Cellular Werner Phenotypes in Mice Expressing a Putative Dominant-Negative Human *WRN* Gene

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ABSTRACT

Mutations at the Werner helicase locus (*WRN*) are responsible for the Werner syndrome (WS). WS patients prematurely develop an aged appearance and various age-related disorders. We have generated transgenic mice expressing human *WRN* with a putative dominant-negative mutation (K577M-WRN). Primary tail fibroblast cultures from K577M-WRN mice showed three characteristics of WS cells: hypersensitivity to 4-nitroquinoline-1-oxide (4NQO), reduced replicative potential, and reduced expression of the endogenous WRN protein. These data suggest that K577M-WRN mice may provide a novel mouse model for the WS.

WERNER syndrome (WS) is an autosomal recessive progeroid syndrome characterized by premature development of an aged appearance and many disorders associated with advanced age, such as bilateral cataracts, scleroderma-like skin, osteoporosis, several forms of arteriosclerosis, hypogonadism, type II diabetes mellitus, and neoplasia. Symptoms appear at puberty and death occurs at a mean age of 47 years, usually as a result of cardiovascular diseases or malignancies (Epstein *et al.* 1966; Martin *et al.* 1970; Tollefsbol and Cohen 1984; Goto 1997).

Primary cultures of somatic cells from WS patients have very limited proliferative potentials and retarded cell cycle progression (Martin *et al.* 1970, 1974; Tollefsbol and Cohen 1984; Salk *et al.* 1985a; Kill *et al.* 1994). Genomic instability is also evident and has been characterized as "variegated translocation mosaicism" (Salk *et al.* 1985b). Hypersensitivity to a genotoxic agent, 4-nitroquinoline-1-oxide (4NQO), has also been documented (Gebhardt *et al.* 1988; Ogburn *et al.* 1997). Lymphoblastoid cell lines (LCLs) from individuals heterozygous for *WRN* mutations exhibit sensitivities that are intermediate between those of the homozygous mutants and wild type (Ogburn *et al.* 1997).

The Werner syndrome is caused by mutations at the *WRN* locus on chromosome 8p (GenBank accession no. L76937; Yu *et al.* 1996). Human WRN protein (hWRNp) contains an N-terminal exonuclease domain, a central helicase domain, and a C-terminal nuclear localization signal (Gray *et al.* 1997; Matsumoto *et al.* 1997; Suzuki *et al.* 1997; Huang *et al.* 1998). More than 20 *WRN* mutations have been identified in WS patients (Yu *et al.* 1996; Moser *et al.* 1999). All result in truncated protein products missing the nuclear localization signal. The mouse *WRN* gene has also been cloned and shown to share the characteristic domains of the human homolog, overall amino acid sequence homology being 76% (Imamura *et al.* 1997).

Activities ($3' \rightarrow 5'$ helicase and $3' \rightarrow 5'$ exonuclease) of the recombinant WRNp have been demonstrated by *in vitro* assays and it has been shown that a single amino acid substitution at position 577 (K577M) in the recombinant human WRNp results in the abolishment of the ATPase and helicase activities, but not the exonuclease activity (Gray *et al.* 1997; Huang *et al.* 1998). The corresponding mutation in *Escherichia coli uvrD* encoding a $3' \rightarrow 5'$ helicase causes a dominant-negative effect on cell growth in response to UV light (George *et al.* 1994).

To examine the role of *WRN* in an animal model, we generated mouse lines overexpressing either the K577M mutant or wild-type human *WRN* using conventional transgenic methodology (Hogan *et* al. 1986). The expression of several cellular phenotypes characteristic of the human WS in transgenic mice suggests a dominant-negative action of the K577M-WRN allele *in vivo.*

MATERIALS AND METHODS

Generation of transgenic lines: Mouse expression vectors were constructed by subcloning full-length cDNA encoding human wtWRNp and human K577M-WRNp into pBSCA (derived from pCAGGS). This vector contains a cytomegalovirus enhancer, a chick β-actin promoter, and a rabbit β-globin polyadenylation site (Gray *et al.* 1998). Transgenic mice were created by pronuclear injection as previously described (Hogan *et al.* 1986). The original founder animals were on

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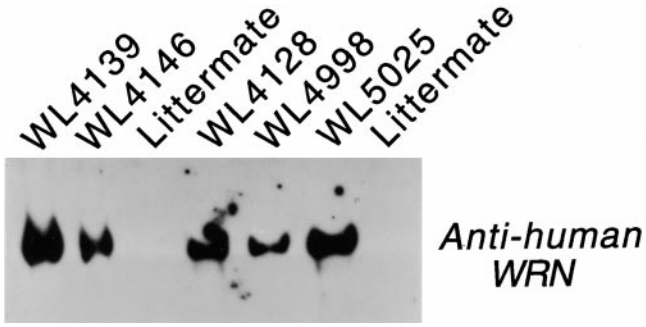

Figure 1.—Expression of wtWRNp and K577M-WRNp in primary tail fibroblasts. Western analysis of nuclear fractions of tail fibroblasts utilized anti-C-terminal human WRN antibody (Gray *et al.*, 1998). WL4139 and WL4146 are the transgenic lines of K577M-WRN, and WL4128, WL4998, and WL5025 are the transgenic lines of wtWRN.

a mixed C57BL/6J × C3HJ background. Three lines of K577M mutant transgenics (WL4128, WL4998, and WL5025) and two lines of wild-type WRN transgenics (WL4139 and WL4146) were generated using traditional pronuclear injection methods and backcrossed with C57BL/6J. Primary fibroblast cultures from the tails of founders and/or first backcross offspring were used for the experiments.

Preparation of cells: Mouse tails were minced and digested with collagenase and primary mouse tail fibroblasts were cultured as described (Martin *et al.* 1996). In brief, aseptically collected biopsies of mouse tails were minced, washed three times in Ca and Mg-free phosphate-buffered normal saline (PBS; pH 7.1), and resuspended in 20 ml of a 1:1 mixture of Type 1 collagenase (1 mg/ml in PBS) and Dulbecco's Modified Eagle Media (DMEM; GIBCO/BRL, Gaithersburg, MD) with 100 units/ml penicillin and 100 μg/ml streptomycin. The suspension was stirred at moderate speed for 1 hr in a 37° incubator to digest tissues. After letting the tissue fragments settle, the supernatant was collected and one-tenth volume of fetal bovine serum (FBS) was added to stop the collagenase action. The supernatant was stored in ice. The remaining tissue fragments were redigested for an additional 1 hr at 37°. At the end of this digest there were few if any tissue fragments remaining. The resulting cell suspension was combined and centrifuged at 2600 × *g* for 5 min. The resulting cell pellet was resuspended in 10 ml of DMEM supplemented with 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 10% FBS that was heat-inactivated at 56° for 30 min. This cell suspension was used for the experiments.

A SV40-transformed normal human fibroblast cell line, GM649, was obtained from Coriell Cell Repositories (Camden, NJ). All cells were maintained in DMEM media supplemented with 10% heat-inactivated FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified, 5% CO_2 incubator at 37°.

Western blot analysis: A total of 2×10^5 primary tail fibroblasts were plated in 100-mm cell culture dishes. Cells were collected 48 hr later, and nuclear and cytoplastic fractions were separated. A total of 100 μg of nuclear protein was resolved by 7% SDS-PAGE and visualized by Western analysis, using anti-human WRN or anti-mouse WRN polyclonal antibodies. Anti-human WRN antibody was made against glutathione *S*-transferase (GST)-fused partial human WRN C terminus, aa 982–1432, and was affinity-purified. Anti-mouse WRN antibody was made against GST-fused partial mouse WRN C terminus, aa 997–1297, and was also affinity-purified (Gray *et al.* 1998). This anti-human WRN antibody usually does not cross-react with mouse WRNp (Figure 1), although in one experiment (Figure 5) it weakly cross-reacted with mouse WRNp.

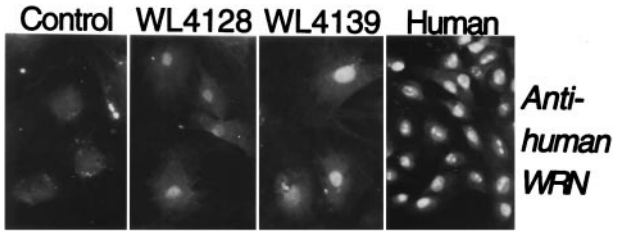

Figure 2.—Subcellular localization of wtWRNp and K577M-WRNp in primary tail fibroblasts. Indirect immunofluorescent staining utilized the same antibody as in Figure 1 in primary tail fibroblasts from littermate control, WL4128, WL4139, and human fibroblasts, GM439, which serves as a positive control (Gray *et al.* 1998).

4NQO sensitivity assay: Aliquots (1 ml) of the collagenase-derived cell suspension from mouse tails were plated in 75-cm^2 flasks. Exponentially growing cells were replated 48–72 hr later in multiple 25-cm^2 tissue culture flasks at 5×10^4 cells/flask in triplicates. The media were replaced 24 hr later with media containing the indicated concentrations of 4NQO (Sigma, St. Louis; stock 3 mg/ml in DMSO). DMSO was added to the control flasks at a level equivalent to that of the highest dose of 4NQO. After 24 hr all flasks were fed with fresh media without 4NQO and incubated for an additional 48 hr. The flasks were then trypsinized and counted using a hemocytometer to determine the survival of cells.

Clone size distribution assay: The collagenase-derived cell suspension (10 ml total per mouse tail) was plated in four 100-mm cell culture dishes at a ratio of 10 μl per dish in 10 ml media and incubated for 10 days. This density provides well-separated individual cells and colonies. Dishes were fed once at between 5 and 7 days with fresh media. Dishes were then rinsed with PBS and stained with 0.5% crystal violet in 20% ethanol. Using a dissecting microscope, the colonies and individual undivided cells were scored for the number of cell divisions that had occurred.

Statistical analysis: Differences in 4NQO sensitivity were tested by two-factor (dose by genotype) ANOVA using Statview (Abacus Concepts, Berkeley, CA). *Post hoc t*-tests were then performed to describe differences in 4NQO sensitivity across genotypes. For clone size distribution assays, statistical comparisons between genotypes were made using *t*-tests comparing the numbers of colonies achieving a given number of cell divisions.

RESULTS

Generation of transgenic mice overexpressing K577M-WRNp and wild-type-WRNp: The degree of expression of the transgenes in the primary tail fibroblasts varied from line to line as determined by Western analysis using an antibody specific for the C terminus of hWRNp (Gray *et al.* 1998; Figure 1). Primary fibroblasts from five transgenic lines showed various levels of expression, while a nonspecific band showed (see Figure 5) similar levels, indicating equal loading (data not shown). Immunofluoresence staining of hWRNp revealed that both wtWRNp and K577M-WRNp were localized to nucleoplasm (Figure 2). This was in contrast to

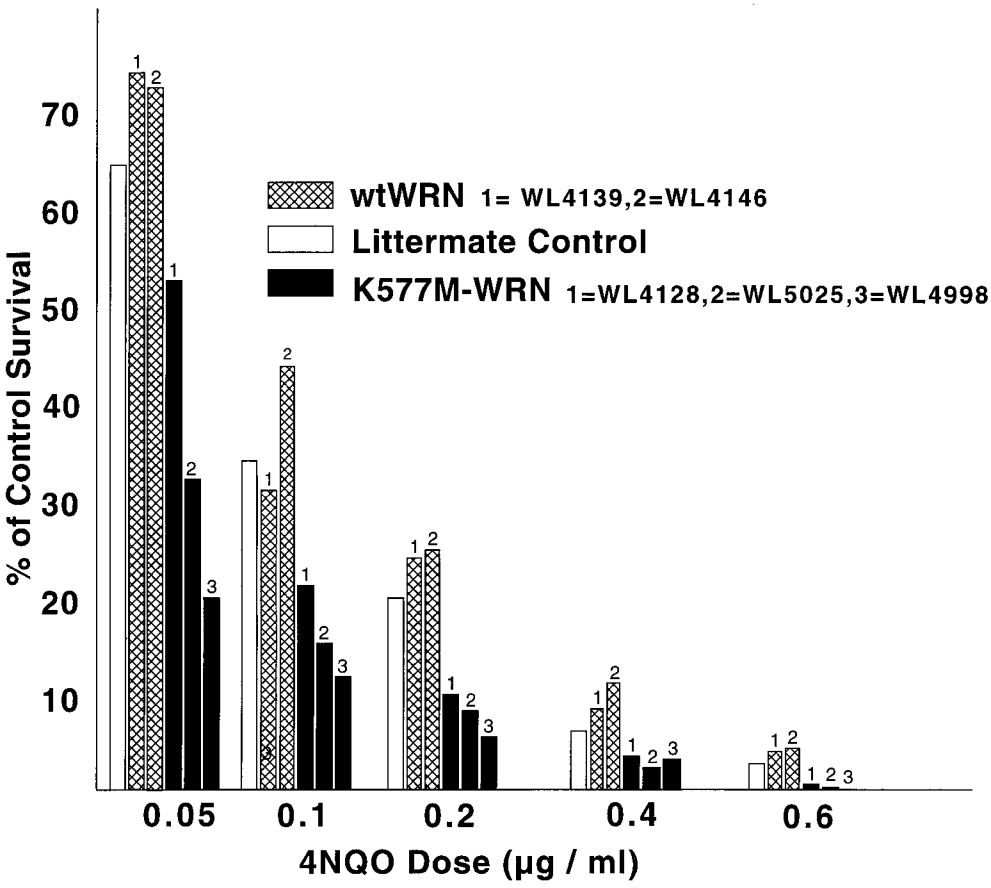

Figure 3.—4NQO sensitivity of primary tail fibroblasts. Cell survival was expressed as the percentage of untreated control cells (control counts ranged from 1 to 2.5 × 10^5 cells) for each dose of 4NQO. Open bars represent the mean survival of nontransgenic littermate control cells (N = 17 mice), crosshatched bars represent the mean survival of cells from transgenic mice expressing wtWRN (1, WL4139, N = 9 mice; 2, WL4146, N = 4 mice), and solid bars represent the mean survival of cells from mice expressing K577M-WRN (1, WL4128, N = 10 mice; 2, WL5025, N = 3 mice; 3, WL4998, N = 1 mouse).

the pattern of localization in replicating populations of human fibroblasts, in which nucleoli were more intensely stained (Figure 2). It has been reported that hWRNp is primarily localized to the nucleoli of actively replicating cells (Gray *et al.* 1998; Marciniak *et al.* 1998), while mWRNp is localized to nucleoplasm (Marciniak *et al.* 1998). The intensities of immunofluorescence staining in the mouse fibroblasts roughly reflected the levels of protein expression as determined by Western analysis. Tail fibroblasts from WL4128 expressed relatively low levels of K577M-WRNp by both Western analysis and immunofluorescence staining, whereas WL4139 expressed relatively high levels of wtWRNp by both methods. In each culture, virtually all cells stained with comparable intensities.

Hypersensitivity to 4NQO and reduced proliferative capacity in K577M-WRN mice: We examined two well-characterized cellular phenotypes observed in WS cells: sensitivity to a genotoxic agent, 4-nitroquinoline-1-oxide (4NQO; Ogburn *et al.* 1997), and replicative potential (Salk *et al.* 1985a). Hypersensitivity to 4NQO was observed in primary tail fibroblasts from all three K577M-WRN mice, whether derived from the founder or the backcrossed offspring (Figure 3). There was no correlation between the amount of K577M-WRNp and the degree of 4NQO sensitivity among the three K577M-WRN lines available for analysis. Significantly reduced survival of cells from K577M-WRN mice was observed when compared to the sensitivities observed in cells from wtWRN transgenics (P = 0.008 by ANOVA analysis). *Post hoc* analyses showed significant differences for four of five dose levels ($P < 0.05$); the comparison at 0.05 µg/ml was not significant (P = 0.079). When sensitivities in K577M-WRN mice were compared to those of nontransgenic littermate controls, the difference was again significant (P = 0.0327 by ANOVA analysis). *Post hoc* analyses showed significant differences for four of five dose levels ($P < 0.05$); the comparison at 0.4 µg/ml was not significant (P = 0.0759). Combined data from the wtWRN transgenics and from nontransgenic littermate controls showed no significant difference in 4NQO sensitivity (P = 0.729 by ANOVA analysis). This indicates that abolishment of the ATPase activity of hWRNp is able to confer a dominant-negative effect at the cellular level and that additional human wtWRNp does not confer resistance to 4NQO challenge.

The replicative potentials of K577M transgenic cell populations, as determined by clone size distribution assays (Smith *et al.* 1978), were significantly decreased, as compared to cell populations from wtWRN transgenics ($P < 0.01$) and nontransgenic littermate controls ($P < 0.01$; Figure 4). There were no statistically significant differences among the three K577M lines, indicating that differences in levels of K577M-WRNp do

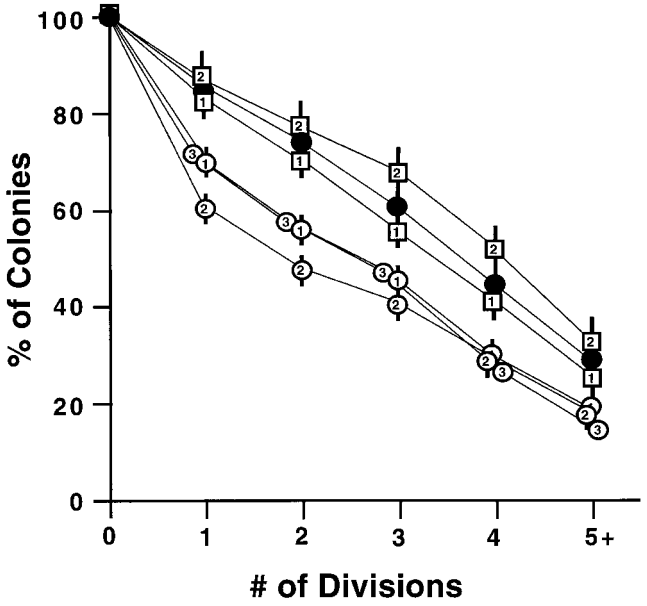

Figure 4.—Clone size distribution of primary tail fibroblasts. Clone sizes are expressed as the mean (±SE of the mean) percentage of cells achieving at least the number of cell divisions indicated. Between 200 and 300 colonies were counted for each animal assayed. Solid circles represent the results for cells from nontransgenic littermate controls (N = 14 mice), numbers in squares represent the results for cells from mice expressing wtWRN (1, WL4139, N = 8 mice; 2, WL4146, N = 2 mice), and numbered circles represent the results for cells from mice expressing K577M-WRN (1, WL4128, N = 8 mice; 2, WL5025, N = 3 mice; 3, WL4998, N = 1 mouse).

not result in detectable differences in cellular replicative potentials. Overexpression of human wtWRNp had no effect on the cellular replicative potential when compared to that of littermate controls (P = 0.158).

Downregulation of the endogenous mouse WRNp in K577M-WRN mice: It has been observed that transcription of reporter genes driven by human *WRN* promoters was reduced to ~40% when these constructs were introduced to WS cells (Wang *et al.* 1998). It has also been shown that *WRN* mRNAs were reduced to less than half of wild-type levels in cells from WS heterozygotes (Yamabe *et al.* 1997). These findings are consistent with the presence of a positive autoregulatory loop for *WRN* gene expression. We therefore performed Western blot assays to determine the expression of endogenous mWRNp levels in the tail fibroblasts used for the above studies. As shown in Figure 5, endogenous mWRNp levels were reduced in tail fibroblasts from K577M-WRN transgenics. By contrast, they were elevated in fibroblasts from wtWRN transgenics. Table 1 summarizes the expression of mouse WRNp in all lines of transgenics including two lines expressing wtWRNp and three lines expressing K577M-WRNp. The results were consistent in replicates, except for one experiment with a culture from a human wtWRN transgenic line (WL4139) in which we observed a 25% reduction of mWRNp. There was no relationship between the degree of expression of the transgenes and the extent of the changes in the expression of endogenous mWRNp. These data suggest that overexpression of either wtWRNp or K577M-WRNp increased or decreased, respectively, the expression of the endogenous mouse gene. Only the K577M-WRNp, however, produced detectable effects at the cellular level.

DISCUSSION

Our data show that expression of a putative dominant-negative human WRNp in transgenic mice conferred three cellular characteristics of WS. First, we documented a significant decrease in replicative potential of fibroblast-like cells from tail skin. This is a particularly well-documented WS phenotype, having been reported by many laboratories and many patients (Martin *et al.* 1970, 1974; Tollefsbol and Cohen 1984; Salk *et al.* 1985a; Kill *et al.* 1994). Second, we established hypersensitivity to a genotoxic agent, 4NQO, shown by two laboratories to be a characteristic of WS (Gebhart *et al.* 1988; Ogburn *et al.* 1997). That 4NQO sensitivity may be a robust marker of WRN helicase deficiency is suggested by the observation of cells from heterozygous carriers that exhibit a sensitivity intermediate between that of wild-type individuals and homozygous WS patients (Ogburn *et al.* 1997). Finally, as shown for human cells deficient in *WRN*, expression from the resident normal allele (in this case two murine alleles) is downregulated.

There are at least two major mechanisms that may be responsible for the observed dominant-negative effects of the mutant form of human WRNp used in our experiments. Because many known helicases function as a multimeric complex, overexpression of a dominant-negative mutant WRNp may cause the WS phenotype

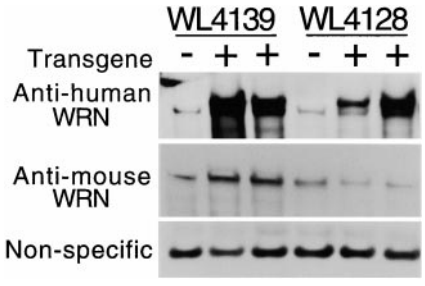

Figure 5.—Expression of endogenous mouse WRNp in primary tail fibroblasts. Western analysis of nuclear fractions of tail fibroblasts in WL4139 (wtWRN transgenic line) and WL4128 (K577M-WRN transgenic line) are shown. +, transgenic mice; −, littermate controls. (Top) Probed with anti-human WRNp antibody, (middle) probed with anti-mouse WRN antibody. (Bottom) The nonspecific bands in the mouse WRNp blot serving as a loading control.

TABLE 1

Endogenous mouse WRN protein (mWRNp) levels in fibroblasts from mice expressing transgenic human wild-type (wtWRN) or mutant WRN (K577M-WRN)

Transgenic lines	Transgene	Relative mWRNp
Control	None	1.00
WL4139	wtWRN	1.61
WL4146	wtWRN	1.22
WL4128	K577M-WRN	0.51
WL4998	K577M-WRN	0.32
WL5025	K577M-WRN	0.66

Western analysis is shown in Figure 5. mWRNp levels were normalized to nonspecific bands of the autoradiographs and to the levels in littermate controls and expressed as the "Relative mWRNp." The averages of two to three experiments are shown.

by disrupting the overall function of such a protein complex. An alternative possibility would be that K577M-WRNp may generate a multimeric complex itself and mouse WRNp may make the complex itself, and the K577M-WRNp complexes and the mouse WRNp complexes compete for WRNp binding sites. If this is the case, the effect of K577M-WRNp would be dose dependent until reaching a plateau. Our data showed that the effect of K577M-WRNp is independent of its expression levels. We therefore favor the first possibility.

Reduced endogenous mouse WRNp expression levels might also be the mechanism producing WS phenotypes in K577M-WRN mice. Cells from humans with heterozygous *WRN* mutations express significantly lower *WRN* mRNA than cells from normal individuals (Yamabe *et al.* 1997). This is thought to be the result of degradation of mutated mRNAs via a specific pathway (Jacobson and Peltz 1996), and the *WRN* promoter appears to have a positive autoregulatory pathway (Wang *et al.* 1998). As a result, steady-state levels of *WRN* mRNA in the heterozygotes, most of which is presumably from the wild-type allele, are on average 30% of the controls (Yamabe *et al.* 1997). Cells from heterozygotes exhibit moderate hypersensitivity to 4NQO (Ogburn *et al.* 1997), topoisomerase inhibitor I, and camptothecin (Poot *et al.* 1999), although they exhibit no clinical phenotypes.

Transgenic lines expressing human wtWRNp showed increased expression of endogenous mWRNp, but only marginally improved cell survival when challenged with 4NQO. This may be because WRNp is not the limiting factor in the functional pathway involved in survival of 4NQO challenge. Alternatively, the amount of wtWRN may not be sufficient to cause cellular biological effects under the experimental conditions employed.

The introduced hWRNp was localized to the nucleoplasm, where mWRNp had previously been shown to be localized (Marciniak *et al.* 1998). These observations suggest that the subcellular localization of WRNp is dictated by host factors. Murine cells may lack the ability to transport WRNp, either human or mouse, to the nucleoli.

Embryonic stem (ES) cell lines with homozygous deletions of the third and fourth motifs of the helicase domains of mWRNp have been synthesized (Lebel and Leder 1998). The altered mWRNp retains the ATPase domain of the helicase and the exonuclease domains. These mice therefore may provide only a partial model of WS. Because all known spontaneous mutations appear to be null (their truncated products being excluded from the nucleus; Moser *et al.* 1999), it may be necessary to eliminate the exonuclease function as well as the helicase function to model WS. Our dominant-negative model is more likely to impair both functions if, in fact, the dominant-negative effect results from functional inactivation of the relevant multimeric complexes.

Our model may have certain complicating factors. Expression of a dominant-negative mutant could impair the functions of other related helicases, which could have at least partially complemented the loss of WRN helicase activity. We have not yet determined the degree to which the introduced K577M-WRNp may affect the expression of other related helicases such as *BLM* (Ellis *et al.* 1995), human *RecQL* (Puranam and Blackshear 1994), human *RecQ4* and *RecQ5* (Kitao *et al.* 1998), or yet-unknown members of this family of proteins. It is also possible that overexpressed K577M-WRNp may "absorb" limiting cofactors necessary for the function of WRNp complex. There may exist some compensatory mechanisms mediated by other members of the RecQ family of helicases when the WRNp function is absent, perhaps particularly so in mice. If that is the case, knockout of only the WRN protein might not be sufficient to reproduce the complete phenotype; experiments designed to limit such compensatory mechanisms might result in a more robust model of the human disease.

It will be important to carry out careful pathological and pathophysiologic studies in aging cohorts of our K577M-WRN transgenics. Many phenotypes, such as cataracts and neoplasms, may not become apparent until more advanced ages. It will be equally important to assess the role of background alleles, to quantitate fecundity, and to assess meiotic recombination rates once the transgenic lines have been backcrossed to defined genetic backgrounds.

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