Unexpectedly Low Loss of Heterozygosity in Genetically Unstable Werner Syndrome Cell Lines

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We have determined the mitotic stability of micro- and mini-satellite DNA sequences in SV40-immortalized Werner syndrome (WS) and control fibroblast cell lines. Five microsatellite loci were genotyped in two WS and two control SV40-immortalized fibroblast cell lines and in 154 independent primary or secondary clones derived from these. We used four minisatellite "core" or individual locus probes in Southern blot hybridization analyses to assess minisatellite stability in WS and control clones. Microsatellite allele length was stably maintained in both WS and control cells, and an upper limit for the generation of new allele lengths was estimated to be $\leq 4.5 \times 10^{-4}$/allele/generation (or $\leq 2.25 \times 10^{-5}$/CA repeat/generation). In contrast to length stability, loss of heterozygosity (LOH) at microsatellite loci ranged up to 76% at the 13 informative locus:cell line combinations. An unexpected, and counterintuitive, finding was a much *lower* frequency of LOH in WS than in control clones at microsatellite loci on three different chromosomes. Minisatellite band alterations (gains, losses, or band intensity differences) were 4-fold lower in WS than in control cells. Our results suggest that the chromosomal and molecular genetic instability displayed by WS cells is unlikely to be the result of a micro- or mini-satellite destabilizing defect. A second, unexpected conclusion is that WS cells may possess a novel means of either suppressing or masking LOH events in the presence of constitutional cytogenetic and molecular genetic instability. *Genes Chromosom. Cancer 18:133–142, 1997.* © 1997 Wiley-Liss, Inc.

INTRODUCTION

Werner syndrome (WS; McKusick catalog number 277700) is an uncommon, autosomal recessive disease whose phenotype caricatures premature aging: WS patients develop bilateral cataracts, graying and loss of hair, osteoporosis, atherosclerosis, and diabetes as young adults, and they have an increased risk of developing cancer (Epstein et al., 1966). The most common malignant neoplasms in WS patients include thyroid carcinoma, malignant fibrous histiocytoma, malignant melanoma, bladder carcinoma, fibrosarcoma, and leiomyosarcoma (Goto et al., 1981, 1996; Sato et al., 1988; Tsuchiya et al., 1991). The WS locus, *WRN*, is located in chromosomal region 8p1.11-2.11 near the markers GSR and D8S339 (Goto et al., 1992; Schellenberg et al., 1992; Thomas et al., 1993; Oshima et al., 1994). It encodes a putative helicase of 1432 animo acids (Yu et al., 1996).

Cells and cell lines from WS patients display several different types of constitutional genetic instability. These include chromosomal instability, with the generation of multiple, clonal chromosome rearrangements (termed variegated translocation mosaicism; Hoehn et al., 1975; Salk et al., 1985); a deletion mutator phenotype in SV40-transformed WS fibroblast cell lines (Fukuchi et al., 1989); and elevated frequencies of hypoxanthine phosphoribosyltransferase (*HPRT*)-deficient T cells and glycophorin-A variant red cells in the blood of WS patients (Fukuchi et al., 1990; Monnat, 1992; unpublished results). One or more of these types of genetic instability may play a role in generating the WS clinical phenotype, including an increased risk of malignancy. An attractive mechanistic explanation for constitutional genetic instability in WS is a heritable defect in DNA repair. In contrast to several other inherited human cancer predispositions, however, no convincing deficit in any DNA repair pathway has been identified in WS cells or cell lines (reviewed in Monnat, 1992).

We have examined the mitotic stability of micro- and mini-satellite sequences in WS cell lines. The stability of both DNA sequence families was estimated by determining how frequently allele length variation or loss occurred per cell division in clonally derived sublines isolated from SV40-immortalized WS and control fibroblast cell lines. We reasoned that identification of genetic instability in either class of repeated DNA sequence might provide a clue to the biochemical defect associated with loss of the WRN protein, and thus provide a means for

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studying the *in vivo* function(s) of the *WS* gene and WRN protein.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The SV40-immortalized WS (WV-1 and PSV-811) and control (GM637 and GM639) SV40 fibroblast cell lines were derived from unrelated individuals. The origins of these lines and the conditions for the growth and isolation of spontaneous, clonally derived 6-thioguanine-resistant mutants from each have been described previously (Fukuchi et al., 1989). Primary TG-resistant clones were used as a source for generating secondary clones by limiting-dilution cloning in 96-well microtiter plates as described previously (Monnat, 1989). The number of cells plated per well, 1 to 16, was adjusted to insure that a large proportion of wells lacked colonies (i.e., that the "P_o" fraction was high; Kraemer et al., 1980), thus ensuring with high probability that secondary colonies arose from single cells. DNA from secondary clones was prepared as described by Higuchi (1989). Aliquots (2.5–5 µl/well) of the resulting lysates were used for PCR genotyping reactions.

Microsatellite Genotyping

Five polymorphic microsatellite loci on three different chromosomes were genotyped by radiolabeled PCR amplification and acrylamide gel electrophoresis: D2S123 (Weissenbach et al., 1992), D8S87 (Weber et al., 1990), D8S255 (Weissenbach et al., 1992), D10S141 (Love et al., 1993), and D10S197 (Weissenbach et al., 1992), with use of the referenced locus-specific primer pairs. One primer in each pair was radiolabeled with T4 polynucleotide kinase and γ-^{32}P-ATP. Amplification reactions were performed in 96-well plates on a PTC-100 Thermal Cycler (MJ Research, Watertown, MA). Twenty-five amplification cycles were performed: each cycle consisted of a 40 s, 94°C denaturation step, a 30 s annealing step (at 55°C for D8S255 and D10S141, 60°C for D8S87 and D2S123, and 62°C for D10S197), and a 20 s, 72°C extension step. Completed amplification reactions were size fractionated on 5.8% polyacrylamide gels (19:1 acrylamide:bis ratio) containing 7 M urea and 29% formamide (v/v) in 3:1 TBE (135 mM Tris, pH 8.3, 45 mM boric acid, 2.5 mM EDTA) buffer (Anderson, 1981). Autoradiograms were scored visually, and genotyping was repeated on all DNA samples that differed in genotype allele length or intensity from parental cell lines. DNAs from parental cell lines and from two CEPH donors (1331-01 and -02, BIOS Corp, New Haven, CT) were used throughout as amplification and electrophoretic mobility controls.

Southern Blot Analysis of Minisatellites

Genomic DNA samples (3.3 µg) were digested with the restriction endonucleases *Alu*I or *Hinf*I, then size-fractionated by electrophoresis through 0.8% agarose gels prior to being transferred onto Magna NT hybridization membrane (MSI, Westborough, MA) as described previously (Monnat, 1989). Minisatellite core probes p33.6 and p33.15 (Jeffreys et al., 1985) and individual locus probes pMS1 and pMS32 (Wong et al., 1987) were radiolabeled with α-^{32}P-dCTP by random priming as described previously (Feinberg and Vogelstein, 1983). Blots were hybridized for 24–48 hr at 42°C in 50% formamide buffer, and then rinsed twice with 5× SSC (150 nM NaCl, 15 mM Na citrate) at room temperature before washing for 1 hr at 65°C in 1× SSC/0.1% sodium dodecyl sulfate (SDS) for core probes or 0.1× SSC/0.1% SDS for pMS1 and pMS32.

Chromosome Painting

Subconfluent cells were treated with Colcemid (0.1 mg/ml) for 15 min, then trypsinized and used for preparation of chromosome spreads. The resulting slides were washed for 30 min at 37°C in 2× SSC and dehydrated with ethanol prior to hybridization with WCP Spectrum-Green probe 2 or 10 (Gibco BRL, Gaithersburg, MD) according to the probe manufacturer's protocols. Metaphase photos were taken on a Zeiss Photomicroscope III with a Chroma 51006 FITC/Texas Red filter and Kodak EPH P1600 film.

Statistical Analyses

Frequencies of loss of heterozygosity (LOH) at microsatellite loci in WS and control cell clones were compared by binomial regression analysis by use of the GEE function of the S-PLUS statistical package. We incorporated an overdispersion parameter (McCullagh and Nelder, 1989) and a robust score test (White, 1982) to adjust the P value for possible lack of independence between observations. The probabilities of the observed LOH frequencies in WS cell clones under a series of hypothetical background LOH frequencies were calculated with the assumption that each observation was an independent binomial trial with a mean equal to an assumed background frequency. We estimated the probability that secondary clones derived from WS cell clones WV1-12R2 and WV1-

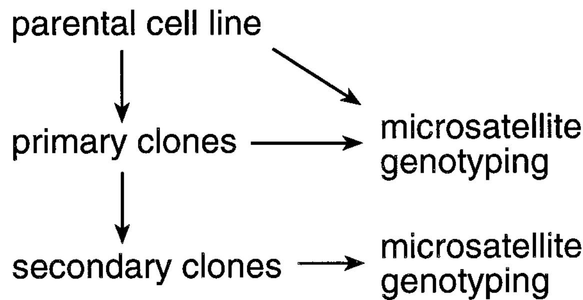

Figure 1. Experimental design. Independent spontaneous 6-thioguanine-resistant primary clones were derived from each of four SV40-immortalized WS or control cell lines, and then genotyped at five microsatellite loci. Secondary clones were isolated from a portion of primary clones by dilution cloning, then genotyped at the same five microsatellite loci.

37R1 and from control clone GM639-22R were derived from single progenitor cells by assuming that the number of cells that survived to form colonies followed a Poisson distribution. The mean parameter for the Poisson distribution for each cell line was estimated from the P_0 fraction (the fraction of wells lacking colony growth) for 60 plate wells.

RESULTS

Microsatellite Length Stability

The mitotic stability of microsatellite allele lengths was assayed by locus-specific PCR amplification and gel-electrophoretic analysis of five microsatellite loci located on three chromosomes. We applied the following scoring criteria in genotyping analyses: length variants 1) were novel-sized alleles that were longer or shorter than the original alleles of the parental cells, 2) were observed in at least two independent PCR amplification reactions of the same template DNA sample, 3) could not be generated by PCR amplification of size-purified, allele-specific products, and 4) were scored under conditions for which the sensitivity of detection of length variants was known. Four parental cell lines and 154 independent primary or secondary clones were used for the analyses shown in outline in Figure 1. An example of PCR genotyping of the D8S255 microsatellite locus in 10 independent, primary WS or control clones is shown in Figure 2.

The experimental protocol that we used to determine the sensitivity of detection of allele length variants is shown in Figure 3. Genomic DNAs known to contain only the longer or shorter of two alleles at a single locus were mixed in different ratios, and then amplified for gel-electrophoretic analysis. The amount of template DNA was constant in each mixture, and amplifications were

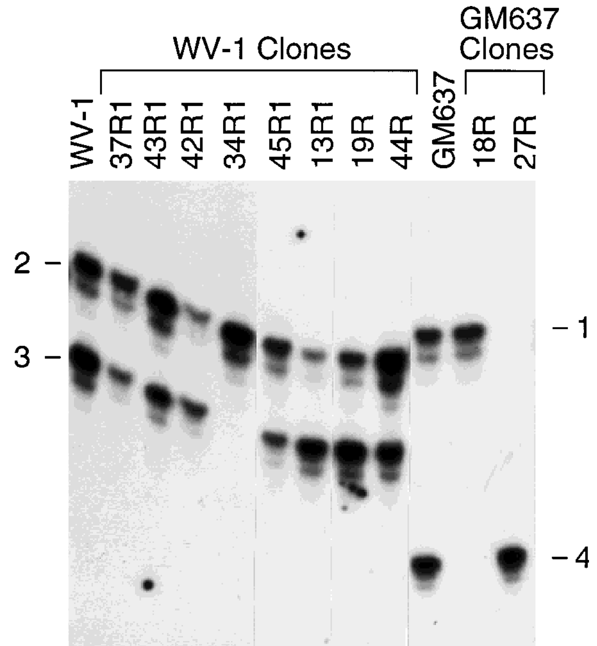

Figure 2. Loss of heterozygosity of D8S255 alleles. Microsatellite allele lengths at the D8S255 locus were examined by a combination of allele-specific PCR amplification and gel electrophoresis. Template DNA sources are indicated across the top of the autoradiogram, and allele lengths are numbered from 1 to 4 (longest to shortest allele length). A shorter D8S255 (#3) allele has been lost from primary WS clone WV-1 34R1 (lane 5), whereas primary control clones GM637-18R and -27R have lost either the shortest (allele 4, lane 11) or longest (allele 1, lane 12) of the D8S255 alleles. Allele intensity differences for D8S255 alleles 2 and 3 are also present in WS WV-1 primary clones (see, e.g., lanes 2, 4, and 7).

performed under conditions identical to those used for genotyping. These experiments indicate that long novel alleles or short novel alleles shorter than "shadow bands" (products of allele length n-2bp, n-4bp, etc. that are commonly and reproducibly seen when microsatellite alleles are genotyped by PCR) could be detected reliably at a level of 2–5% in template DNA, whereas short novel alleles of the same size as "shadow bands" could be detected when they were present at a level of ≥25% in 24 ng template DNA samples.

We observed no novel allele lengths at any of five microsatellite loci in 154 WS or control clones. These analyses did, however, reveal a preexisting D2S123 allele length variant in the WS cell line WV-1. This longer variant allele was faintly visible in the parental cell line and was stably transmitted as a full-intensity variant allele to 26 of 35 WV-1-derived primary clones (Fig. 4A). This longer variant allele could not be generated by PCR amplification of any of the other WV-1-derived D2S123 PCR products (Fig. 4B), and it was subsequently shown to contain two additional CA repeats

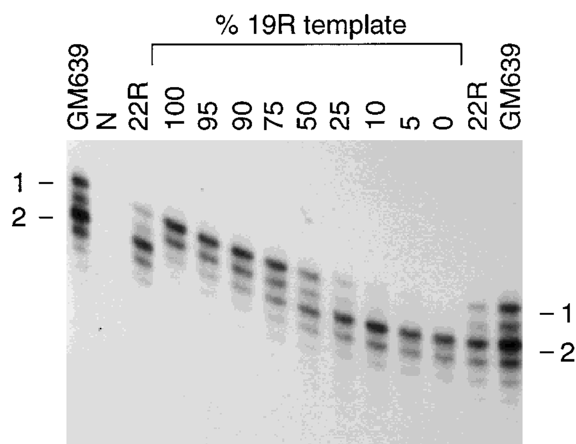

Figure 3. Sensitivity of detection of allele length variants by PCR genotyping. DNAs isolated from two control clones that contained only 1 D10S197 allele were mixed in different ratios for determination of the ability to detect microsatellite length variants by PCR genotyping . The template DNA source is indicated across the top of the autoradiogram. The two predominant allele lengths are indicated to the left of the figure. *% 19R template* (lanes 4–12) indicates the percent of GM639-19R DNA (containing only D10S197 allele 1) that was mixed with GM639-8R DNA (containing only D10S197 allele 2) and used for genotyping reactions. The amount of template DNA was constant in all amplification reactions. Genotyped in parallel were DNAs from clones GM639-19R (lane 4), GM639-8R (lane 12), and GM639-22R (lanes 3 and 13) and the parental GM639 cell line (lanes 1 and 14). Lane 2 (**N**) is a no-DNA control.

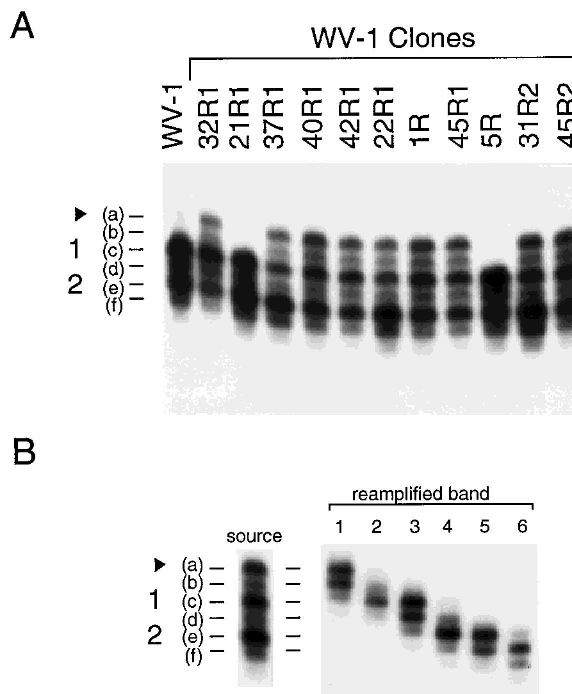

Figure 4. Analysis of a preexisting D2S123 allele length variant in Werner syndrome cell line WV-1. **A:** Two predominant D2S123 allele lengths, indicated to the left of the figure (1c and 2e), were present in the WV-1 parental cell line (lane 1) and all 11 primary WV-1 clones. A longer variant D2S123 allele [▶, **(a)**] was observed in 9 of the 11 primary clones and was faintly visible in the parental WV-1 cell line (lane 1). "Shadow" bands **(b)**, **(d)**, and **(f)** are frequently observed in dinucleotide repeat PCR genotyping. **B:** Each of the six different major and minor D2S123 allele length products generated during D2S123 genotyping of WS primary clone WV-1 40R1 were isolated and reamplified. The predominant reamplification products (reamplified bands, lanes 1–6, corresponding to isolated primary amplification fragments **a-f**) consisted of fragments of the same length or 1 repeat longer or shorter than the primary amplification fragment.

as compared with D2S123 allele 1 (Fig. 4, and DNA sequencing data not shown).

A combination of genotyping data and detection sensitivity determinations allowed us to estimate an upper limit for the *rate* of generation of new microsatellite length variants in WS and control cells of ~ 4.5×10^{-4} per allele per generation. This rate is the reciprocal of the product of three numbers: the number of cell divisions that can be reliably assayed (7 divisions or the first three doublings), the number of alleles per locus in a cell (assumed here to be 2), and the number of clones examined (154). The corresponding rate of generation of novel-length alleles per CA repeat is ~ 2.2×10^{-5} per CA repeat per generation for a 40 bp (20 repeat) microsatellite, the average size of the microsatellite loci that we studied. This estimated rate of generation of novel-length alleles is conservative in that it is based on the assumption that novel allele lengths would be detected reliably when present at a level of ≥12.5% (or 1 in 8) in a cell population.

Loss of Heterozygosity at Microsatellite Loci

In contrast to microsatellite length stability, we observed loss of heterozygosity (LOH) at all 5 microsatellite loci in WS and control cell clones (Table 1). An example of LOH at the D8S255 locus in WS and control clones is shown in Figure 2: complete loss of a longer or shorter allele is seen in three of ten primary clones derived from the parental WS WV-1 or the control GM637 cell line.

An unanticipated finding in these analyses was that LOH was *less* frequent at every locus in WS (0–5% per locus) than in control clones (12–40% per locus; Table 1). This difference is highly statistically significant ($P<0.0002$). We also determined the probability of finding the observed WS LOH frequencies by chance alone at assumed background LOH frequencies ranging from 1 to 60%. These probabilities fell below 0.05 (or 5%) at assumed background LOH frequencies of ≥ 4% for WS line WV-1, and of ≥ 12% for WS line PSV-811. The control SV40-transformed fibroblast cell lines used in this study, in contrast, had average LOH frequencies of 33–40% over five loci (Table 1).

We used dilution subcloning of WS-derived primary clones WV1-12R2 and WV1-37R1 and of

TABLE 1. Loss of Heterozygosity at Microsatellite Loci in Werner Syndrome and Control Primary Clones

Cell line	Microsatellite locus[a]					Cell line LOH (%)[d]
	D2S123	D8S87	D8S255	D10S141	D10S197	
WV-1 (WS)[b]	1/35[c]	1/35	1/35	0/35	0/35	2/105 (1.9)
PSV-811 (WS)	2/31	n.i.	n.i.	1/31	n.i.	3/62 (4.8)
GM637 (control)	n.i.	2/5	2/5	n.i.	n.i.	2/5 (40)
GM639 (control)	2/17	3/17	2/17	n.i.	13/17	17–18/51 (33.3–35.3)
Locus LOH[e] (%)	5/83 (6)	7/57 (12.3)	6/57 (10.5)	1/66 (1.5)	13/52 (25)	

[a]Five microsatellite loci in 88 independent, 6-thioguanine-resistant primary clones isolated from SV-40-transformed Werner syndrome (WV-1 and PSV-811) or control (GM637 and GM639) cell lines were PCR-genotyped as described in Materials and Methods.
[b]WS, Werner syndrome cell line; control, control cell line.
[c]The proportion of independent clones that have lost one of two alleles at each microsatellite locus is indicated. n.i., a locus that was homozygous, and thus not informative, in a given cell line.
[d]Cell line loss of heterozygosity (LOH) is the number of allele losses in a cell line divided by the number of primary clones assayed. In cell lines where two physically linked loci were informative (e.g., D8S87 and D8S255 or D10S141 and D10S197 in WS cell line WV-1), we used data from one of each pair of linked loci to calculate average cell line LOH. The range of cell line LOH was calculated for GM639, as there was a difference in the frequency of LOH at D8S87 and D8S255 (three vs. two occurrences, respectively). LOH frequencies between WS and control cells were compared by a binomial regression analysis that incorporated an overdispersion parameter and a robust score test to compensate for possible lack of independence between observations (see Materials and Methods).
[e]Locus loss of heterozygosity is the number of alleles lost at a given microsatellite locus divided by the number of primary clones analyzed.

control primary clone GM639-22R to determine whether differences in microsatellite allele intensity represented LOH differences between cells within a clone. WS-derived primary clones WV1-12R2 and WV1-37R1 demonstrated consistent and reproducible D8S87 allele intensities of 1:4 and 2:1 (longer:shorter allele ratio), respectively, as compared with a 1:1 allele intensity ratio in the parental WV-1 cell line. Five of six WV1-12R2-derived secondary clones retained the 1:4 allele intensity ratio seen in the WV1-12R2 primary clone. The remaining secondary clone lacked the longer allele. Comparable results were seen in a second WS primary clone, WV1-37R1: 29 of 31 secondary clones retained the 2:1 D8S87 allele intensity ratio seen in the WV1-37R1 primary clone, whereas the two remaining clones had either a 1:1 ratio or had lost the shorter D8S87 allele. The probabilities that secondary clones arose from one or at most two cells were 63% and 90%, respectively, for WV1-12R2, and 73% and 95%, respectively, for WV1-37R1. In contrast, only 8 of 26 GM639-22R-derived secondary control clones retained the 1:2 ratio of longer: shorter D10S197 allele intensities observed in GM639-22R (Fig. 3). The remaining 18 secondary clones demonstrated a 1:1 allele intensity ratio (12 clones) or loss of the longer D10S197 allele (6 clones). The probabilities that the GM639-22R secondary clones used in these analyses arose from one or at most two cells were 93% and 99%, respectively.

Chromosome painting analyses of chromosomes 2 and 10 conducted in conjunction with these secondary clone analyses revealed the more frequent occurrence of chromosomal rearrangements in WS than in control primary clones. WS cell lines and clones had $\geq$5 chromosomal or subchromosomal hybridization signals with each chromosome-specific probe in all scorable metaphases. In contrast, GM639 control cells and two GM639-derived primary clones had $\leq$3 hybridization signals on what appeared to be whole chromosomes in each metaphase cell with both of the chromosome-specific probes. Representative chromosome 2 painting results for 2 WV-1 and 2 GM639-derived primary clones are shown in Figure 5.

Minisatellite Stability

The mitotic stability of DNA sequences detected by minisatellite core and individual locus probes was determined by Southern blot hybridization analysis of DNA from parental cell lines and primary clones. Four minisatellite probes were used: the core probes p33.6 and p33.15 and individual locus probes pMS1 and pMS32. For blot hybridization analyses we used the same set of clone DNAs that were used for microsatellite analyses, with the exception of one GM639-derived primary clone for which we lacked enough DNA for blots.

We determined the frequencies of altered bands detected by the four minisatellite probes to allow comparisons among the cell lines that differed in the number of bands detected by each probe. Three types of alterations were observed in *Hinf*I-digested DNAs: band losses, the appearance of novel bands, and band intensity differences (Fig. 6). A four-fold lower frequency of altered bands was

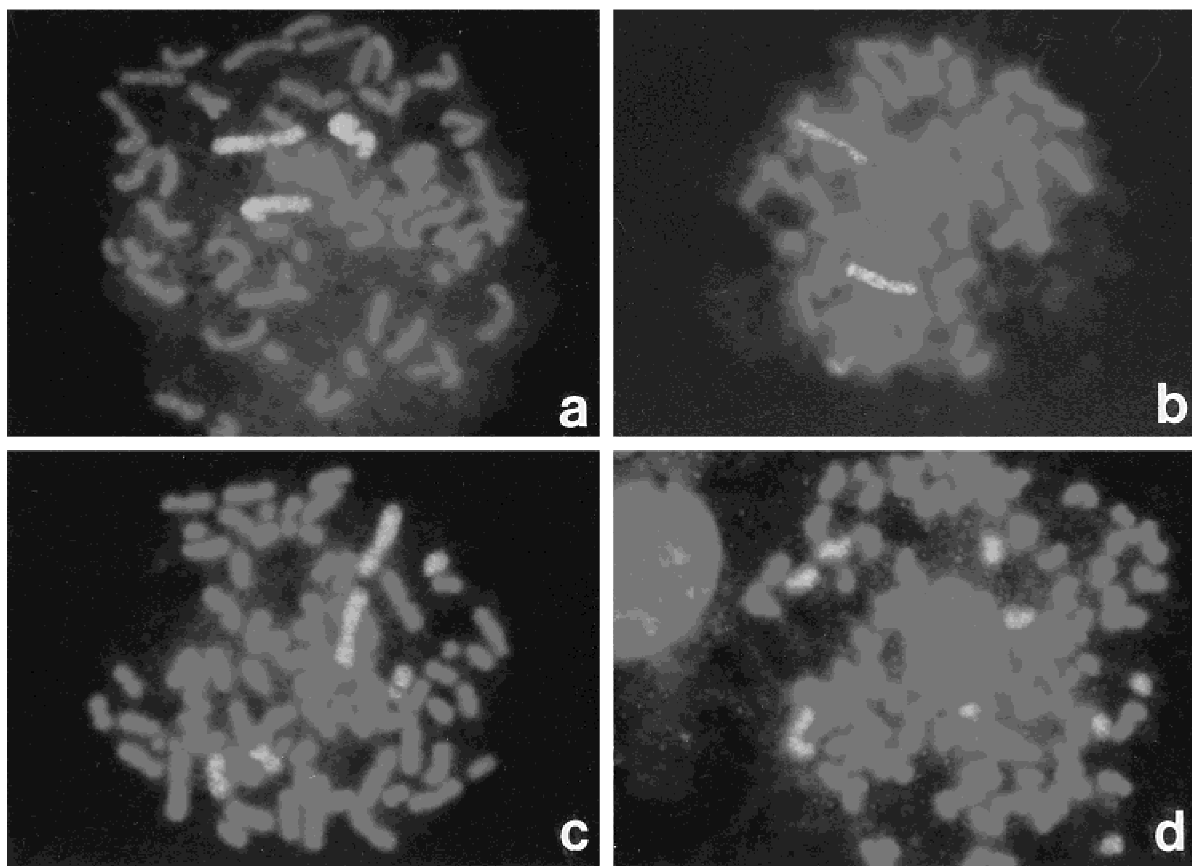

Figure 5. Chromosome painting reveals frequent chromosomal rearrangement in Werner syndrome cells. Chromosome 2 and 10-specific paints were used on metaphase cells of WS and control cell lines and primary clones. Representative chromosome 2 paints are shown for control clones GM639-19R (**a**) and GM639-9R (**b**), which revealed ≤3 hybridization signals in each metaphase cell, with most of the hybridizing signal in chromosome-sized segments. In contrast, chromosome 2 paints of WS primary clones WV1-22R1 (**c**) and WV1-28R (**d**) revealed ≥5 hybridization signals in all metaphase cells, with most of the hybridizing signal in subchromosomal fragments.

observed in WS as compared with control clones (9/1886 or 0.5% in WS compared with 10/528 or 1.9% in control clones). This difference is statistically significant (χ^2 = 8.9 with df=1; P=0.003). In these analyses, the locus probes pMS1 and pMS32 detected alterations as often as core probes, despite their detecting a smaller number of bands (8 alterations in 891 scorable bands for the locus probes, vs. 11 alterations in 1523 scorable bands for core probes using the same samples). This may reflect the higher mutation rates that have been reported for these locus probes (Wong et al., 1987).

DNAs in which alterations were detected after *Hinf*I digestion were reanalyzed after digestion with *Alu*I, a restriction enzyme that will cleave both methylated and unmethylated DNA. In most cases, alterations seen in *Hinf*I-digested DNA were also detected in *Alu*I-digested DNA, indicating that alterations were not due to DNA methylation differences (see, e.g., Fig. 6).

DISCUSSION

We have examined the mitotic stability of CA-repeat microsatellite and minisatellite DNA sequences in WS and control cell lines. The rationale for this study was provided by the identification of microsatellite length instability and a mutator phenotype, in association with mutations in genes encoding DNA mismatch repair proteins, in several different types of human tumor cells (reviewed in Loeb, 1994; Eshleman and Markowitz, 1995; Karran, 1996; Modrich and Lahue, 1996). The genetic control of minisatellite stability is less well understood, although both meiotic and somatic variations have been well documented (reviewed in Jeffreys et al., 1995). We reasoned that identification of a satellite DNA-destabilizing defect might provide a mechanistic basis for the constitutional genetic instability displayed by WS cells and provide clues to the *in vivo* function(s) of the *WRN* gene product

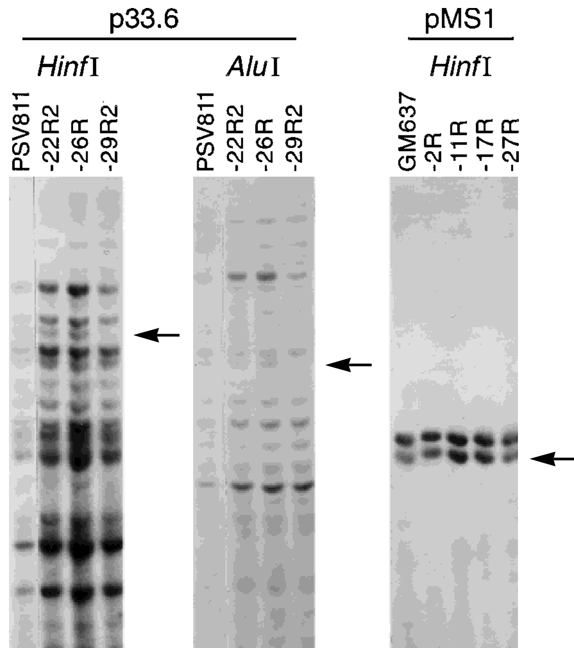

Figure 6. Minisatellite mutations in SV40-immortalized Werner and control cells. Southern blot analyses, with minisatellite core or individual locus probes, were performed with *Hinf*1 or *Alu*I-digested DNA from WS or control primary clones. Representative examples of fragment losses are shown for minisatellite core probe 33.6 in WS primary clone PSV-811-29R2 after *Hinf*1 or *Alu*I digestion (arrows), and for the fragment intensity differences for minisatellite locus probe pMS1 in control primary clone GM637-27R after *Hinf*1 digestion in control clones GM637-11R and GM637-17R.

(Hoehn et al., 1975; Salk et al., 1985; Fukuchi et al., 1989, 1990; Monnat, 1992; Yu et al., 1996).

The mitotic stability of microsatellite sequences was assayed by locus-specific PCR amplification and gel-electrophoretic analysis. Five microsatellite loci on chromosomes 2, 8, and 10 were examined, including three (D2S123, D8S255, and D10S197) that are unstable in colorectal tumor tissue from hereditary nonpolyposis colon cancer (HNPCC) patients (Aaltonen et al., 1993; Lothe et al., 1993). We observed no novel allele lengths that would indicate mitotic instability or length variation at any of these loci in 154 WS or control clones. These results and knowledge of the detection sensitivity of the genotyping assay allowed us to estimate an upper limit for the *rate* of generation of new microsatellite length variants in WS and control cells of $\sim 4.5 \times 10^{-4}$ per locus per generation. This upper rate limit for the generation of new allele length variants is comparable to estimated rates in control cells and cell lines. It is, however, lower than rates observed in DNA mismatch repair-deficient cell lines and tumor cells (see, e.g., Bhattacharyya et al., 1994; Farber et al., 1994; Shibata et al., 1994; Karran, 1996).

During these analyses, we found one preexisting D2S123 allele length variant in the Werner cell line WV-1. This variant was two CA repeats longer than the longest D2S123 allele observed in the WV-1 parental cell line. Genotyping analyses indicated that this variant allele was present in a minority of cells in the parent cell line, but could be inherited as a full-intensity variant allele in primary clones (Fig. 4). Comparable additional alleles have been observed at low frequency ($\leq 10^{-4}$/generation) in other primary and immortalized human cells (see, e.g., Banchs et al., 1994; Hackman et al., 1995).

Our genotyping results also suggest that coincident microsatellite length mutations are uncommon in WS or control cell clones containing mutations at the X-linked *HPRT* locus. This is of some importance because the primary clones used in these analyses were originally selected as spontaneous *HPRT* mutants (Fukuchi et al., 1989), and coincident microsatellite mutations have been seen more frequently that expected by chance alone in human lymphoblastoid cell clones containing mutations at the thymidine kinase locus (Li et al., 1994).

In contrast to the apparent mitotic stability of microsatellite length, we observed LOH at all five microsatellite loci in WS and control cell clones. An unanticipated finding in these analyses was that LOH was *less* frequent at every locus in WS than in control clones (0–5% per locus in WS vs. 12–40% in control clones; Table 1). We had anticipated, from previous work that identified chromosomal instability and deletion mutator phenotypes in WS (Hoehn et al., 1975; Salk et al., 1985; Fukuchi et al., 1989) and from the high correlation between frequency of LOH and chromosomal aneuploidy in many human tumors (reviewed in Cavanee et al., 1990; Lasko et al., 1991; Rodriguez et al., 1994) that LOH would be *more* frequent in chromosomally unstable WS clones. The opposite, however, was observed.

The statistical significance of this difference in the frequency of LOH was reinforced by two different types of analysis. First, in a regression analysis for correlated outcomes, the observed difference in LOH frequency between WS and control clones was highly statistically significant ($P < 0.0002$). We also determined the probability of observing the WS LOH frequencies by chance alone under different background probabilities ranging from 1 to 60%: these probabilities fell below 0.05 (or 5%) at assumed background LOH probabilities of $\geq 4\%$ for WS cell line WV-1 and $\geq 12\%$ for WS cell line PSV-811. The control SV40-transformed fibroblast cell lines that we used in this study, in contrast, had LOH frequencies of 33–40%

over the five loci that were genotyped (see Table 1). This range of LOH is commonly observed at unselected loci or chromosome arms in a wide variety of human tumors and tumor cell lines (see, for example, Peiffer et al., 1995; Barrett et al., 1996). A total of 315 of 440 (or 72%) of the primary clone and locus pairs were informative in these analyses. This high average heterozygosity argues that the LOH, which we observed in WS and control cells, arose during clonal growth, rather than before or at the time cell lines were established. Comparable, although less dramatic results, i.e., a lower frequency of alterations, were seen in blot hybridizations with a combination of core and individual locus minisatellite probes. These results argue that WS cell clones have a consistently low LOH frequency at different chromosomal loci, and that this difference is highly statistically significant when compared with control clone, human tumor, and human tumor cell line LOH frequency data.

Two models could explain the low frequency of LOH in WS cells. First, there could be active suppression of LOH in WS cells. Alternatively, WS cells could contain additional copies of microsatellite and other loci that would effectively "mask," or limit the detection of, LOH. The first of these alternatives, LOH suppression, cannot be ruled out, although it seems biologically implausible, given the diversity of mechanisms that may generate LOH (reviewed in Lasko et al., 1991; Li et al., 1992) and evidence for several types of constitutional genetic instability in WS cells or cell lines (Monnat, 1992). The second mechanism, copy number "masking" of LOH, is more attractive and plausible, given what is known about the WS defect and the effects of SV40 transformation.

Karyotypic and molecular genetic instability associated with WS mutations and with SV40 transformation may promote low levels of gene amplification at multiple, independent chromosomal loci. LOH events observed in WS cell lines would then represent allele losses that occurred *before* amplification, or as a result of chromosomal and/or molecular alterations that eliminated all copies of a given allele at a locus. Consistent with this model are the allele intensity differences observed in PCR genotyping assays, the difficulty we experienced in segregating these differences in WS as compared with control clones, and our chromosome painting results.

In our analyses, we used SV40-transformed, rather than primary, fibroblasts because of the severely limited growth potential of primary fibroblast strains from WS patients (Martin et al., 1970; Tollefsbol and Cohen, 1984). SV40 transformation suppresses or partially complements the WS defect but can promote genetic instability in human fibroblasts (Sack, 1981; Hanaoka et al., 1983; Matsumura et al., 1985; Huschtscha et al., 1986; Fukuchi et al., 1989; Saito and Moses, 1991; Woods et al., 1994). We chose, as comparable controls, two SV40-transformed fibroblast cell lines from unrelated control individuals (Fukuchi et al., 1989). The difference in LOH frequency that we observed between WS and control SV40-transformed fibroblast clones is thus most likely to be a consequence of mutations at the *WRN* locus *in conjunction with* SV40 transformation, rather than of SV40 transformation alone.

A comparable analysis of the mitotic stability of microsatellite and minisatellite lengths in Bloom syndrome (BS) cells and cell lines was recently reported (Foucault et al., 1996). BS is an autosomal recessive disease characterized by growth retardation, sun sensitivity with facial erythema, immunodeficiency, and an increased risk of malignancy. Cells and cell lines from BS patients display genomic instability in the form of markedly elevated sister chromatid exchanges, chromatid breaks and gaps, and a mutator phenotype (reviewed in German, 1993). It is of particular interest that the BS gene product, BLM, encodes a putative helicase that is similar in terms of size and sequence organization to the *WRN* gene product (Ellis et al., 1995; Yu et al., 1996). Foucault et al. found that the lengths of microsatellite and minisatellite sequences were mitotically stable in BS cells. They did not, however, look explicitly at the frequency of LOH in these analyses.

Our results indicate that the mutator phenotype of WS cells is not accompanied by the presence of micro- or mini-satellite length instability. A surprising finding in these analyses was an unexpectedly low frequency of LOH at microsatellite loci, and of minisatellite alterations, in WS cells that display high levels of constitutional chromosomal and molecular genetic instability. These results indicate that LOH is not an obligatory consequence of cytogenetic or molecular genetic instability nor of SV40 immortalization. The interplay of molecular and cytogenetic instability may thus provide ways for either promoting or suppressing LOH in human cells.

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