Molecular Biology of the Cell
Vol. 10, 2655–2668, August 1999

The Werner Syndrome Protein Is Involved in RNA Polymerase II Transcription

Adayabalam S. Balajee,*† Amrita Machwe,*† Alfred May,* Matthew D. Gray,‡ Junko Oshima,‡ George M. Martin,‡ Jan O. Nehlin,* Robert Brosh,* David K. Orren,* and Vilhelm A. Bohr*§

*Laboratory of Molecular Genetics, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224; and ‡Pathology Department, University of Washington School of Medicine, Seattle, Washington 98195-7470

Submitted February 23, 1999; Accepted May 27, 1999
Monitoring Editor: Joseph Gall

Werner syndrome (WS) is a human progeroid syndrome characterized by the early onset of a large number of clinical features associated with the normal aging process. The complex molecular and cellular phenotypes of WS involve characteristic features of genomic instability and accelerated replicative senescence. The gene involved (*WRN*) was recently cloned, and its gene product (WRNp) was biochemically characterized as a helicase. Helicases play important roles in a variety of DNA transactions, including DNA replication, transcription, repair, and recombination. We have assessed the role of the *WRN* gene in transcription by analyzing the efficiency of basal transcription in WS lymphoblastoid cell lines that carry homozygous *WRN* mutations. Transcription was measured in permeabilized cells by [^{3}H]UTP incorporation and in vitro by using a plasmid template containing the RNA polymerase II (RNA pol II)–dependent adenovirus major late promoter. With both of these approaches, we find that the transcription efficiency in different WS cell lines is reduced to 40–60% of the transcription in cells from normal individuals. This defect can be complemented by the addition of normal cell extracts to the chromatin of WS cells. Addition of purified wild-type WRNp but not mutated WRNp to the in vitro transcription assay markedly stimulates RNA pol II–dependent transcription carried out by nuclear extracts. A nonhelicase domain (a direct repeat of 27 amino acids) also appears to have a role in transcription enhancement, as revealed by a yeast hybrid–protein reporter assay. This is further supported by the lack of stimulation of transcription when mutant WRNp lacking this domain was added to the in vitro assay. We have thus used several approaches to show a role for WRNp in RNA pol II transcription, possibly as a transcriptional activator. A deficit in either global or regional transcription in WS cells may be a primary molecular defect responsible for the WS clinical phenotype.

INTRODUCTION

Werner syndrome (WS) is a homozygous recessive disease characterized by early onset of many signs of normal aging, such as atrophy of the skin, graying of the hair, cataracts, diabetes, and osteoporosis (Martin, 1997). Other features include short stature, hyperpigmentation, hyperkeratosis, telangiectasia, bird-like facies, hypogonadism, and reduced reproductive capacity. Cancers, particularly sarcomas, occur in these patients with increased frequency. The symptoms of WS begin to appear around the age of puberty, and most patients die before age 50. As a segmental progeroid syndrome, WS does not exhibit all of the features of normal aging but nevertheless is a very useful model system for the molecular study of normal aging.

Cells from WS patients grow more slowly and senesce at an earlier population doubling than age-matched normal cells. A hallmark molecular defect of WS is genomic instability arising from karyotypic abnormalities including inversions, translocations, and chromosome losses (Martin, 1997). These effects could potentially be the result of defects in DNA repair, replication, and/or recombination, although the actual biochemical defect remains unknown. WS cells are characterized by elevated rates of mutations at specific genes (Fukuchi *et al.*, 1989). They also show enhanced nonhomologous recombination and error-prone ligation of linearized plasmid (Runger *et al.*, 1994). The spontaneous hypermutability and genomic instability of WS cells could be the result of error-prone ligation of DNA strand breaks that

† These authors contributed equally to this work.
§ Corresponding author. E-mail address: vbohr@nih.gov.

© 1999 by The American Society for Cell Biology

Table 1. Summary of the WRN mutations in the WS cell lines used in this study

Cell line	Type	WRN mutation status	Codon	Exon	Nucleotide sequence	Mutation type
KO375	LCL-EBV	Homozygous	369	9	CGA (Arg) to TGA (terminator)	Substitution
AG4103	LCL-EBV	Homozygous	369	9	CGA to TGA	Substitution
JO1050	LCL-EBV	Homozygous	1047-78	26	tag-GGT to tac-GGT (splice donor)	Substitution
AG9387	LCL-SV	Wild type				
GM1310B	LCL-SV	Wild type				
SNW646	LCL-EBV	Wild type				
HeLa	ST	Wild type				

Based on Yu *et al.* (1997). LCL, lymphoblastoid cell line; EBV, EBV transformed; SV, SV40 transformed; ST, spontaneous transformation.

arise during replication and recombination of genomic DNA. It has been hypothesized that the clinical features and high probability of tumors in nonepithelial tissue of WS patients is a consequence of increased genomic instability.

Although genomic instability is well documented at various biological levels in WS, the cells are not generally sensitive to DNA-damaging agents. Transcription-coupled repair of UV light–induced cyclobutane pyrimidine dimers is reduced in WS lymphoblastoid cells but not in primary fibroblast cells (Webb *et al.*, 1996). A mismatch repair defect has been observed only in fibroblastoid cells of WS (Bennett *et al.*, 1997). Hence, the DNA repair defects observed in WS seem to be specific for certain cell types rather than generalized. This raises the possibility that a primary molecular defect other than DNA repair may be the cause of this rare disease.

The gene that is defective in WS, the *WRN* gene, has recently been identified (Yu *et al.*, 1996). The predicted amino acid sequence indicates that the WRN protein (WRNp) is a member of a large family (RecQ) of helicases with the ability to unwind DNA duplexes. Recently, WRNp has been demonstrated to have intrinsic helicase activity (Gray *et al.*, 1997; Suzuki *et al.*, 1997). In addition, the WRN protein contains several other interesting features, including a 27-amino-acid direct repeat sequence, a nuclear localization signal sequence, and a putative exonuclease domain. Also, a recent report indicates that WRNp has $3' \rightarrow 5'$ exonuclease activity (Huang *et al.*, 1998).

Helicases are required for various DNA metabolic activities, including replication, transcription, DNA repair, and recombination (Duguet, 1997). The critical role of helicases in transcription, combined with previous suggestions that defects in transcription could lead to severe clinical phenotypes (Bootsma and Hoeijmakers, 1993), prompted us to investigate the role of WRNp in transcription. In this report, we have undertaken a broad approach to assess the relative role of the WRNp in transcription using a number of WS lymphoblastoid cells that have homozygous mutations in the *WRN* gene. Transcription was measured in several ways: 1) RNA synthesis in permeabilized cells, 2) RNA synthesis by chromatin prepared from Triton X-100–extracted cells, and 3) RNA polymerase II (RNA pol II)–specific transcription carried out by nuclear extracts on a plasmid template. Our results indicate that the transcription efficiency in various WS lymphoblastoid cells is reduced to 40–60% of the transcription observed in wild-type cells. This defect can be complemented in the WS chromatin by addition of nuclear extract from wild-type cells. The RNA pol II–dependent transcription can be stimulated by the addition of purified wild-type but not mutant WRNp in the plasmid-based in vitro assay. Additionally, using a yeast reporter assay, we found a region consisting of a direct repeat of 27 amino acids located proximally to the helicase motifs of the WRNp that functions as a strong transcriptional activation domain.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The lymphoblastoid cells from normal individuals (GM1310B and AG9387) and WS patient AG4103 were obtained from Coriel Cell Repository (NJ). Epstein–Barr virus (EBV)-transformed lymphoblastoid cells of normal (SNW646) and WS (JO1050 and KO375) were from the International Repository of Werner Syndrome. Lymphoblast cultures were grown in RPMI 1640 medium (Life Technologies, Gaithersburg, MD), supplemented with 2 mM L-glutamine (Life Technologies), 1% penicillin-streptomycin (Life Technologies), and 15% heat-inactivated FBS (Life Technologies). All the cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. All cell lines used in this study had cell viabilities of >90%, as judged by the trypan dye exclusion assay. Table 1 summarizes the mutational status of the cell lines used in this study.

Transcription in Permeabilized Cells

Measurement of transcription in permeabilized cells involved the following steps: 1) encapsulation of exponentially growing cells in agarose microbeads, 2) permeabilization with lysolecithin (20 μg/ml) at 4°C in modified physiological buffer, 3) incubation of beads with NTPs and [^{3}H]UTP at 37°C, 4) collection of beads at 5, 15, and 30 min of reaction, and 5) reaction termination, lysis with 2% SDS, trichloroacetic acid (TCA) precipitation, and scintillation counting.

Encapsulation of Cells. The cells were grown in the presence of [^{14}C]thymidine (0.1 μCi/ml; specific activity, 55.3 mCi/mmol; New England Nuclear, Boston, MA) to uniformly label the DNA. The procedure for encapsulation has been described by Jackson *et al.* (1993). Briefly, 2.5% agarose (type VII; Sigma, St. Louis, MO) in PBS was melted and cooled to 39°C. Five milliliters of cells (2–4 $\times$ 10^6 cells/ml) in complete medium were mixed with 1.25 ml of molten agarose in a conical flask at 39°C. After the addition of 15 ml of liquid paraffin oil (Fisher Scientific, Pittsburgh, PA), the mixture was vortexed for 30 s and kept on ice with constant swirling for 2 min. Fifteen milliliters of complete medium were added to the flask, and the contents were transferred to a 50-ml centrifuge tube. The agarose beads were pelleted using a bench-top centrifuge (500 $\times$ *g*, 5 min). After the removal of paraffin and excess aqueous phase, the

agarose-encapsulated cells were thoroughly washed three times in PBS.

Lysis of Encapsulated Cells. The beads containing the cells were lysed either in lysolecithin (20 μg/ml) or 0.5% Triton X-100 in a modified physiological buffer (PB) for 15 min on ice. The PB buffer contained 10 mM Na_2HPO_4, 2.5 mM $MgCl_2$, 65 mM KCl, 65 mM $KC_2H_3O_2$, 1 mM Na_2ATP, 1 mM dithiothreitol, and 0.2 mM PMSF. We used lysolecithin instead of streptolysin O in the transcription experiments because the permeabilization of the cells was consistently better than with streptolysin O. After lysis, cells were washed several times in PB to remove the detergent, and the in vitro reaction was carried out. The chromatin prepared by extraction with Triton X-100 was found to replicate in vitro at 85% of the rate found in vivo in an S-phase–specific manner (Jackson *et al.*, 1993).

Transcription. The reaction was started by the addition of 50 μl of 10× concentrated transcription mixture to 450 μl of beads. The transcription mixture contained a final concentration of 0.1 mM CTP, 0.1 mM GTP, 5 μM cold UTP, [^{3}H]UTP (80–100 μCi/ml; specific activity, 60 Ci/mmol; Amersham, Arlington, Heights, IL), 10 μM *S*-adenosylmethionine, 1 mM ATP, and 2.5 mM $MgCl_2$ and RNasin (40 U/ml; Boehringer Mannheim, Indianapolis, IN). The beads were incubated at 37°C, and 100 μl beads were collected at 0, 5, 15, and 30 min. The beads were washed four times in ice-cold PB to remove the unincorporated nucleotides. Lysis was carried out in 2% SDS for 2 h at 37°C. After lysis, an equal volume of 20% TCA was added to the beads, and the samples were spotted onto glass fiber discs (GF/C; Whatman, Maidstone, United Kingdom). The filters were washed sequentially with 5% TCA, 70% ethanol, and acetone. They were then dried under a infrared lamp, the radioactivity incorporated into acid insoluble material was scintillation counted, and the incorporated radioactivity was expressed as UMP incorporated per 10^6 cells. In some experiments, the lysed cells in the beads were incubated with either α-amanitin (5 μg/ml) or α-amanitin and actinomycin D (2 μg/ml) for 30 min on ice before the transcription reaction.

Transcription Complementation Studies. Nuclear extracts from normal and lymphoblastoid cells were prepared by the method of Dignam *et al.* (1983). The extracts were dialyzed overnight at 4°C against 25 mM HEPES-KOH, pH 7.9, 1 mM DTT, 1 mM EDTA, 17% glycerol, and 12 mM $MgCl_2$, 0.1 M KCl, aliquoted, and stored at −80°C. The transcription procedure was essentially the same as above, except that the chromatin was prepared from normal and WS cells after lysis with 0.5% Triton X-100. The chromatin was preincubated with 50 μg of nuclear extracts for 15–30 min, and the transcription was measured using [^{3}H]UTP.

In Vitro Transcription

Preparation of Nuclear Extracts. Nuclear extracts were prepared essentially by the method of Dignam *et al.* (1983). Briefly, 1 l of actively growing cells (10^6/ml) was harvested by centrifugation at 1850 × *g* for 10 min at 4°C and then washed two times with PBS at 4°C and subsequently resuspended in 5 packed cell pellet volumes (PCV) of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT). The cells were collected by centrifugation and resuspended in three PCV of hypotonic buffer. The cells were then allowed to swell on ice for 10 min and lysed using a Bellco Glass (Vineland, NJ) Dounce homogenizer (B type pestle). The resulting material was centrifuged at 3300 × *g* for 15 min to pellet nuclei. The nuclear pellets were resuspended in 0.5 PCV of low-salt buffer (20 mM HEPES, pH 7.9, 25% [vol/vol] glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) to which an equal volume of high-salt buffer (20 mM HEPES, pH 7.9, 25% [vol/vol] glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) was added. The suspension was stirred for 30 min at 4°C and then centrifuged at 25,000 × *g* for 30 min at 4°C. The supernatant was dialyzed twice against 50 volumes of the storage buffer (20 mM HEPES, pH 7.9, 20% [vol/vol] glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT). The dialysate was clarified by centrifugation at 25,000 × *g* for 20 min, and the supernatant was frozen and stored at −80°C.

WRN (Wild-Type and Mutant) Proteins. His-tagged recombinant wild-type and mutant WRNp were purified as previously described (Gray *et al.*, 1997). Briefly, *Spodoptera frugiperda* (*Sf9*) cells grown in suspension at 27°C were infected (at a multiplicity of infection of 10) with baculovirus containing *WRN* (wild-type or mutant) cDNA sequence with an upstream sequence coding for a hexahistidine tag at the amino terminus of the recombinant protein. Infected *Sf9* cells were harvested after incubation for ~72 h and tested for overproduction of recombinant protein. Cells producing significant quantities of WRNp were lysed in buffer containing 0.15 M Tris, pH 8.0, 0.15 M NaCl, 10% glycerol, 1 mM PMSF, and 0.5% NP-40. The resulting lysate was clarified by centrifugation (10,000 × *g* for 10 min) and then bound to Ni-nitrioltriacetic acid resin (Qiagen, Hilden, Germany) in batch with mixing for 1 h at 4°C. The resin was washed initially with buffer containing 50 mM Tris, pH 8.0, and 0.5 M $LiCl_2$ and then with buffer containing 10 mM 1,4-piperazinediethanesulfonic acid, pH 7.0, 50 mM NaCl, 10% glycerol, and 1 mM PMSF. WRNp was eluted using the latter buffer containing 0.5 M imidazole and stored at −80°C. As a mock control, parallel purifications were done using lysates from insect cells infected with baculovirus lacking *WRN* sequences. The WRN-K577M mutant contains a single amino acid substitution changing the invariant lysine residue to methionine (K577M) in motif I of the seven conserved helicase domains (see Figure 8A). The WRN-ΔR mutant lacks the 27-amino-acid direct repeat upstream to the helicase domain (see Figure 8A). To confirm WRNp activity and purity, wild-type, mutant, and mock protein preparations were tested for ATPase and helicase activity. Wild-type and WRN-ΔR had comparable ATPase and helicase activities, whereas neither the WRN-K577M protein nor the mock preparation demonstrated detectable ATPase or helicase activity.

DNA Template for In Vitro Transcription. Plasmid pML(C_2AT) containing the adenovirus major late promoter (AdML) plus a G-less cassette (Sawadogo and Roeder, 1985) was provided by Dr. Roger Kornberg (Stanford University, Stanford, CA). The plasmids, propagated in *Escherichia coli*, were isolated by lysozyme treatment. Plasmid DNA was recovered after passing the lysate through prepared columns (Qiagen) following the conditions specified by the manufacturer. The plasmid DNA was further purified by CsCl/ethidium bromide gradient centrifugation and sucrose gradient centrifugation (Sambrook *et al.*, 1989; Biggerstaff *et al.*, 1991).

In Vitro RNA pol II Transcription. Transcription was carried out in 50-μl reactions containing 2 μg of supercoiled plasmid pML(C_2AT), 37.5 mM HEPES-KOH, pH 7.9, 1.5 mM DTT, 0.5 mM EDTA, 8.5% glycerol, 8.5 mM $MgCl_2$, 50 mM KCl, 8 mM phosphocreatine, 2.5 μg creatine phosphokinase (type I; Sigma), 500 μM ATP and CTP, 5 μM UTP, 20 μCi [^{32}P]UTP (3000Ci/mmol; Amersham), 20 U of RNase inhibitor (RNasin; Promega, Madison, WI), and 20–100 μg of nuclear extracts. Fifty to 100 ng of partially purified WRNp (wild-type and mutant forms) were added to the transcription reaction where indicated. Reactions were incubated at 30°C for 1 h and subsequently treated with RNase T_1 (10–20 U) for 10 min at room temperature. SDS (12.5 μl of a 10% solution) and proteinase K (10 μl of a 5 mg/ml solution) were added, and the samples were incubated at 30°C for 20 min. After addition of 2 μl of tRNA (10 mg/ml) and 0.6 ml of ethanol, RNA was precipitated at −20°C for 20 min, pelleted by centrifugation for 15 min, and washed with 70% ethanol. The pelleted RNA was dried under vacuum, dissolved in 20 μl of formamide dye solution, and heated at 95°C for 5 min, and an aliquot was loaded onto a 5% polyacrylamide-7 M urea gel. The

transcription products were electrophoresed in Tris-borate-EDTA buffer at 15 W, and gels were rinsed in distilled water for 20 min, dried, and subjected to phosphorimaging. Under the conditions of the in vitro transcription reaction, two transcripts of ~400 nucleotides in length are observed. The presence of two unique transcripts is typical of this assay (Sawadogo and Roeder, 1985) and could be due to contamination of nucleotides with trace amounts of GTP or due to two independent transcription start sites.

Yeast Hybrid–Reporter Assay

L40 *Saccharomyces cerevisiae* reporter strain pBTM116 (LexA DNA-binding domain) fusion vector and pLexA-VP16 (DNA binding-domain + herpes simplex virus VP16 transcriptional activation domain) positive control vector were kindly provided by Dr. Stanley Hollenberg. Yeast media were purchased from BIO 101 (La Jolla, CA), and 3-amino-1,2,4-triazole (3-AT) was from Sigma. LexA-WRN fusion protein constructs were created using the expression vector pBTM116 (Vojtek *et al.*, 1993). This plasmid carries the *TRP1* gene for selection in yeast and a multiple cloning site inserted after the 202-amino-acid ORF of the *E. coli lexA* gene, which encodes an SOS function regulatory protein and is driven by a constitutive yeast *ADH* promoter in this vector. In-frame fusions between the C terminus of the LexA protein and specified domains of the human WRNp were created by insertion of PCR-generated *WRN* cDNA domains into the multiple cloning sites of pBTM116, as indicated in Table 3. The full-length *WRN* cDNA fusion, construct F+, was created by insertion of the unique *Bam*HI–*Ssp*I *WRN* cDNA fragment into the *Bam*HI and *Sal*I (filled) sites of *WRN* domain within pBTM116. To create F1/2 a single copy of the acidic 27-amino-acid repeat was removed from construct F+ by deletion of the unique *Afl*II–*Afl*II fragment of the *WRN* cDNA, which occurs within the repeated sequence. To create the full deletion of the repeated sequence, F−, a PCR fragment was generated from *WRN* cDNA using primers TH1 (see Table 3) and THΔR (5′-CGG CTT AAG CTC AGT AGA TTT A-3′), which was digested with *Bam*HI and *Afl*II and then substituted for the *Bam*HI–*Afl*II *WRN* fragment of construct F1/2. This PCR product results in the addition of a novel *Afl*II site located just before the acidic repeat sequence, which becomes fused upon ligation to the distal *Afl*II site within the endogenous *WRN* message. To create the R+, R1/2, and R− WRN fusion protein constructs the indicated primers were used to amplify the wild-type or deleted repeat domains directly from the corresponding full-length constructs, F+, F1/2, and F−, respectively. These PCR products were then inserted into the pBTM116 vector at the restriction sites indicated in Table 3. All constructs were confirmed by manual sequencing to be in frame and devoid of mutations.

S. cerevisiae LexA reporter strain L40 (*MATa his3Δ200 trp1-901 leu2-3112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-LacZ GAL4 gal80*) was transformed individually with pBTM116, pLexA-VP16 control vectors, and each LexA-WRN fusion protein construct. To assess the relative reporter activities of each construct, positive transformants were selected on yeast media plates lacking tryptophan, and multiple colonies were restreaked for even growth and subjected to a β-galactosidase (β-gal) filter assay, performed essentially as described (Breeden and Nasmyth, 1985). An equal inoculum of each transformant was streaked onto media plates lacking both tryptophan and histidine and containing 0, 5, 10, 25, 50, 75, 150, 200, 250, 300, 350, 400, 450, or 500 mM 3-AT. Relative growth on 3-AT–containing plates was assessed after 10 d of incubation at 30°C. All reporter assays were repeated in at least three different experiments. Reporter strength was invariable for each construct in all 3-AT growth experiments.

Immunofluorescence Detection of WRN Helicase in Interphase Nuclei

Exponentially growing normal primary fibroblast cells (GM38A) and SV40-transformed WS (AG 11395) cells were fixed in acetone: methanol (1:1) for 10 min at 25°C. A rabbit polyclonal antibody to WRNp, kindly provided by Dr. L. Guarente (Massachusetts Institute of Technology, Cambridge, MA), was used, and the staining procedure was as described (Marciniak *et al.*, 1998). The mouse monoclonal antibody to nucleoli was obtained from Chemicon (Temecula, CA). The nucleolus antibody was used at a dilution of 1:50 in a immunological buffer (PBS containing 0.5% Tween 20). Fluorescein isothiocyante–conjugated anti-mouse immunoglobulin A (1:50 dilution) was used to detect the nucleolar regions.

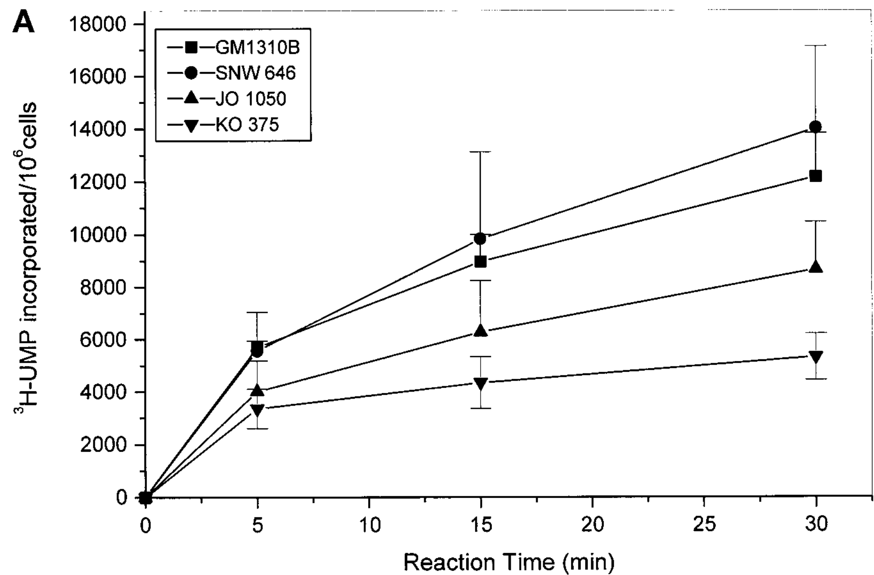
RESULTS

Transcription in Permeabilized Cells

The technique of Jackson *et al.* (1993) was used for analysis of transcription. In the initial experiments, different concentrations of lysolecithin were used to permeabilize the normal (wild-type) human and WS lymphoblastoid cells and to determine the optimal concentration for high transcription efficiency. A concentration of 20 μg/ml lysolecithin was found to be optimal, because the transcription measured by incorporation of [^{3}H]UTP was highest at this concentration (our unpublished results). This concentration was used for comparison of transcription rates between normal and WS cells.

We next measured the transcription efficiency in exponentially growing wild-type and WS lymphoblastoid cell lines (Figure 1). The transcription rate was similar in the wild-type cell lines GM1310B and SNW646, whereas both WS cell lines, KO375 and JO1050, showed reduced incorporation of UTP into RNA (Figure 1A). The different rates of transcription were not due to differences in growth rates between wild-type and WS lymphoblasts, because the doubling times of the cells used in these experiments were very similar (our unpublished data). Because the permeabilized cells cannot initiate new rounds of transcription in this type of experiment, our results reflect transcription elongation after in vivo initiation. Figure 1B shows the relative transcription rate measured over 30 min in three WS cell lines compared with the wild-type cell line SNW646, again revealing reduced transcription in all three WS lines. Mutational analysis of the *WRN* gene had shown that the cell line AG4103 is a confirmed homozygote harboring mutations in the proximity of the helicase motifs producing a highly truncated WRNp (Yu *et al.*, 1997) (Table 1). Both the KO375 and JO1050 cell lines, derived from Japanese patients, are homozygous for the *WRN* gene mutations involving base substitutions, which prematurely truncate WRNp at exons 9 and 26, respectively (Table 1).

The relative contribution of RNA pol I, II, and III in transcription was next determined in WS and non-WS cells. When permeabilized wild-type (non-WS) human cells were incubated with α-amanitin, a potent inhibitor of RNA pol II, transcription was reduced by ~70% (Figure 2A). Incubation with both α-amanitin and actinomycin D completely abolished transcription, as would be expected (our unpublished results). These results suggest that, under the conditions of this assay, RNA pol II is responsible for ~70% of the total transcription in normal cells. To determine the relative contribution of RNA pol II to the transcription in WS cells, α-amanitin–resistant RNA synthesis was also measured in WS cells. Based on levels measured in untreated WS and wild-type cells, transcription in the WS cells was reduced to a lesser degree by α-amanitin than in the wild-type cell line (Figure 2B). When RNA pol II transcription was abolished by the addition of α-amanitin, the



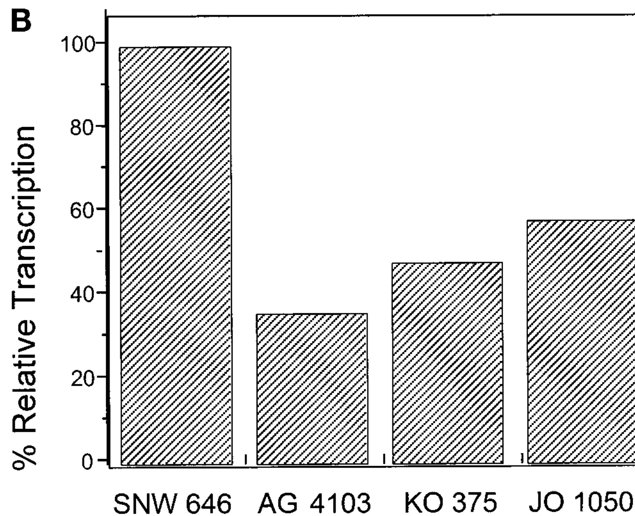


Figure 1. (A) Transcription in permeabilized cells from two wild-type lymphoblastoid cells (SNW646 and GM1310B) and two WS lymphoblastoid cells (JO1050 and KO375). The cells in exponential growth were prelabeled with [^{14}C]thymidine and permeabilized with lysolecithin (20 μg/ml), and transcription was carried out at 37°C. The radioactivity incorporated was determined by scintillation counting and expressed as [^{3}H]UMP incorporated per 10^6 cells. Error bars indicate the SEM of six independent experiments. (B) Comparison of the rate of transcription in wild-type and WS lymphoblastoid cell lines. The cells were prelabeled with [^{14}C]thymidine, encapsulated, and lysed with lysolecithin, and transcription was carried out for 30 min at 37°C. Transcription levels for each cell line were normalized to the level for the wild-type (non-WS) SNW646 cell line.

residual transcription (RNA pol I and III) measured from wild-type (SNW 646) and WS (KO375) cells was very similar (Figure 2, A and B). This suggests that differences in pol II transcription between wild-type and WS cells are not due to global cellular metabolic differences and that the transcription defect in WS appears to be mainly associated with RNA pol II–dependent transcription.

Complementation of Chromatin Transcription in WS Cells

We next determined whether the reduced transcription observed in WS cells could be complemented with nuclear extracts from HeLa cells that are highly potent in supporting transcription. For our complementation experiments, we used chromatin prepared from permeablilized Werner (KO375) and non-Werner (GM1310B) cells that were depleted of soluble proteins by extraction with 0.5% Triton X-100. We have previously shown that chromatin prepared from wild-type cells after extraction with Triton X-100 is competent for transcription despite the loss of the majority of cellular proteins (Balajee *et al.*, 1997). In the non-WS cell line GM1310B, these chromatin preparations are competent for transcription, and addition of 50 μg of HeLa nuclear extract did not affect the transcription rate (Figure 3). In contrast, the transcription rate in the chromatin from the WS cell line KO375 was markedly deficient when compared with the non-WS chromatin (Figure 3 and Table 2). However, this defect in chromatin from KO375 could be complemented by the addition of HeLa nuclear extract (Figure 3). In contrast, addition of KO375 extract back to KO375 chromatin did not alter the transcription rate (Table 2). These results suggest that chromatin isolated from WS cells is deficient in transcription because it lacks certain factors, including

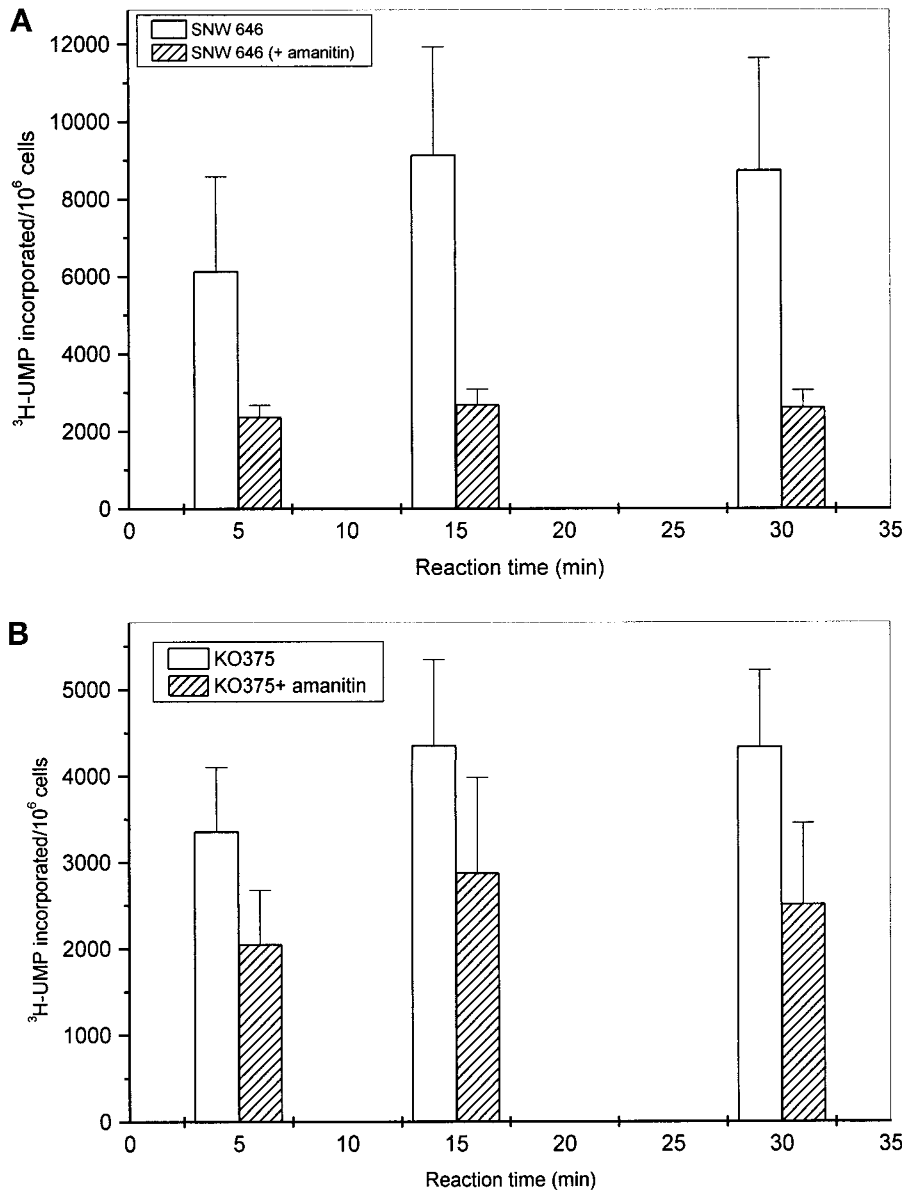


Figure 2. RNA pol I–III transcription in normal and WS lymphoblastoid cell lines. The cells were labeled with [^{14}C]thymidine, encapsulated, lysed, and incubated with α-amanitin (10 μg/ml) on ice for 30 min before the transcription reaction. The amount of [^{3}H]UMP incorporation was measured by scintillation counting. The α-amanitin–resistant synthesis represents the transcription by RNA pol I and III. (A) Effect of α-amanitin in a normal cell line (SNW646); (B) effect of α-amanitin in a WS cell line (KO375).

WRNp. Efficient transcription from WS chromatin can be restored by readdition of these factors that are present in wild-type but not in WS nuclear extracts.

Reduced Levels of RNA pol II Transcription In Vitro in WS Nuclear Extracts

Transcription was also measured in vitro using nuclear extracts to perform transcription on a plasmid containing the RNA pol II–specific AdML promoter. Nuclear extracts were prepared from EBV-transformed WS lymphoblast cell lines (JO1050 and KO375) and an EBV-transformed wild-type lymphoblast cell line (SNW646). Wild-type and WS extracts contained comparable amounts of several proteins necessary for RNA pol II–specific transcription as measured by Western blotting (our unpublished results). These extracts also were comparably active in both RNA pol I transcription and DNA repair incorporation assays (our unpublished data), attesting to the metabolic states of the cell lines and extracts. After incubation of the extracts with the pol II–specific plasmid, the transcripts (~400 nucleotides in length) generated from the AdML promoter were purified and analyzed by electrophoresis and phosphorimaging. Addition of α-amanitin to the in vitro transcription reaction completely abolishes the production of these transcripts, attesting to the utility of this assay for measuring RNA pol II–specific transcription (Dianov *et al.*, 1997). Compared with the level of transcription observed from the human wild type (SNW646), the two homozygous WS cell lines JO1050 and KO375 showed a considerable reduction in transcription from the AdML promoter (Figure 4A). The reduced tran-

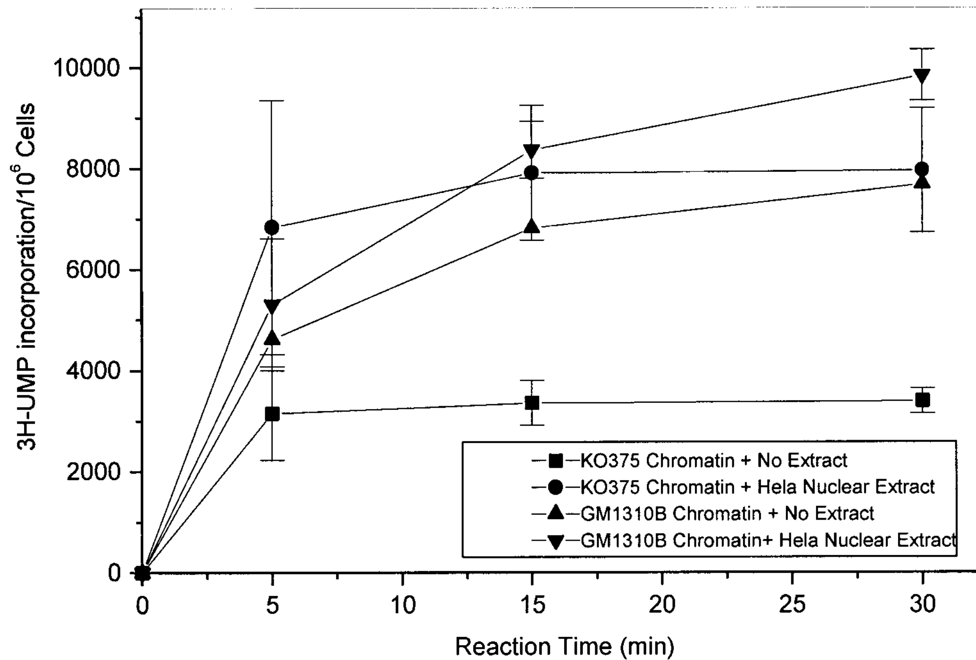


Figure 3. Complementation of transcription defect in chromatin from the homozygous mutant cell line KO375 with nuclear extracts of HeLa cells. KO375 and GM1310B cells were prelabeled with [^{14}C]thymidine, encapsulated, and lysed with 0.5% Triton X-100 for 15 min on ice. The lysed cells were washed several times with PBS and incubated with 50 μg of nuclear extracts prepared from HeLa cells in separate reactions. The transcription measured in the chromatin of KO375 cells without the addition of nuclear extracts served as a control to determine the transcription enhancement by HeLa nuclear extracts. The transcription rate is expressed as [^{3}H]UMP incorporated per 10^6 cells based on the specific activity of [^{14}C]thymidine per cell.

scription catalyzed by WS nuclear extracts, compared with wild-type nuclear extracts, was observed over a broad range (20–80 μg) of extract protein concentration (Figure 4B). Lower extract concentrations are unable to support significant levels of transcription, presumably because of insufficient amounts of the required factors. At concentrations of >100 μg, transcription becomes inhibited by factors in both WS and wild-type extracts. In multiple experiments using independently prepared extracts, each of the WS cell lines (JO1050 and KO375) showed a 50–60% reduction in transcription compared with the wild-type control (Figure 4C). Nuclear extracts from WS cell lines (JO1050 and KO375) also showed an even greater reduction in transcription compared with nuclear extracts from other non-WS controls, including an SV40-transformed lymphoblast cell line AG09387 and a commercially available HeLa nuclear extract (our unpublished results). Combined with the earlier studies in permeabilized cells, our results indicate that WS cells are partially deficient in pol II transcription. We also tested the levels of RNA pol I transcription using a linear plasmid, prHu3, containing the human RNA pol I promoter and a portion of the rRNA gene (Learned *et al.*, 1985). In contrast to the results with RNA pol II transcription, there was no significant difference in the RNA pol I transcription catalyzed by normal and WS cell extracts (our unpublished results).

Table 2. Complementation of transcription in WS (KO375) cells by nuclear extracts

	Transcription (%)	
Cell line	15 min	30 min
GM1310B chromatin[a]	100	100
KO375 chromatin	37.4	32.8
KO375 chromatin + Hela nuclear extract	116	103
KO375 chromatin + KO375 nuclear extract	39.7	30.9

[a] The transcription measured as [^{3}H]UMP incorporated per 10^6 cells in the normal cell line (GM1310) was considered 100%. The transcription measured in WS (KO375) chromatin supplemented with 50 μg of either Hela or KO375 nuclear extracts was expressed as percent of control.

WRN Contains a Strong Transcriptional Activation Domain

A yeast hybrid–protein reporter system was used to determine whether the human WRNp contains specific domains capable of activating transcription in vivo. The WRNp domains and full-length cDNAs diagrammed in Figure 5 and Table 3 were cloned into the yeast expression vector pBTM116 to create LexA (DNA binding domain)-WRN fusion protein constructs. LexA-WRN fusion proteins were expressed in *S. cerevisiae* strain L40, which contains transcriptional reporters for the expression of LacZ and His3 coding sequences driven, respectively, by minimal *GAL1*

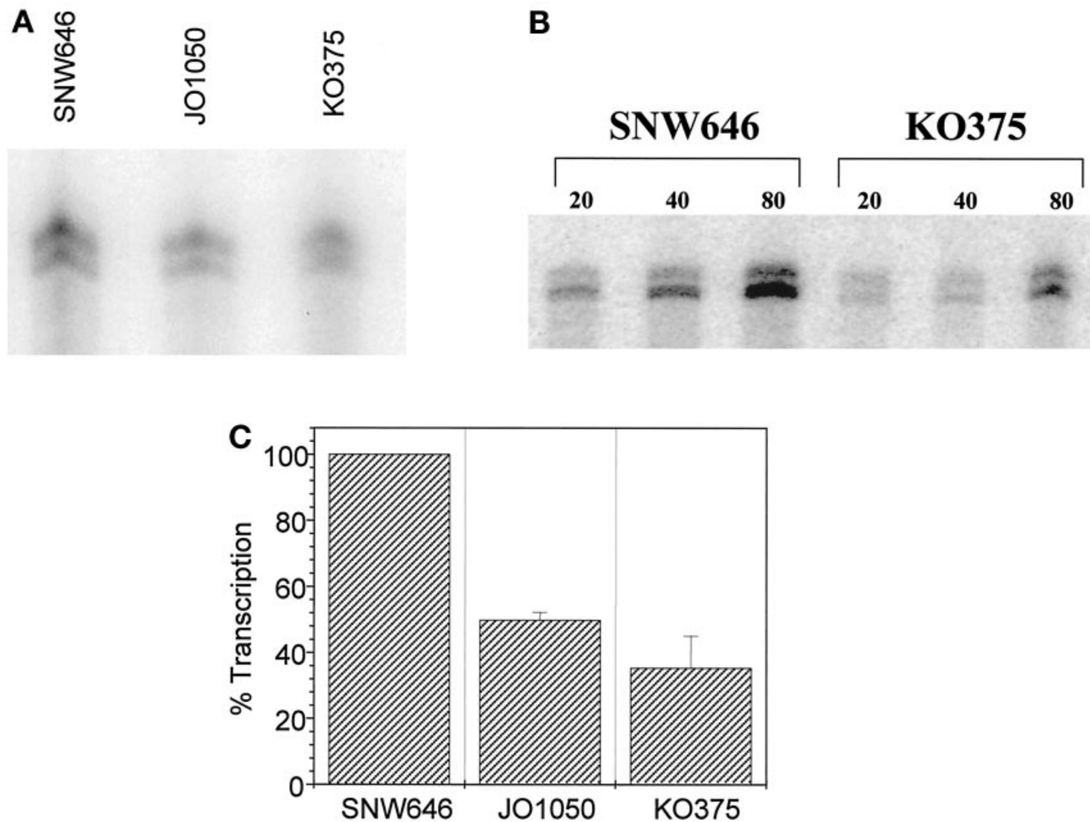


Figure 4. In vitro RNA pol II transcription from the AdML promoter in extracts of wild-type and WS cells. (A) Transcription from the AdML promoter carried on a supercoiled plasmid with a G-less cassette was assayed using nuclear extracts from wild-type (SNW 646) and WS (JO1050 and KO375) lymphoblasts. Incubation of plasmid DNA was carried out with nuclear extracts (75 μg of total protein) for 1 h, and transcription was monitored by electrophoresis and phosphorimaging (see MATERIALS AND METHODS for details). RNA pol II-specific transcription is evidenced by the production of two transcripts ~400 nucleotides in length. (B) Transcription using the nuclear extracts from wild-type (SNW646) and WS(KO375) cells was assayed over a range of extract concentrations (20, 40, and 80 μg of protein). (C) Relative levels of transcription in WS nuclear extracts. Transcription was measured using multiple independently prepared nuclear extracts and normalized to the transcription levels using nuclear extracts from a wild-type cell line (SNW646). The error bars represent the SEM between individual experiments.

and *HIS3* promoters fused to multimerized LexA DNA binding sites (Vojtek *et al.*, 1993). Each fusion protein was assessed for its ability to activate these LexA-driven β-gal and histidine reporter constructs. The ability of pBTM116 expression vectors containing LexA-WRN fusion protein constructs to allow the L40 (his$^-$) strain to grow on media lacking histidine indicates a significant level of expression of LexA fusion proteins. LexA and a LexA-VP16 (activation domain) fusion protein were used as negative and positive controls, respectively.

All LexA-WRN constructs containing the repeated sequence of 27 amino acids (Figure 5) were strongly positive in the β-gal filter assay after only 2 h of incubation of the filters with the X-gal substrate similar to the LexA-VP16–positive control (our unpublished results). After overnight incubation of the filters, weak β-gal activity could be detected from mildly acidic domains 2 and 6 and from a full-length WRN construct, F−, in which the repeated sequences had been deleted. In repeated assays the relative rate of β-gal development was consistent for each clone but varied consistently between different clones. To determine the relative strength of each construct in activating transcription, 3-AT, a potent inhibitor of histidine biosynthesis, was used to quantitate histidine prototrophy. In this assay, all LexA-*WRN* constructs could grow on the basal histidine-deficient media, but only domains with visible β-gal activity were able to grow in the presence of 3-AT (Figure 6). The wild-type full-length WRNp, F+, and the repeat containing domain, R+, were as strong activators of transcription as the VP16-positive control. Surprisingly, constructs containing only a single copy of the repeated sequence, F1/2 and R1/2, were able to efficiently stimulate histidine biosynthesis in the presence of 3-AT, although to a lesser extent than two copies. No other WRN domain appeared to display even remotely comparable transcriptional activating capability in this assay. WRN domains unable to significantly stimulate the histidine reporter presumably are not strong activators of transcription. However, we cannot rule out disparities in expression and LexA fusion protein stability as potential causes for lack of activation by such domains. Nevertheless,

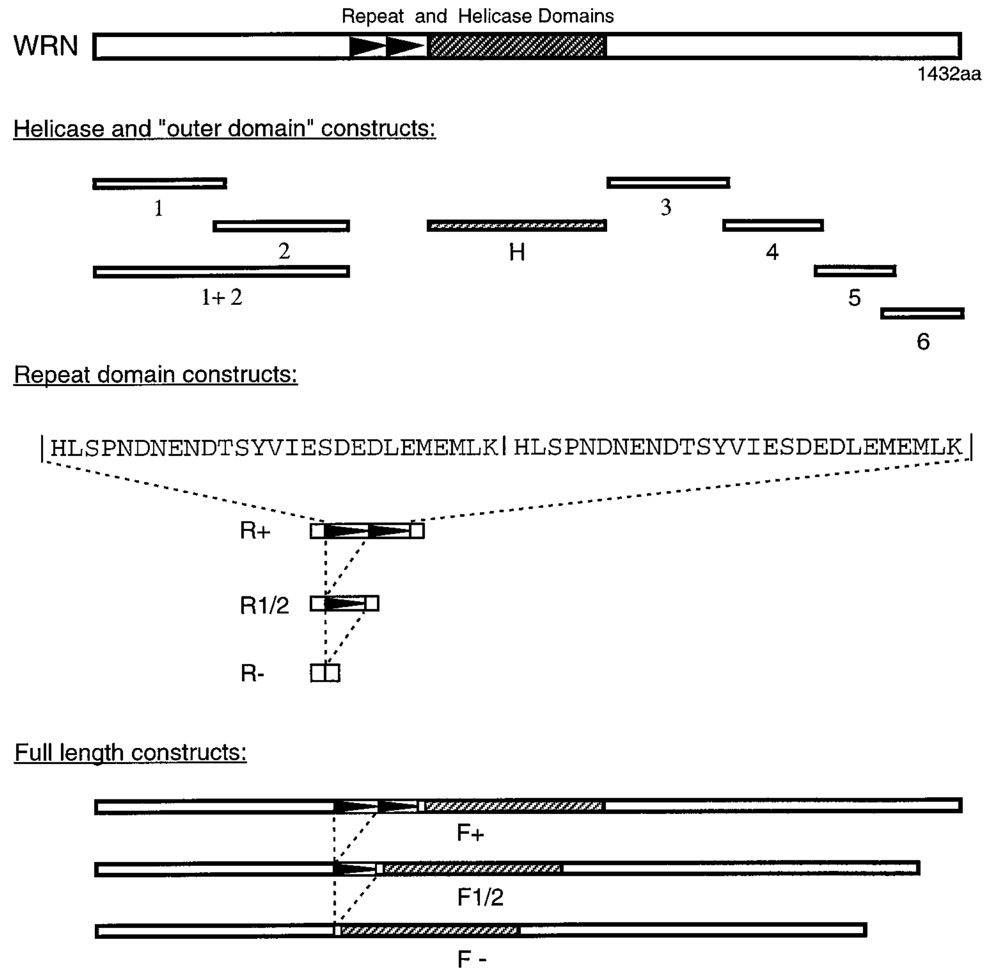


Figure 5. Schematic diagram of LexA-WRN fusion protein constructs. The full-length human WRNp is shown at the top with the location of repeat (arrows) and helicase (shaded box) domains indicated. Each bar represents the WRN portion of an individual LexA hybrid construct, and each is aligned according to its relative position within the full-length protein (repeats are not to scale). The sequence of the 27-amino-acid direct repeat is shown, and dotted lines delineate successive deletions of this region made in the particular repeat and full-length constructs. The specific WRNp coding domains contained within each construct are presented in Table 3.

a strong positive stimulation of histidine biosynthesis, as seen in all constructs containing the 27-amino-acid repeated sequence, is indicative of a strong transcriptional activation domain. Our results suggest a role for this 27-amino-acid domain, alone or in the context of the entire WRNp, in general or specific activation of transcription.

Effects of Purified WRNp (Wild-Type or Mutant) on In Vitro Transcription

We assayed whether the observed defective transcription in WS extracts could be complemented by the reintroduction of wild-type WRNp to the in vitro system. His-tagged WRNp (both wild-type and mutant forms) were overexpressed in *Sf9* insect cells and purified by Ni affinity chromatography. The addition of WRNp (wild type [wt]) to the transcription reaction resulted in a significant stimulation of transcription in both the wild-type (SNW646) and WS (KO375) extracts (Figure 7A). As a mock control, parallel identical protein purifications were done using lysates from insect cells infected with baculovirus lacking *WRN* sequences. The addition of this mock protein preparation to the SNW646 nuclear extracts (Figure 7B) or to KO375 nuclear extracts (our unpublished results) did not stimulate transcription, indicating that the previously observed stimulation is attributable to WRNp (wt). Although the WRN protein does not exclusively complement the observed transcription deficiency of WS nuclear extracts, our results suggest that the WRN protein strongly stimulates RNA pol II transcription in this in vitro assay. This is in close agreement with data obtained from the hybrid reporter assay that shows that the WRN protein contains specific domains capable of transcriptional activation in vivo.

To determine whether specific domains within the WRN protein might be involved in the observed stimulation of transcription, the effect of mutant WRN proteins on in vitro transcription was studied. We examined two mutant proteins of particular interest (shown in Figure 8A): 1) WRN-ΔR lacking the 27-amino-acid direct repeat that was shown to function as a transcriptional activation domain in the yeast reporter assay (see above), and 2) WRN-K577M completely lacking ATPase/helicase activity because of a single amino acid substitution changing the invariant lysine residue to methionine (K577M) in motif I of the seven conserved helicase domains. Again, these mutant proteins were purified in an identical manner as the wild-type WRNp. Notably, the WRN-ΔR mutant retains ATPase and helicase activity (our

Table 3. pLexA-WRN fusion protein constructs

Name of construct	WRN Protein coding Domain[a]	Primer set (Forward/reverse)	Primer sequence site (5′→3′)	pBTM116 cloning site
1	6–220	TH1	*Bgl*II (GCC AGA TCT TGG AAA CAA CTG CAC)	
		TH2	*Sal*I (GCC GTC GAC ACC AGC ATA AGC ATC)	*Bam*HI-*Sal*I
2	205–412	TH3	*Eco*RI (CCG GAA TTC CTC ACT GAG GAC CAG)	
		TH4	*Sal*I (GCC GTC GAC TTC TTC CTG AGA CTG)	*Eco*RI-*Sal*I
1+2	6–412	TH1	*Bgl*II (GCC AGA TCT TGG AAA CAA CTG CAC)	
		TH4	*Sal*I (GCC GTC GAC TTC TTC CTG AGA CTG)	*Bam*HI-*Sal*I
R+	406–525	TH13	*Eco*RI (TGA GAA TTC GGA CAG CAG TCT CAG)	
		TH14	*Sal*I (CGC GTC GAC CTT ATC ATC ATC TTC)	*Eco*RI-*Sal*I
R1/2	406–525	TH13	*Eco*RI (TGA GAA TTC GGA CAG CAG TCT CAG)	
	(450–476)	TH14	*Sal*I (CGC GTC GAC CTT ATC ATC ATC TTC)	*Eco*RI-*Sal*I
R−	406–525	TH13	*Eco*RI (TGA GAA TTC GGA CAG CAG TCT CAG)	
	(424–475)	TH14	*Sal*I (CGC GTC GAC CTT ATC ATC ATC TTC)	*Eco*RI-*Sal*I
H	522–868	TH15	*Eco*RI (CGC GAA TTC GAT GAT GAT AAG GAC)	
		TH16	*Sal*I (ATT GTC GAC CCA GAG GAC GTG ACA)	*Eco*RI-*Sal*I
3	861–1,062	TH5	*Eco*RI (CCG GAA TTC AGT TCT TGT CAC GTC)	
		TH6	*Sal*I (GCC GTC GAC GAT GAG GCT GTG AGA)	*Eco*RI-*Sal*I
4	1,014–1,179	TH7	*Eco*RI (CCG GAA TTC TGG AAG GCT TTT TCC)	
		TH8	*Sal*I (GCC GTC GAC TGC CAG AAT AGC TGG)	*Eco*RI-*Sal*I
5	1,162–1,306	TH9	*Eco*RI (CCG GAA TTC GTA GAA GCT AGG CAG)	
		TH10	*Sal*I (GCC GTC GAC TGC TCG CTC CAA ATC)	*Eco*RI-*Sal*I
6	1,272–1,432	TH11	*Eco*RI (CCG GAA TTC CCT TTG AAG AGC ATA)	
		TH12	*Sal*I (GCC GTC GAC GCT GAT TTA AGA TGC)	*Eco*RI-*Sal*I
F+	1–1,432	TH1	*Bgl*II (GCC AGA TCT TGG AAA CAA CTG CAC)	
		TH2[b]	*Sal*I (GCC GTC GAC GCT GAT TTA AGA TGC)	*Bam*HI-*Sal*I
F1/2	1–1,432	TH1	*Bgl*II (GCC AGA TCT TGG AAA CAA CTG CAC)	
	(450–476)	TH2[b]	*Sal*I (GCC GTC GAC GCT GAT TTA AGA TGC)	*Bam*HI-*Sal*I
F−	1–1,432	TH1	*Bgl*II (GCC AGA TCT TGG AAA CAA CTG CAC)	
	(424–475)	TH2[b]	*Sal*I (GCC GTC GAC GCT GAT TTA AGA TGC)	*Bam*HI-*Sal*I

[a] WRNp domains are relative to amino acid positions 1–1432 of the wild-type human WRNp (accession number L76937). Internal amino acid deletions are indicated within parentheses and were constructed as described in MATERIALS AND METHODS.

[b] Full-length constructs were made by insertion of the remaining 3′-WRN coding sequence into pLexA-WRN domain 1, as described in MATERIALS AND METHODS.

unpublished results), indicating correct folding and stability of this protein despite the deletion of 54 amino acids upstream of the helicase domain. The mutant proteins were compared with the wild-type WRN protein for their ability to stimulate transcription catalyzed by SNW646 nuclear extracts (Figure 8B). Reactions contained 75 μg of SNW646 nuclear extract to which 50 ng of WRN (wt) or mutant proteins were added. The addition of WRN-ΔR protein to the SNW646 extract did not result in a significant stimulation of transcription over the basal level. The addition of WRN-K577M mutant protein resulted in only a marginal stimulation of transcription. For each mutant protein, similar results were observed in at least three independent experiments. The stimulation by the WRN-K577M protein was significantly less than that observed after the addition of the WRNp (wt), which consistently resulted in a seven- to eight-fold stimulation of transcription over the basal level (Figure 8B). Our results strongly suggest that the 27-amino-acid direct repeat functions as a transcriptional activation domain in the in vitro transcription assay, in agreement with the results using the yeast reporter system. In addition, the ATPase/helicase activity of the WRN protein may also play a role in stimulation of transcription in the in vitro assay. The lack of stimulation by the mutant WRN proteins and mock preparation also confirms that stimulation of pol II–specific transcription in vitro is a bona fide property of wild-type WRNp.

Immunofluorescent Localization of WRN Helicase in the Nucleoplasm of Human Cells

Proliferating primary human fibroblasts (GM38A) and SV40-transformed WS fibroblasts (AG11395) were immunostained using affinity-purified polyclonal antibody to WRN helicase. The WS fibroblasts did not show any staining for WRNp (Figure 9A). In the majority of the GM38 cells, distinct punctuated patterns of staining were observed in the nucleoplasm (Figure 9B). In a small fraction of the cells (~20% of the total population) we observed distinct fluorescent speckles both in the nucleoplasm and nucleoli. We immunostained the cells simultaneously with both nucleolus (see MATERIALS AND METHODS) and WRN antibody for colocalization (Figure 9C) and found that WRN helicase was localized in discrete foci throughout the nucleoplasm.

DISCUSSION

We have used several approaches to measure the transcription capacity of WS cells and nuclear extracts and to deter-

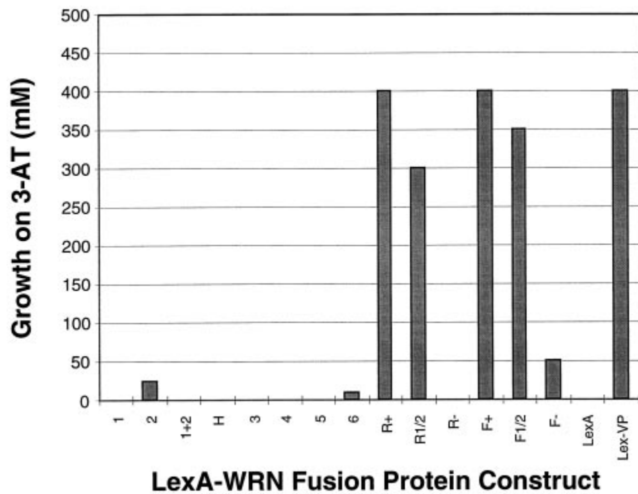


Figure 6. The WRNp direct repeat domain drives transcription in a histidine prototrophy assay. LexA-WRN fusion protein constructs (see Figure 5 and Table 3) were transformed into L40 yeast and assessed for their ability to grow on media plates containing a range of 3-AT concentrations. Relative growth was scored 10 d after plating in the presence of the indicated amount of 3-AT. LexA is expressed by pBTM116 with no insert (negative control), and LexA-VP is a fusion protein between LexA and the transcriptional activation domain of the herpes simplex virus viral protein 16, VP16 (positive control).

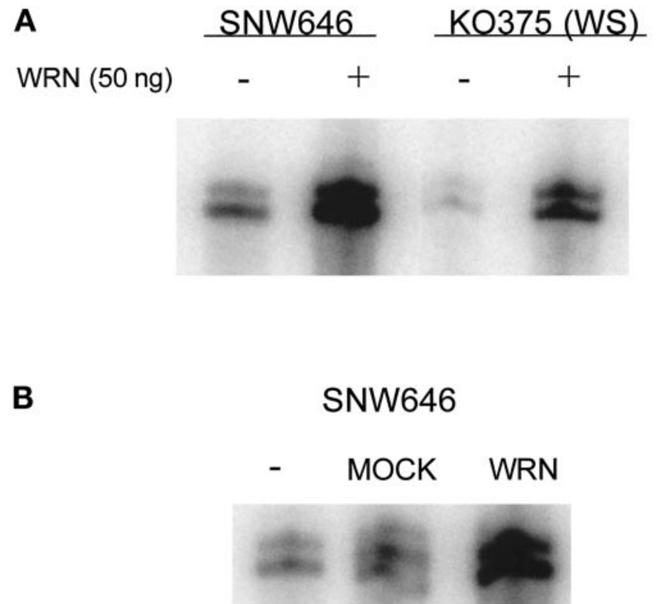


Figure 7. Stimulation of in vitro transcription by addition of WRNp to extracts derived from wild-type and WS lymphoblastoid cell lines. (A) Transcription was assayed in nuclear extracts prepared from wild-type (SNW646) and WS (KO375) cells. WRNp (~50 ng) was added to the reaction (as indicated), and transcription was monitored after incubation at 30°C for 1 h as described earlier. (B) As a control for the above experiment, equivalent volumes of WRNp and mock-purified protein preparation were added to aliquots of the wild-type extract (SNW646), and transcription was measured as above.

mine whether the WRNp has a role in transcription. Because of the extremely poor growth of primary WS cells, it was necessary to use lymphoblastoid cell lines for our experiments. Also, these cell lines have been used for many of the previous studies on WS. Notably, the growth kinetics of the wild-type and WS lymphoblasts used in these studies are similar. The WS lymphoblastoid cell lines have defined mutations in the *WRN* gene (Table 1) and have been used previously for characterization of the biochemical properties of WS. Studies using permeabilized cells indicate a decrease in transcription efficiency of ~50% or more in three different WS lymphoblastoid cell lines that harbor homozygous mutations in the *WRN* gene compared with normal cells. The nuclear extracts prepared from these cell lines also showed reduced RNA pol II transcription in vitro from a plasmid containing the RNA pol II–specific AdML promoter. The consistency in the results obtained by these two different approaches strongly suggests that the observed transcription deficiency might be at least partially responsible for the WS phenotype. This transcription deficiency is not likely to be due to a replication deficiency, because we did not observe any difference in cell cycle progression between the wild-type and WS lymphoblastoid cells.

We also demonstrated that transcription catalyzed by chromatin from WS cells is significantly lower than that catalyzed by chromatin from wild-type cells. This transcriptional deficiency in chromatin of permeabilized WS cells can be complemented by the addition of extracts from normal cells. Also, the addition of purified WRNp to the in vitro plasmid-based assay significantly stimulates transcription. This stimulation is specific to the wild-type WRNp, because both the mock protein preparation and two mutant WRN proteins failed to stimulate transcription. The mutation in the ATPase/helicase motif of WRN-K577Mp greatly reduces its activity in transcriptional stimulation, suggesting that the ATP/helicase activity is involved with the transcriptional activator function. Also, the WRN-ΔRp that lacks the 27-amino-acid direct repeat but retains ATPase/helicase activity did not stimulate the in vitro transcription reaction. Our studies with the yeast reporter system also indicate that the direct repeat sequence has a role in WRN-specific transcriptional activation. Thus, both the direct repeats and the helicase domain may be involved in the stimulation of transcription in the in vitro system.

Results from the yeast reporter system also support the idea that WRNp may have a role in transcription. In this assay, full-length WRNp fused to the LexA DNA binding domain was able to stimulate transcription to levels comparable with the positive control (the VP16 transcriptional activation domain fused to LexA). This activation presumably occurs via the recruitment of particular component(s) of the transcriptional machinery to the LexA DNA binding sites upstream of the yeast reporter genes. LexA-WRN constructs deleting various regions of WRNp demonstrate that the 27-amino-acid direct repeat domain (amino acids 424–477) is likely to be responsible for the robust transcriptional activation function of WRNp in the reporter assay. The WRNp homologues from *Xenopus laevis* (Yan *et al.*, 1998) and mouse (Imamura *et al.*, 1997) also contain an almost identical 27-amino-acid sequence, although notably this sequence is

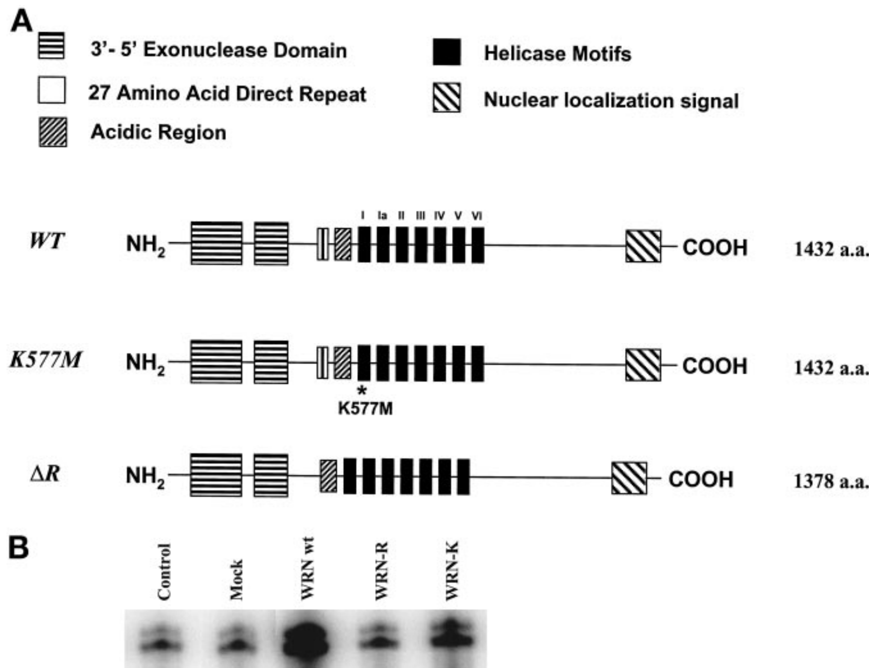


Figure 8. (A) Schematic map (not to scale) of the WRNp domain structure and the mutations in the WRN-ΔR and WRN-K577M proteins. Domains are depicted by uniquely shaded boxes as indicated and an asterisk marks the site of the amino acid (a.a.) change in helicase motif I of WRN-K577M. (B) Effect of addition of wt WRN and mutant proteins on RNA pol II transcription. The effect of addition of wt WRNp and mutant proteins (WRN-ΔR andWRN-K577M) on the transcription from the AdML promoter by nuclear extracts from wild-type SNW646 was assayed as described earlier. Reactions contained 75 μg of nuclear extract to which 50 ng of WRN (wt) or mutant proteins (WRN-ΔR or WRN-K577M) were added. As a control, an equivalent amount of the mock-purified protein preparation was used in one reaction. The reactions were incubated at 30°C for 1 h, and transcription was monitored by electrophoresis and phosphorimaging. Similar results were observed in at least three independent experiments.

not repeated as in human WRNp. However, our results also indicate that the presence of a single copy of this repeated sequence can also activate transcription, suggesting that the WRNp homologues in *Xenopus* and mouse may also have similar roles in transcription.

The yeast reporter assay showed that the helicase domain alone did not stimulate transcription in that system as did the direct repeats proximal to the helicase domain. Although the 27-amino-acid direct repeat activation domain can apparently stimulate transcription independently of the rest of the WRNp in the yeast reporter assays, it appears unable to do so in the context of the helicase-deficient mutant (K577M) in the in vitro transcription assay. It is possible that the WRN helicase requires other functional domains to actively participate in transcription in the reporter assay. Notably, the direct repeat domain has an acidic character. Acidic domains of proteins have been known to show activation in the reporter system, indicating the ability of the particular domain, when fused to the LexA-DNA binding domain, to help recruit the yeast transcriptional machinery to the LexA promoter. In this context alone, it is not possible to interpret the significance of this activation with respect to the functioning of the domain when it is present in its naturally occurring location within the protein. Importantly, in both the reporter assay and the in vitro assay, the repeat domain within the context of the wild-type protein still retains this ability to provide activation. The amount of histidine reporter activation provided by the WRN repeat in this one-hybrid analysis is significantly strong considering that the 3-AT concentrations overcome by activation of the histidine reporter in these experiments are 1–2 orders of magnitude greater than those routinely used to increase sensitivity in two-hybrid studies.

Recently, Ye *et al.* (1998) studied the transcriptional activation of the GAL1 promoter by WRN helicase in a yeast assay system using different regions of the WRNp. They identified a region of 315–403 aa, which may function as a transcriptional activation domain. They also found that another region of the protein may activate transcription when the former region is present. These observations are consistent with our results and with the notion that both the helicase domain and the direct repeat regions of WRNp have functions in transcriptional activation.

Although we cannot rule out the participation of other domains, our experiments indicate that the direct repeat domain is involved in the transcriptional stimulation and activation observed with the wild-type WRNp. It is also possible that the acidic direct repeat of WRNp may normally recruit these activating factors to the DNA for reasons other than, or in addition to, transcription, such as DNA replication, recombination, and other types of repair, or perhaps even to maintain the structure of chromatin in some general manner during some, or all, of these processes. Recent studies imply that WRNp also has a function in replication (Lebel and Leder, 1998; Yan *et al.*, 1998). In either case, there are likely to be related gene products such as BLMp and RECQLp that can, in the absence of WRNp, complement for some aspects of normal WRNp functions. The pol II–specific deficit exhibited by WS cells is thus presumably the product of both loss of *WRN* gene functions and the remaining genome's inability to completely complement for these losses.

Our results using the plasmid-based in vitro assay suggest that the helicase function of WRNp might also be involved in transcription. Many studies have demonstrated important roles for helicases in various DNA metabolic activities (Duguet, 1997). The replication and transcription processes require unwinding of the DNA duplex so that the DNA is made accessible to various enzymes. This can be accomplished by disruption of nucleosome assembly through the

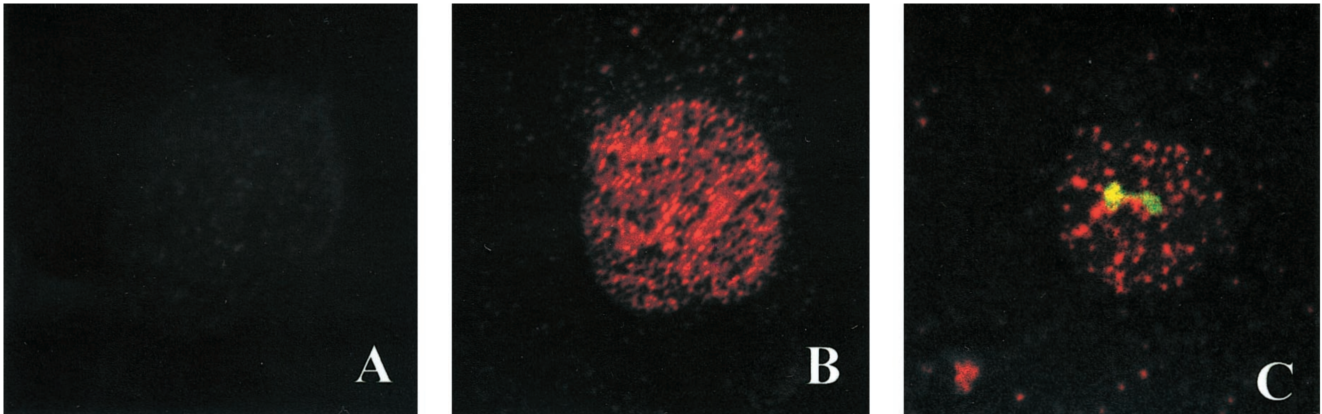


Figure 9. Immunofluorescent staining of WRN helicase in the nucleoplasm of WS (AG11395) and normal fibroblasts (GM38A). Exponentially growing normal human and WS fibroblasts were fixed in acetone:methanol. Cells were stained with a rabbit polyclonal antibody to WRNp and a Texas Red–conjugated secondary antibody to rabbit immunoglobulin G. The red dots indicate the positions of WRNp in the nucleoplasm. WS fibroblasts did not show any staining (A), whereas the normal cells showed distinct staining of WRNp (B). (C) Colocalization of both nucleolar (green fluorescence) and WRNp (red fluorescence) in the interphase nuclei of GM38 cells detected with fluorescein- and Texas Red–conjugated secondary antibodies.

positive supercoiling produced by a helicase tracking through a DNA duplex (Ramsperger and Stahl, 1995). The involvement of helicases in transcription is also exemplified by the observation that one of the basal transcription factors, TFIIH, exhibits DNA unwinding activity (Schaeffer *et al.*, 1993). Mutations in the ATP binding domain of Rad25 (the yeast homologue of the human XPB subunit of TFIIH) helicase appear to be incompatible with cell viability and RNA pol II transcription (Guzder *et al.*, 1994). Hence, a mutational inactivation of the ATPase/helicase activity may be expected to disrupt vital cellular processes and could lead to genomic instability.

Recent studies (Gray *et al.*, 1998; Marciniak *et al.*, 1998) have reported that WRNp is enriched in the nucleolus. This is also supported by observations in yeast, in which the WRNp homologue SGS1p also localizes to the nucleolar region (Sinclair *et al.*, 1998). The nucleolus is the site of rDNA gene clusters that are transcribed by RNA pol I. Our results using α-amanitin to inhibit RNA pol II transcription suggest that RNA pol I transcription is not affected in WS cells, and that the defect in WS cells is primarily restricted to RNA pol II transcription. Also, we have conducted experiments using a promoter for RNA pol I transcription to study rDNA transcription in vitro. No major defect in RNA pol I transcription was observed in the WS cell lines used in this study (our unpublished data). This would suggest that the role of the WRNp in the nucleolar region might not be primarily one of transcriptional regulation. The role(s) of WRNp in nucleolar metabolism remains to be defined.

By immunofluorescence we observe distinct staining for WRNp in the nuclei of fibroblasts (Figure 9). A similar observation was very recently reported by Shiratori *et al.* (1999), who showed that the majority of WRNp is in the nucleoplasm rather than in the nucleolus. Thus, our localization studies and transcription studies both suggest that the primary function of the WRNp is in the nucleus rather than in the nucleolus.

The consequences of a transcriptional defect in cells could span from minor to very severe. It has been postulated that there are human disorders called "transcription syndromes" in which the multiple clinical manifestations are due to transcription defects (Bootsma and Hoeijmakers, 1993). Cockayne syndrome, another segmental progeroid syndrome (Martin, 1978), belongs to this category of diseases. We have recently reported a defect in basal transcription in cells from individuals with this disease (Balajee *et al.*, 1997). The present work suggests that WS, the prototype of segmental progeroid syndromes (Martin, 1978), may also be a member of the group of transcription syndromes.

The transcription defect may be global or may affect certain genes or categories of genes within the genome. In a previous study, the transcripts of metalloproteases were reduced by 50% in WS cells (Millis *et al.*, 1992). This supports our observation of a defect but does not determine whether it is localized to certain genes or to the entire genome. If global transcription is reduced by 50%, and if it is due to defects in transcription of certain genes or regions of genes, it would be expected that the reduction in those regions is much greater than the 50%, which could result in serious detriments and clinical outcomes. A transcription defect, whether global or regional, could produce many of the complex clinical features of WS and thus may represent the major molecular defect underlying the disease.

ACKNOWLEDGMENTS

We thank Drs. P. Gearhart and R. Selzer for comments, and we appreciate the interaction with the Danish Center for Molecular Gerontology. We thank Irina Dianova for help with cell cultures. This work was supported by grant AG-01751, AG-14446, and R24-CA-78088 from the National Institute on Aging.

REFERENCES

Balajee, A.S., May, A., Dianov, G.L., Friedberg, E.C., and Bohr, V.A. (1997). Reduced RNA polymerase II transcription in intact and permeabilized Cockayne syndrome group B cells. Proc. Natl. Acad. Sci. USA *94*, 4306–4311.

Bennett, S.E., Umar, A., Oshima, J., Monnat, R.J.J., and Kunkel, T.A. (1997). Mismatch repair in extracts of Werner syndrome cell lines. Cancer Res. *57*, 2956–2960.

Biggerstaff, M., Robins, P., Coverly, D., and Wood, R.D. (1991). Effect of exogenous DNA fragments on human cell extract-mediated repair synthesis. Mutat. Res. *254*, 217–224.

Bootsma, D., and Hoeijmakers, J.H. (1993). DNA repair. Engagement with transcription [news, comment]. Nature *363*, 114–115.

Breeden, L., and Nasmyth, K. (1985). Regulation of the yeast HO gene. Cold Spring Harbor Symp. Quant. Biol. *50*, 643–650.

Dianov, G.L., Houle, J.F., Iyer, N., Bohr, V.A., and Friedberg, E.C. (1997). Reduced RNA polymerase II transcription in extracts of cockayne syndrome and xeroderma pigmentosum/Cockayne syndrome cells. Nucleic Acids Res. *25*, 3636–3642.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in soluble extract from isolated mammalian nuclei. Nucleic Acids Res. *11*, 1475–1489.

Duguet, M. (1997). When helicase and topoisomerase meet! J Cell Sci. *110*, 1345–1350.

Fukuchi, K., Martin, G.M., and Monnat, R.J. (1989). Mutator phenotype of Werner syndrome is characterized by extensive deletions. Proc. Natl. Acad. Sci. USA *86*, 5893–5897.

Gray, M.D., Shen, J.C., Kamath-Loeb, A.S., Blank, A., Sopher, B.L., Martin, G.M., Oshima, J., and Loeb, L.A. (1997). The Werner syndrome protein is a DNA helicase. Nat. Genet. *17*, 100–103.

Gray, M.D., Wang, L., Youssoufian, H., Martin, G.M., and Oshima, J. (1998). Werner helicase is localized to transcriptionally active nucleoli of cycling cells. Exp. Cell Res. *242*, 487–494.

Guzder, S.N., Sung, P., Bailly, V., Prakash, L., and Prakash, S. (1994). RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription. Nature *369*, 578–581.

Huang, S., Li, B., Gray, M.D., Oshima, J., Mian, I.S., and Campisi, J. (1998). The premature ageing syndrome protein, WRN, is a $3'\rightarrow5'$ exonuclease. Nat. Genet. *20*, 114–116.

Imamura, O., Ichikawa, K., Yamabe, Y., Goto, M., Sugawara, M., and Furuichi, Y. (1997). Cloning of a mouse homologue of the human Werner syndrome gene and assignment to 8A4 by fluorescence in situ hybridization. Genomics *41*, 298–300.

Jackson, D.A., Hassan, A.B., and Cook, P.R. (1993). Visualization of focal sites of transcription within human nuclei. EMBO J. *12*, 1059–1065.

Learned, R.M., Cordes, S., and Tjian, R. (1985). Purification and characterization of a transcription factor that confers promoter specificity to human RNA polymerase I. Mol. Cell. Biol. *5*, 1358–1369.

Lebel, M., and Leder, P. (1998). A deletion within the murine Werner syndrome helicase induces sensitivity to inhibitors of topoisomerase and loss of cellular proliferative capacity. Proc. Natl. Acad. Sci. USA *95*, 13097–13102.

Marciniak, R.A., Lombard, D.B., Johnson, F.B., and Guarente, L. (1998). Nucleolar localization of the Werner syndrome protein in human cells. Proc. Natl. Acad. Sci. USA *95*, 6686–6892.

Martin, G.M. (1978). Genetic syndromes in man with potential relevance to the pathobiology of aging. Birth Defects Orig. Artic. Ser. *14*, 5–39.

Martin, G.M. (1997). The Werner mutation: does it lead to a "public" or "private" mechanism of aging? Mol. Med. *3*, 356–358.

Millis, A.J., Hoyle, M., McCue, H.M., and Martini, H. (1992). Differential expression of metalloproteinase and tissue inhibitor of metalloproteinase genes in aged human fibroblasts. Exp. Cell Res. *201*, 373–379.

Ramsperger, U., and Stahl, H. (1995). Unwinding of chromatin by the SV40 large T antigen DNA helicase. EMBO J. *14*, 3215–3225.

Runger, T.M., Bauer, C., Dekant, B., Moller, K., Sobotta, P., Czerny, C., Poot, M., and Martin, G.M. (1994). Hypermutable ligation of plasmid DNA ends in cells from patients with Werner syndrome. J. Invest. Dermatol. *102*, 45–48.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sawadogo, M., and Roeder, R.G. (1985). Factors involved in specific transcription by RNA polymerase II: analysis by rapid and quantitative in vitro assay. Proc. Natl. Acad. Sci. USA *82*, 4394–4398.

Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J.H., Chambon, P., and Egly, J.M. (1993). DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. Science *260*, 58–63.

Shiratori, M., *et al*. (1999). Detection by epitope-defined monoclonal antibodies of Werner DNA helicases in the nucleoplasm and their up-regulation by cell transformation and immortalization. J. Cell Biol. *144*, 1–9.

Sinclair, D.A., Mills, K., and Guarente, L. (1998). Accelerated aging and nucleolar fragmentation in yeast *sgs1* mutants. Science *277*, 1313–1316.

Suzuki, N., Shimamoto, A., Imamura, O., Kuromitsu, J., Kitao, S., Goto, M., and Furuichi, Y. (1997). DNA helicase activity in Werner's syndrome gene product synthesized in a baculovirus system. Nucleic Acids Res. *25*, 2973–2978.

Vojtek, A.B., Hollenberg, S.M., and Cooper, J.A. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell *74*, 205–214.

Webb, D.K., Evans, M.K., and Bohr, V.A. (1996). DNA repair fine structure in Werner's syndrome cell lines. Exp. Cell Res. *224*, 272–278.

Yan, H., Chen, C.-Y., Kobayashi, R., and Newport, J. (1998). Replication focus-forming activity 1 and the Werner syndrome gene product. Nat. Genet. *19*, 375–378.

Ye, L., Nakura, J., Morishima, A., and Miki, T. (1998). Transcriptional activation by the Werner syndrome gene product in yeast. Exp. Gerontol. *33*, 805–812.

Yu, C.E., *et al*. (1996). Positional cloning of the Werner's syndrome gene. Science *272*, 258–262.

Yu, C.E., *et al*. (1997). Mutations in the consensus helicase domains of the Werner syndrome gene. Am. J. Hum. Genet. *60*, 330–341.