation (C → T) at nucleotide 1336 in exon 9 of the *WRN* gene, which results in a premature termination at codon 369 and produces a truncated WRN protein. SV40-transformed PSV811 cells, formerly reported as a WS cell line (21), and their microcell hybrids in which human chromosome 8 has been introduced were also used. PSV811 cells have a common polymorphism at nucleotide 3453 (T → G) of the *WRN* gene that results in a substitution of amino acid from phenylalanine to leucine in codon 1074 but in no mutations (22). SV40-transformed GM638 cells were used as a control. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical, Tokyo) supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml). All microcell hybrids were cultured in DMEM medium supplemented with 800 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Determination of expression of the WRN gene. To distinguish transcripts of the exogenous *WRN* gene from transcripts of the endogenous *WRN* gene in the WS microcell hybrids, *WRN* cDNA fragments including codon 369 (808 bp) for WS780 cells and those including codon 1074 (934 bp) for PSV811 cells were amplified by a reverse transcriptase-PCR (RT-PCR), subcloned into the pCR-Script Amp SK(+) vector (Stratagene, La Jolla, CA) and sequenced as previously described (5). In order to determine the relative expression of the *WRN* gene among the cell lines used, a cDNA fragment (345 bp) corresponding to c-*myc* gene, which is located on chromosome 8q24, was simultaneously amplified using the c-*myc* primer set (Stratagene, La Jolla, CA) in the same reaction mixture and used to normalize the expression of the *WRN* gene. The PCR products were separated on 1% NuSieve agarose gels (FMC Bioproducts, Rockland, ME), and the gel was stained with SYBR green (FMC Bioproducts, Rockland, ME). The band intensities were measured densitometrically with a scanning imager. The relative expression of the *WRN* gene was calculated by dividing the signal intensity of the band corresponding to the *WRN* gene by the intensity of the band corresponding to the c-*myc* gene.

RESULTS

Expression Levels of the WRN Gene

We examined expressions of the *WRN* gene and the c-*myc* gene in GM638 cells, WS780 cells, PSV811 cells, and their microcell hybrids by the RT-PCR method, and then determined the relative expression of the *WRN* gene normalized by the expression of the c-*myc* gene. We adopted *c-myc* gene as a control for gene expression because its expression was relatively constant throughout cell cycle (23) and its gene dosage was the same as that of *WRN* gene based on the fact that

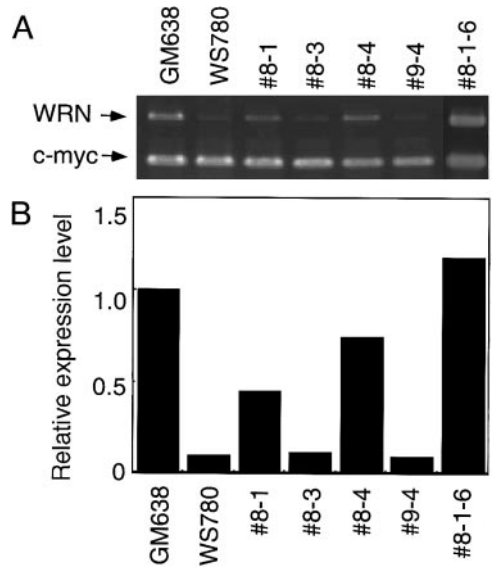

FIG. 1. Expression of the *WRN* gene in WS780 cells and their microcell hybrids. (A) The expression level of the *WRN* gene was measured by RT-PCR. The cells used were as follows: GM638, a human control cell line; WS780, a WS cell line; #8-1 and #8-4, WS780-derived microcell hybrids containing an extra chromosome 8; #8-3, a WS780-derived microcell hybrid containing an extra chromosome 8 deleted in a short arm (del(8)p12-ter); #9-4, a WS780-derived microcell hybrid containing an extra chromosome 9; #8-1-6, a WS780-derived microcell hybrid containing two extra chromosomes 8. (B) The relative expression of the *WRN* gene was determined as described under Materials and Methods. Two independent experiments resulted in the same result.

both genes located on chromosome 8. The very similar results were obtained by measuring expressions of β-actin and GAPDH genes as a control (data not shown). The presence of an extra copy of chromosome 8 in the microcell hybrids derived from WS780 and PSV811 cells were confirmed by the whole chromosome painting method (data not shown; see ref. 5). As shown in Fig. 1, the expression of the *WRN* gene in WS780 cells was very low, showing only 9.2% of that in the control GM638 cells, although those two types of cells expressed the c-*myc* gene at very similar levels. Similar low levels of expression of the *WRN* gene were also observed in WS780-derived microcell hybrids 8-3 and 9-4. On the other hand, increased expression of the *WRN* gene was observed in WS780-derived microcell hybrids 8-1 and 8-4, at levels 36 and 61% of that in the control cells, respectively. These results were consistent with the former analysis by Western blotting (5), in which the expression of WRN protein was detectable in WS780-derived microcell hybrids 8-1 and 8-4, but not in 8-3 and 9-4. Interestingly, the expression of the *WRN* gene in WS780-derived microcell hybrid 8-1-6, which contained two extra copies of chromosome 8, was higher than levels observed in microcell hybrids 8-1 and 8-4, and was comparable to that in the control cells (Fig. 1).

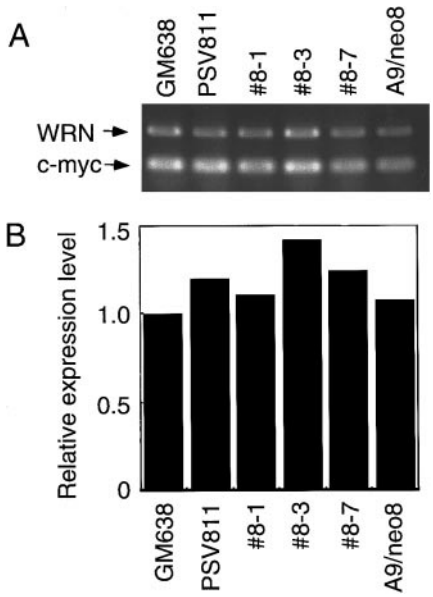

FIG. 2. Expression of the *WRN* gene in PSV811 cells and their microcell hybrids. (A) The expression level of the *WRN* gene was measured by RT-PCR. The cells used are as follows: GM638, a human control cell line; PSV811, a cell line formerly reported as WS; #8-1, #8-3, and #8-7, PSV811-derived microcell hybrids containing an extra chromosome 8; A9/neo8 cells, a mouse cell line containing a normal human chromosome 8. (B) The relative expression of the *WRN* gene was determined as described under Materials and Methods. Two independent experiments resulted in the same result.

On the other hand, PSV811 cells expressed the *WRN* gene at a level comparable to that of the control GM638 cells, as shown in Fig. 2. The relative expression of the *WRN* gene in mouse A9 (neo8) cells that contained a single copy of human chromosome 8 was also comparable to that in the control cells. However, in contrast with the result observed in WS780 cells, introduction of an extra copy of chromosome 8 did not significantly influence the expression of the *WRN* gene (Fig. 2).

Expressions of Endogenous and Exogenous WRN Genes

Direct sequencing of the *WRN* cDNA fragment was a simple and useful method to distinguish between a cDNA derived from the exogenous *WRN* gene and one derived from the endogenous *WRN* gene in the microcell hybrids containing an external chromosome 8. Five to twenty-one cDNAs were prepared from each cell line and sequenced. The results obtained for WS780 cells and their microcell hybrids are shown in Table 1 and those obtained for PSV811 cells and their microcell hybrids are shown in Table 2. As reported previously, WS780 cells only expressed the mutated *WRN* gene (Table 1). WS780-derived microcell hybrids 8-3 and 9-4 expressed the endogenous (mutated) *WRN* gene (Table 1). In contrast, both endogenous (mutated) and exogenous (intact) *WRN* genes were expressed in WS780-derived microcell hybrids 8-1, 8-4, and 8-1-6. Of particular interest was the preferential expression of the exogenous (intact) *WRN* gene in these microcell hybrids, which showed that 85% or more of the transcripts were derived from the intact *WRN* gene (Table 1).

TABLE 1

Expressions of Endogenous and Exogenous *WRN* Genes in WS780 Cells and WS780-Derived Microcell Hybrids

Cell line	No. of sequenced plasmids containing *WRN* cDNA fragment	No. of clones	
		end[a] TGA (%)	exo[b] CGA (%)
A9/neo8	6	0 (0)	6 (100)
WS780	5	5 (100)	0 (0)
#8-1	20	3 (15)	17 (85)
#8-3	9	9 (100)	0 (0)
#8-4	19	1 (5)	18 (95)
#9-4	7	7 (100)	0 (0)
#8-1-6	18	1 (6)	17 (94)

[a] Endogeneous *WRN* cDNA fragment containing codon 369 (TGA).
[b] Exogenous *WRN* cDNA fragment containing codon 369 (CGA).

On the other hand, the expressions of endogenous and exogenous *WRN* genes were almost equivalent in PSV811-derived microcell hybrids (Table 2). These results indicate that the preferential expression of exogenous *WRN* gene is only observed in the WS cell lines that have a nonsense mutation in the endogenous *WRN* gene.

DISCUSSION

The present study demonstrated the preferential expression of the intact *WRN* gene introduced exogenously as compared with that of the endogenous *WRN* gene containing a nonsense mutation in WS780-derived microcell hybrids. Other investigators previously reported the reduced expression of WRN mRNA or WRN protein in cell lines from *WRN* heterozygous individuals (17, 18). In those studies, however, they did

TABLE 2

Expressions of Endogenous and Exogenous *WRN* Genes in PSV811 Cells and PSV811-Derived Microcell Hybrids

Cell line	No. of sequenced plasmids containing *WRN* cDNA fragment	No. of clones	
		end[a] TTG (%)	exo[b] TTT (%)
PSV811	9	9 (100)	0 (0)
#8-1	11	5 (45)	6 (55)
#8-3	21	9 (43)	12 (57)
#8-7	17	8 (47)	18 (53)

[a] Endogeneous *WRN* cDNA fragment containing codon 1074 (TTG).
[b] Exogenous *WRN* cDNA fragment containing codon 1074 (TTT).

not distinguish the expression of mutant *WRN* gene from that of intact *WRN* gene. Thus, this is the first report revealing the differential expression levels between intact and mutated *WRN* genes in WS cells. This differentiability can be achieved by establishment of the WS cell line in which a human chromosome 8 has been introduced (5). In contrast to this, there has been no previous report of establishment of a proliferative WS cell line expressing the WRN protein by the introduction of a cloned *WRN* gene, suggesting that overexpression of the *WRN* gene under the influence of an artificial promoter in an expression vector might be disadvantageous for the growth of WS cells.

Accumulated evidence has shown that mRNAs are monitored for errors by an RNA surveillance mechanism, with the result that imperfect messages containing premature translation termination codons (PTCs) are rapidly degraded (19, 20). This pathway, known as NMD, reduces the accumulation of truncated or even deleterious proteins. NMD plays a significant role in the etiology of human genetic diseases including β-thalassemia (19), ataxia telangiectasia (AT) (24, 25), Marfan syndrome (26), and inherited cancers (27, 28). In β-thalassemia, PTC mutations in the two 5′ proximal exons of the β-globin gene are common, and the PTC-mutated mRNA is degraded by NMD (28). It is noteworthy that the majority of mutations in the *ATM* gene that cause AT and those in *BRCA1* that are associated with breast cancer include PTCs (24, 25, 29). The important role of NMD is to serve as an mRNA surveillance mechanism to protect against adverse effects resulting from a truncated dominant-negative protein in the heterozygotes of recessive diseases. For example, the majority of β-thalassemia heterozygotes are phenotypically healthy, whereas homozygotes suffer from severe symptoms of anemia (19). Therefore, this function of NMD serves as a quality control of gene expression.

It is intriguing that all of the *WRN* mutations thus far identified are predicted to truncate the WRN protein with a loss of the C-terminal nuclear localization signal (NLS) from the 1432 residue native protein (9, 16). The present study indicates that these mutated WRN mRNAs are eliminated possibly by NMD in WS cells, supporting the idea that NMD plays a significant role in the etiology of WS. Alternatively, it is possible that the expression of the aberrant *WRN* gene is selectively suppressed in WS cells as a reason for reduced levels of the mutated WRN mRNAs. However, so far as we know, the selective suppression of aberrant *WRN* gene expression has not been evidenced. Although it is evidenced that WRN promoter activity is specifically depressed in WS cells, this reduced promoter activity is not distinctive for mutated *WRN* gene (30). The evidence that shows that reduced levels of an aberrant mRNA are caused by NMD but not related to promoter function has also been demonstrated in an anemia that shows a deficiency in the glycolytic enzyme, triosephosphate isomerase (TPI) (31).

NMD might eliminate putative adverse effects induced by the mutant WRN in WS heterozygotes. Wang *et al.* (32) demonstrated that that fibroblasts derived from transgenic mice expressing human *WRN* gene with a putative dominant-negative mutation showed three characteristics of WS cells such as hypersensitivity to 4NQO, reduced replicative potential, and reduced expression of the endogenous WRN protein. This clearly indicates that the expression of *WRN* gene with a dominant-negative mutation is deleterious for cells, suggesting that the role of NMD eliminates premature aberrant *WRN* mRNA to avoid adverse effects in WS heterozygotes.

However, several studies have demonstrated the difference in phenotypes of cells from WS heterozygotes as compared with normal cells. For example, B cell lines from clinically normal heterozygous carriers exhibit intermediate sensitivity to 4NQO between normal individuals and WS patients (7). In addition, genetic instability as measured by the frequency of loss of the GPA O/N allele variant *in vivo* is increased in heterozygous family members (33). Presumably, an insufficient amount of the WRN protein might be responsible for these abnormal responses, because the NMD surveillance system possibly eliminates premature WRN mRNA, preventing adverse effects by the mutant WRN protein. We (5) previously demonstrated that expression of normal WRN protein did not rescue the abnormal phenotypes of WS 780 cells, suggesting that WS phenotypes develop through a multistep process initiated by WS mutations. Therefore, further studies are needed to elucidate the phenotypic differences between normal cells and WS heterozygous cells.

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