Mutator Phenotype of Werner Syndrome is Characterized by Extensive Deletions

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Genetics

Mutator phenotype of Werner syndrome is characterized by extensive deletions

(aging/genomic instability syndrome/somatic mutation/simian virus 40-transformed fibroblasts/hypoxanthine phosphoribosyltransferase)

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ABSTRACT **Werner syndrome (WS) is a rare autosomal-recessive disorder characterized by the premature appearance of features of normal aging in young adults. The extensive phenotypic overlap between WS and normal aging suggests they may also share pathogenetic mechanisms. We reported previously that somatic cells from WS patients demonstrate a propensity to develop chromosomal aberrations, including translocations, inversions, and deletions, and that WS cell lines demonstrate a high spontaneous mutation rate to 6-thioguanine resistance. We report here the biochemical and molecular characterization of spontaneous mutations at the X chromosome-linked hypoxanthine phosphoribosyltransferase (*HPRT*) locus in 6-thioguanine-resistant WS and control cells. Blot hybridization analysis of 89 independent spontaneous *HPRT* mutations in WS and control mutants lacking HPRT activity revealed an unusually high proportion of *HPRT* deletions in WS as compared with control cells (76% vs. 39%). Approximately half (58%) of the deletions in WS cells consisted of the loss of greater than 20 kilobases of DNA from the *HPRT* gene. These results suggest that an elevated somatic mutation rate, and particularly deletions, may play pathogenetically important roles in WS and in several associated age-dependent human disease processes.**

Werner syndrome (WS; McKusick catalog no. 27770) is a rare autosomal-recessive human disorder characterized by the premature appearance in young adults of several features of normal aging (1–3). The extensive phenotypic overlap between WS and normal aging suggests that they may share pathogenetic mechanisms as well. This possibility has been explored by comparing the properties of cells and tissues from WS patients with those derived from control donors (3, 4). For example, skin fibroblast cells derived from WS patients demonstrate a severely limited capacity to divide *in vitro*. The limited *in vitro* division potential of WS fibroblasts is similar to, though more extreme than, that displayed by fibroblasts from aged normal donors (4).

We have reported two lines of evidence that suggest somatic mutations may play a role in the pathogenesis of WS and of associated age-dependent disease processes: somatic cells from WS patients display a propensity to develop chromosomal translocations, inversions, and deletions; and simian virus 40 (SV40)-transformed fibroblast cell lines from unrelated WS patients display an elevated spontaneous mutation rate at the X chromosome-linked hypoxanthine phosphoribosyltransferase (*HPRT*; EC 2.4.2.8) locus (5, 6).

We report here the biochemical and molecular characterization of 89 independent spontaneous *HPRT* mutations in 6-thioguanine (Sgu)-resistant mutants isolated from two WS and two control SV40-transformed fibroblast cell lines. These results confirm our previous identification (6) of a mutator phenotype in cell lines derived from WS patients and identify a high proportion of deletions among spontaneous *HPRT* mutations in WS cells. These experiments also provide a rationale for attempting to identify the biochemical defect(s) in WS by using transformed cells from WS patients. Previous efforts using nontransformed cells have been compromised by the severely limited division potential of WS cells (4).

MATERIALS AND METHODS

Cells and Cell Culture. WS SV40-transformed fibroblast cell lines were derived from a 45-year-old male WS patient (cell line W-V) (7) and from an unrelated 29-year-old female WS patient (cell line PSV811) (8). Control SV40-transformed fibroblast cell lines derived from an 8-year-old male with galactosemia (cell line GM639) and from a normal 18-year-old female (cell line GM637) were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ). All four cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 6% (vol/vol) fetal bovine serum (GIBCO, Grand Island, NY, or Biologos, Naperville, IL), penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2/95% air, at 37°C. Cell lines were tested on several occasions using a 4′,6-diamidino-2-phenylindole (DAPI) fluorescence assay and found to be negative for the presence of mycoplasma.

Determination of Spontaneous Mutation Rates. The spontaneous mutation rates of WS and control cells to Sgu resistance were determined by the fluctuation test of Luria and Delbrück (9), as described (6), and by the serial sampling method of Newcombe (10, 11). In mutation rate determinations by the method of Newcombe (10, 11), replicate cultures that contained no Sgu-resistant colonies in an initial portion plated in Sgu (i.e., initial mutation frequency, $F_1 = 0$) were replated after 6–7 weeks of additional cell growth to determine the frequency of Sgu-resistant colonies (final mutation frequency, F_2). Independent Sgu-resistant mutants isolated during mutation rate determinations in the presence of 60 μM Sgu were subsequently grown for biochemical and molecular analysis.

Biochemical and Molecular Characterization of *HPRT* Mutations. HPRT activity in Sgu-resistant WS and control cells was measured by the method of Bakay *et al.* (12) as described (13). The parent cell lines and a Lesch–Nyhan lymphoblastoid cell line (GM2292A) (14) were used as positive and negative controls for HPRT enzyme activity assays.

Isolation and blot hybridization analysis of nuclear DNA and RNA from Sgu-resistant WS and control cells using a human HPRT cDNA probe were performed as described (13). Localization of deletions within the *HPRT* gene and the identification of exons contained in novel-sized restriction fragments derived from the X chromosome-linked *HPRT* locus were performed with probes consisting of a 5′ portion of the first *HPRT* intron (p1.7; ref. 15) and subclones of a 908-base-pair HPRT cDNA probe (16) that contained *HPRT*

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; Sgu, 6-thioguanine; WS, Werner syndrome; SV40, simian virus 40.
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Table 1. Determination of spontaneous mutation rates of WS and control cells to Sgu resistance

Method	Cell line			
	W-V	PSV811	GM639	GM637
Serial sampling (Newcombe)*				
Initial cell number (N_1)	1.75×10^6	2.5×10^6	2.4×10^6	4.4×10^6
Final cell number (N_2)	3.6×10^{12}	6.55×10^{11}	3.3×10^{14}	9.2×10^{12}
Cells plated, n	1.6×10^7	2.56×10^7	2.0×10^7	4.4×10^7
Sgu^r colonies (F_2)	21	27	4	1
Mutation rate, no. per cell per generation	8.4×10^{-7}	4.9×10^{-7}	2.8×10^{-8}	7.9×10^{-9}
Fluctuation (mean method)†				
Mutation rate, no. per cell per generation	1.1×10^{-6}	2.8×10^{-7}	5.4×10^{-9}	9.2×10^{-9}

Sgu^r, Sgu resistant.

*Mutant colony frequencies for each cell line were corrected by dividing by colony-forming efficiency for the mutation rate calculations. The spontaneous mutation rate, a, was calculated using the following formula (11): $a = (\ln 2)\,(F_2)/\ln(N_2/N_1)$.

†Mean number of Sgu-resistant colonies per replicate culture, r, was corrected by the cloning efficiency of the cell line used and by the ratio of the number of cells plated to the final cell number in each replicate. A total of 116 replicate cultures in all were used. The mutation rate, a, was calculated from the following formula of Luria and Delbruck (9): $r = aN_t \ln(N_t Ca)$, where C is the number of replicate cultures used and N_t is the final cell number per replicate.

exons 1, 3, 6, 7 plus 8 or exon 9. A 700-base-pair chicken β-actin cDNA was used as a positive control probe in Northern blot analyses.

RESULTS

Spontaneous mutation rates to Sgu resistance ranged from 2.8×10^{-7} to 1.1×10^{-6} per cell per generation for the WS cell lines W-V and PSV811 and from 5.4×10^{-9} to 2.8×10^{-8} per cell per generation for the control cell lines GM637 and GM639 (Table 1). Both WS and control cell lines were comparably sensitive to Sgu. Plateau survival frequencies were reached for each cell line at Sgu concentrations between 12 and 30 μM (data not shown), and 56 of 57 independent Sgu-resistant sublines isolated in 60 μM Sgu contained less than 1% of control HPRT activity (see Fig. 2). The HPRT activity assay used can detect 0.5% of control (wild type) HPRT activity (data not shown).

The structure of the *HPRT* gene in 66 independent Sgu-resistant WS and 23 independent Sgu-resistant control sublines was analyzed by Southern blot hybridization with a human HPRT cDNA probe (Figs. 1 and 2). Seventy-six percent (50/66) of WS and 39% (9/23) of control Sgu-resistant sublines contained an abnormal *HPRT* gene. *HPRT* gene alterations identified in WS and control cells consisted of deletion of either part (23/50 WS or 4/9 control sublines) or all (27/50 WS or 5/9 control sublines) of the *HPRT* gene. A restriction site polymorphism consisting of the loss of the *Eco*RI site in *HPRT* intron 5 was also identified in the parent W-V WS cell line and in Sgu-resistant W-V sublines (data not shown). Southern blot hybridization results were unambiguously interpretable in Sgu-resistant sublines isolated from the male-derived WS (W-V) and control (GM639) cell lines and from the female-derived PSV811 cell line, in which Sgu-resistant sublines appear to contain a single X chromosome. Only 1 of 6 Sgu-resistant sublines isolated from the female-derived control cell line GM637 contained an identifiable alteration in the *HPRT* gene (Fig. 1, GM637-27R).

Northern blot hybridization analysis of steady-state HPRT mRNA levels in Sgu-resistant WS and control sublines revealed three patterns of hybridization (summarized in Fig. 2; data not shown): production of a normal-size [1.6 kilobases (kb); refs. 14 and 16] HPRT mRNA (16 sublines), production of a shorter-than-normal HPRT mRNA (WS sublines PSV811-24R and -29R), and the absence of detectable HPRT mRNA (34 sublines). Northern blot hybridization analysis of Sgu-resistant WS and control sublines that contained an apparently normal *HPRT* gene revealed that 9 of 13 WS and 7 of 12 control Sgu-resistant sublines produced a normal-size

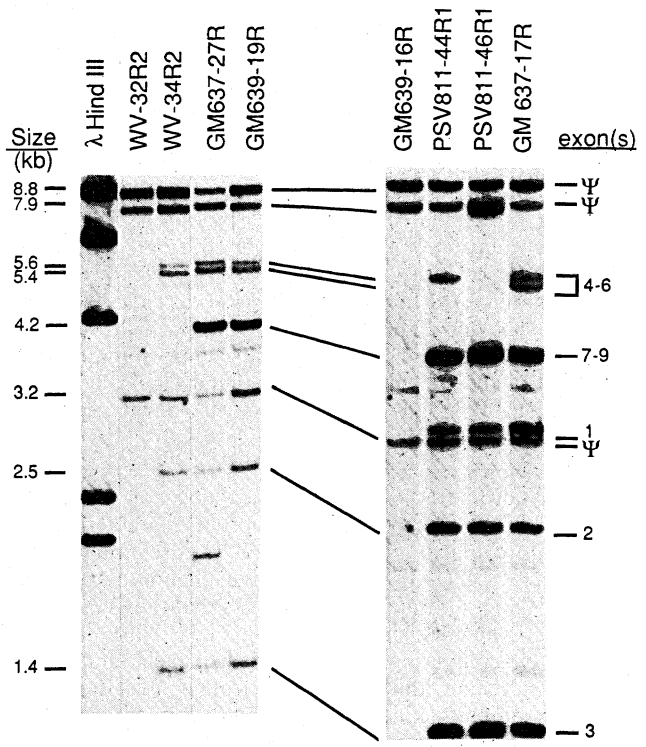

FIG. 1. *HPRT* gene structure in Sgu-resistant WS and control cells. Control sublines GM639-19R and GM637-17R demonstrate the expected pattern and number of fragments produced by *Pst* I from the X chromosome-linked *HPRT* gene and autosomal *HPRT* pseudogenes (Ψ) (13, 14). Sublines WV-32R2 and GM639-16R demonstrate complete deletion of the X chromosome-linked *HPRT* gene with loss of all X chromosome-linked exon-containing fragments. Sublines WV-34R2 and PSV811-44R1 demonstrate loss of one X chromosome-linked exon-containing fragment each, whereas sublines GM637-27R and PSV811-46R1 demonstrate partial deletions of the *HPRT* gene with the appearance of additional fragments of 1.85 kb (GM637-27R) and 7.7 kb (PSV811-46R1). The 1.85-kb novel band identified in subline GM637-27R was produced by a deletion in the region of *HPRT* between exons 2 and 3 from the genetically active X chromosome in the female-derived GM637 cell line. The expected 2.5-kb exon 2- and 1.4-kb exon 3-containing *Pst* I fragments from the genetically inactive X chromosome in subline GM637-27R can be seen as bands of half intensity. The presence of the 3.2-kb exon 1-containing fragment in sublines (as shown on the left) was confirmed on blots washed at a slightly lower stringency (as shown on the right). A human HPRT cDNA probe (16) was used for both analyses. Fragment sizes and exon or pseudogene (Ψ) assignments are indicated. A *Hin*dIII-digested λ size standard is shown.

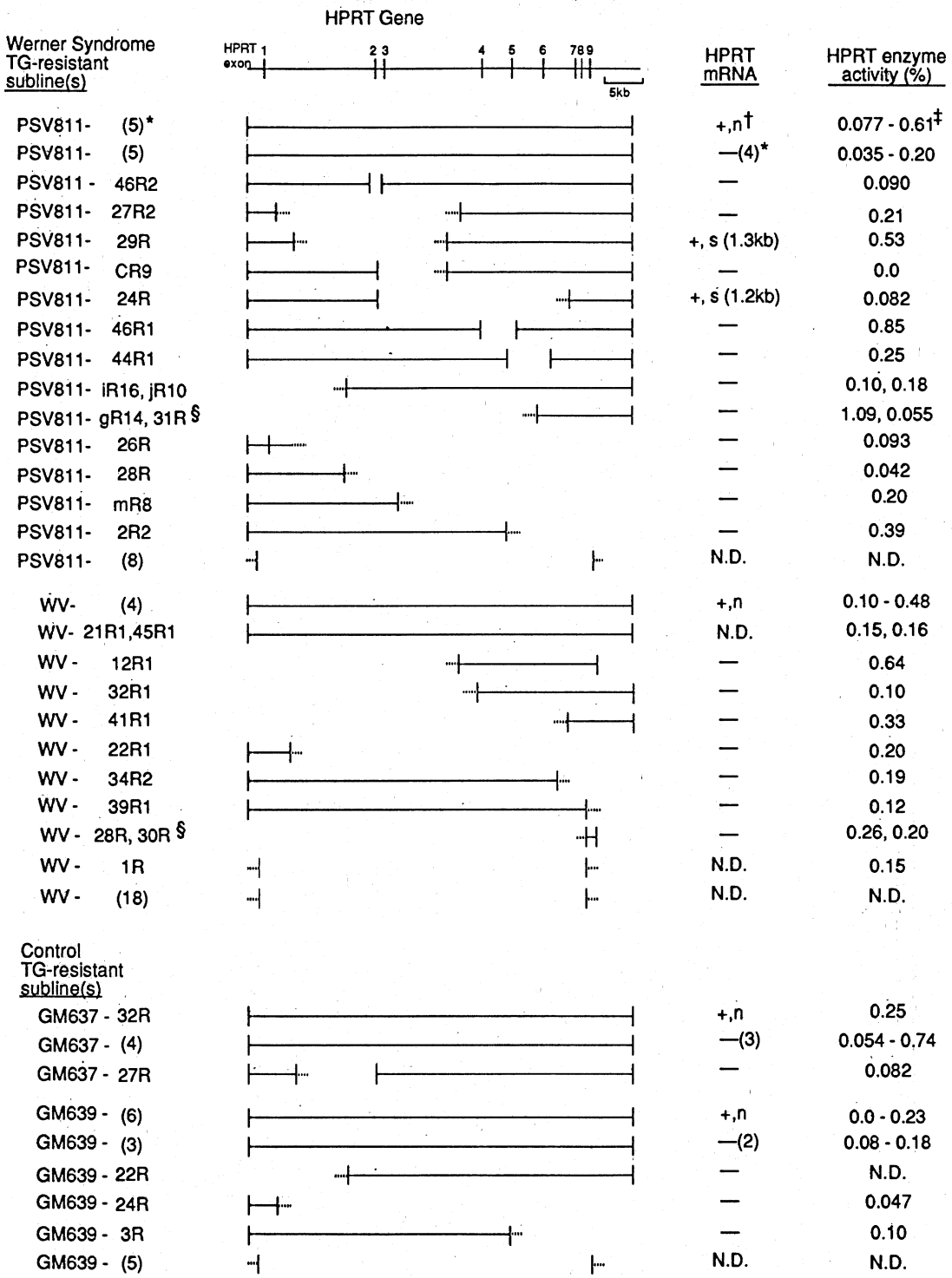

FIG. 2. *HPRT* mutation spectrum and HPRT activity in Sgu-resistant WS and control cells. The structure of the normal human *HPRT* gene is represented by the horizontal line at the top of the figure. The positions of *HPRT* exons are indicated by vertical lines. The 5′ end of each subline restriction map is marked by the 5′ end of the *Pst* I fragment containing *HPRT* exon 1. The 3′ end of each map is indicated by the 3′ end of the restriction fragment containing *HPRT* exons 5–9 (*Hin*dIII) or exons 7–9 (*Pst* I). The structure of the *HPRT* gene in 89 independent Sgu-resistant WS and control sublines is shown below the *HPRT* gene map. The genetically active X chromosome only is displayed for the female-derived control line GM637. Deleted segments in individual sublines are bounded by vertical uprights. Uncertainty in the position of deletion end points of >1 kb or in the extent of deletion is indicated by a dotted line. Subline designations in parentheses (*) indicate the number of independent Sgu-resistant sublines from a given cell line that demonstrated identical blot hybridization patterns. The Sgu-resistant WS subline pairs WV-28R/-30R and PSV811-gR14/-31R (§) contain novel bands of different sizes and thus represent different deletions. The presence and size of HPRT mRNA and amount of HPRT enzyme activity in Sgu-resistant WS and control sublines are shown to the right of the *HPRT* gene maps. Steady-state HPRT mRNA (†) was present (+) and of normal size (n; 1.6 kb) or shorter than 1.6 kb (s) or absent (—). The amount of HPRT enzyme activity (‡) in Sgu-resistant WS and control sublines is indicated as a percent of the activity found in Sgu-sensitive parental cell lines. For Sgu-resistant sublines having the same blot hybridization patterns, the range of HPRT activities in the individual sublines is given. N.D., not determined.

HPRT mRNA. The blot hybridization assay used can detect 1% of control HPRT mRNA levels (data not shown).

DISCUSSION

Our results demonstrate that cell lines derived from unrelated WS patients have an elevated spontaneous mutation rate at the *HPRT* locus and that a high proportion of spontaneous *HPRT* mutations in Sgu-resistant WS cell lines consists of *HPRT* gene deletions. The mutation rates of WS and control cell lines were determined by two methods. Both methods demonstrated a greater than 10-fold elevation in the rate of forward mutation at the *HPRT* locus in WS cells (Table 1). These results confirm, using two additional control cell lines, the previous identification (6) of a mutator phenotype in WS cells. We would suggest caution in interpreting the absolute mutation rate differences between WS and control cells, however, due to inherent difficulties in determining mutation rates in mammalian cells (17) and the small number of Sgu-resistant cells recovered from control cell lines (Table 1).

The most surprising finding of the blot hybridization analysis of *HPRT* mutations in Sgu-resistant WS and control cells was the high proportion of deletions in WS cells (Fig. 2). Fifty of 66 (or 76%) independent WS mutants demonstrated a partial or complete deletion of the *HPRT* gene. Only half as many deletions were observed in control cultures (9 of 23 mutants or 39%). This difference in the proportion of deletions is statistically significant ($P < 0.0025$, $\chi^2 = 8.67$ with 1 degree of freedom in a single-tail test) and may be greater than the observed 2-fold difference for at least two reasons. The stringent selection conditions we used (60 μM Sgu) will select against *HPRT* mutants that contain residual HPRT activity. Mutants containing residual HPRT activity are likely to represent a larger fraction of mutants in control cultures, and thus their loss during selection would lead to an overestimate of the proportion of remaining deletions in control cultures. Second, the blot hybridization assays we used will not detect most microdeletions (<100 bp) in the 45-kb human *HPRT* gene (18–20). If microdeletions are present in greater proportion in WS as compared with control cells, we would not have detected this difference.

The WS mutator phenotype appears to have a strong specificity for deletions. This preference suggests at least two mutational pathways that may be responsible for the WS mutator phenotype and the production of *HPRT* deletions in WS cells. WS cells could promote recombination between DNA sequences within and between chromosomes to produce deletions, inversions, and transpositions. Alternatively, WS cells might mimic the effects of x- and γ-irradiation by producing high endogenous levels of reactive oxygen intermediates (21). This intriguing possibility is suggested by the high proportion of deletions seen in collections of x- and γ-ray-induced *HPRT* mutations (21–24). X- and γ-radiation can also induce cytogenetic changes that occur spontaneously at high frequency in WS cells (5, 25) and the premature appearance of selected features of aging (26).

One concern in using SV40-transformed WS cell lines to further investigate the WS mutator phenotype is that SV40 transformation alone or in concert with the WS mutation might alter cellular mutagenic pathways. SV40 transformation has been shown in selected instances to induce sister-chromatid exchanges and mutations and to increase protein translational error rates (27–30). It is unlikely that SV40 transformation alone is contributing to mutagenesis in the control cell lines we used: the observed mutation rates in control cells were quite low, and the mutational spectrum of control cells was similar to spontaneous *HPRT* mutational spectra determined using other human cell lines that were not derived by SV40 transformation (13, 31, 32). It will be necessary to determine the rate and spectrum of spontaneous *HPRT* mutations in primary WS cells to rule out the formal possibility that SV40 transformation and the WS mutation interact to alter mutagenesis in WS cells.

Two lines of evidence suggest that the mutator phenotype we observed using SV40-transformed WS cell lines also occurs in WS patients. We have demonstrated translocation mosaicism *in vitro* in fibroblasts and lymphocytes from different WS patients and in primary fibroblast cultures derived from one WS patient (33). In addition, an 8-fold elevation in the frequency of Sgu-resistant peripheral blood lymphocytes in five unrelated WS patients as compared with controls has been demonstrated using an autoradiographic assay (unpublished data, and ref. 34). The molecular analysis of spontaneous *HPRT* mutations in independent T-cell clones will be required to determine whether deletions are the predominant class of mutation in these cells.

The elevated rate and altered spectrum of *HPRT* mutations we observed in WS cells suggest that somatic mutations, and perhaps the preferential accumulation of deletions, may play a role in the pathogenesis of WS and of associated age-dependent human disease processes, such as neoplasia and atherosclerosis (35–37). Deletions could initiate or promote these processes by altering the structure or expression of genes or the structure and mitotic transmission fidelity of chromosomes.

The mutator phenotype of WS and the limited cell division potential of WS cells *in vitro* may represent useful targets for gene transfer to complement, and thus identify, the genetic defect in WS. In addition, the detection of consistent deletions or chromosomal mutations in WS cells might allow genes that interact with the WS mutation to be identified. The identification and molecular characterization of the WS locus and of genes that interact with it may be of practical importance: these genes appear to modulate the rate of appearance of aging and of several clinically important age-associated human disease processes.

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