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The transcriptional response to distinct growth factors is impaired in Werner syndrome cells

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ABSTRACT

The Werner syndrome protein (WRN) is mutated in Werner syndrome (WS) and plays a role in telomere maintenance, DNA repair and transcription. WS represents a premature aging syndrome with severe growth retardation. Here we show that WRN is critically required to mediate the stimulatory effect of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-b) and epidermal growth factor (EGF) on the activity of RNA polymerase I (Pol I). Recombinant WRN specifically reconstitutes RNA polymerase I transcription in extracts from Werner syndrome fibroblasts in vitro. In addition, we identified a critical role for WRN during promoter clearance of Pol I transcription, but not in elongation. Notably, WRN was isolated in a complex with Pol I and was crosslinked to the unmethylated, active proportion of rDNA genes in quiescent cells suggesting a so far unknown role for WRN in epigenetic regulation.This together with alterations in Pol I transcription provide a novel mechanism possibly underlying at least in part the severe growth retardation and premature aging in Werner syndrome patients.

1. Introduction

Mutations in the Werner syndrome protein (WRN) are responsible for the Werner syndrome (WS), an autosomal recessively inherited disorder. The first symptom is the striking lack of growth during puberty. In the second and third decade patients develop signs of premature aging with atrophy and wrinkling of the skin, impaired tissue repair, arteriosclerosis, osteoporosis, diabetes mellitus and malignant tumors. The WRN gene encodes a DNA helicase of the ReQ family and the WRN protein is critically involved in DNA metabolism and maintenance including telomere maintenance, DNA repair, recombination, replication and transcription by RNA polymerases I and II (Cox and Faragher, 2007). Cells from Werner syndrome patients are characterized by slow growth rates, premature senescence (Faragher et al., 1993) and genomic instability. Telomere dysfunction is a cause of genomic instability in Werner syndrome. In this regard telomere elongation by stable overexpression of telomerase can reduce the number of aberrant chromosomes to a similar extent as complementation of WS cells with WRN (Crabbe et al., 2007) findings that indicate a protective function for WRN in telomere maintenance and chromosomal stability.

WRN is localized in transcriptionally active nucleoli of cycling human cells (Gray et al., 1998; Marciniak et al., 1998). The nucleolar accumulation of WRN in serum-stimulated cells depends on the ongoing transcription of RNA polymerase I and WRN translocates from the nucleolus to the nucleus upon serum starvation or Pol I inhibition (Shiratori et al., 2002). WRN is involved in RNA polymerase II-dependent transcription as permeabilized WS cells and extracts from WS cells display a 40–60% reduction in the efficacy of Pol II transcription compared to cells from normal individuals. Notably, RNA polymerase II-dependent transcription can be stimulated by the addition of recombinant WRN protein (Balajee et al., 1999). This has raised the question whether the WS transcription defect is global or specific for defined genes. A study addressing this issue compared transcription profiles in fibroblast cell lines derived from young donors, old donors and WS patients. Microarray analyses revealed that the transcription defect is specific for defined genes. Also transcriptional alterations in WS reveal a striking similarity to changes during normal aging (Kyng et al., 2003), thus validating WS as a suitable aging model.

A former study established that there is a deficiency in the cellular response to growth factors in WS cells. A full mitogenic response as measured by DNA synthesis was observed in WS cells after addition of fetal bovine serum (FBS), but did not occur after stimulation with whole human serum (WHS). In line with these data, DNA synthesis was not stimulated following application of PDGF and FGF when compared to the strong mitogenic response in control cells. These data suggest a partial defect in the growth factor response of Werner syndrome cells (Bauer et al., 1986).

Even though these observations suggest that the transcriptional response of RNA polymerase I to growth factors may be affected in

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Werner syndrome cells and may even contribute to the development of clinical features of Werner syndrome, the underlying molecular base has not yet been addressed in sufficient detail. Here we show a defect in the Pol I transcription in response to distinct growth factors in Werner syndrome cells. WRN stimulates promoter clearance of RNA polymerase I transcription and binds to the active fraction of rDNA in quiescent cells.

2. Materials and methods

2.1. Cell growth

Primary foreskin fibroblasts, HeLa, HEK293 and Werner syndrome derived fibroblasts (AG11395) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS). A different strain of Werner syndrome derived fibroblasts (AG05229) was grown in DMEM supplemented with 15% FCS. Sf9 insect cells were cultured in TC100 medium, supplemented with 10% FCS, 2 mM L-glutamine and 100 U/ml penicillin and streptomycin.

AG11395 is a Werner syndrome patient-derived fibroblast cell line immortalized by SV 40 transformation. AG05229 represent primary skin fibroblasts from a Werner Syndrome patient. Both fibroblast cell strains were obtained from Coriell Cell Repositories (Camden, US).

For growth factor stimulation cells were synchronized in DMEM without serum for 3 days. Cells were then stimulated with 10% FCS or single growth factors like VEGF (10 ng/ml), EGF (100 ng/ml), FGF-b (50 ng/ml), PDGF-AB (100 ng/ml) and IGF-I (100 ng/ml). All growth factors were obtained from Cell Concepts GmbH (Umkirch, Germany).

2.2. Nuclear extracts

Native nuclear extracts from an immortalized fibroblast cell line derived from a WS patient (AG11395) and HeLa cells (technical control) were harvested according to an earlier published protocol (Dignam et al., 1983).

2.3. Protein expression

Baculovirus was amplified in Sf9 cells for 8–12 days after transfection. The supernatant was harvested when the cytopathic effect reached 50% and used to transfect Sf9 for protein expression in 1:100 dilution. 72 h after transfection cells were lysed by sonification and the lysate was incubated with anti-Flag conjugated Agarose beads (Sigma) overnight. After sequential washing with buffer AM (20 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol) containing 1 M and 400 mM KCl and 0.5% NP40 (Fluka), flag-WRN was eluted 2×2 h in buffer AM containing 300 mM KCl and 0.1% NP40 and 0.25 mg/ml flag-peptide (Sigma). Due to low volumes, the eluate was not dialysed, but aliquoted and snap frozen in liquid nitrogen.

2.4. Northern blot analysis

Total RNA was harvested 12 h after stimulation with peqGOLD Trifast (Peqlab, Erlangen, Germany) and prepared according to the manufacturer's instructions. RNA was separated on 0.9% MOPS-agarose gels, photographed and transferred onto nylon Hybond N^+ membranes (Amersham Bioscience, Buckinghamshire, UK). After UV-crosslinking the 47S rRNA was visualized by hybridization to ^{32}P labeled anti-sense RNA comprising the 5′-terminal rDNA sequence from −170 to +155. Hybridisation was performed in 50% formamide, 5× SSC, 50 mM sodium phosphate pH 6.5, 8× Denhardt's, 0.5 mg/ml yeast tRNA, 0.1% SDS at 68 °C overnight. Filters were washed in 0.2× SSC, 0.1% SDS for 10 min, 2×. Ethidiumbromide stained 28S rRNA was used as loading control.

2.5. In vitro transcription

The $pHrP_2$ template containing the human rDNA sequence from −411 to +375 was linearized with EcoRI. The pUVAI vector contains the RNA polymerase III gene sequence from an 231 bp sized SalI/AluI VA1-fragment of the pVA plasmid. The 267 bp sized fragment of pUC18 plasmid was isolated by NdeI and EcoRI restriction and served as a template in the elongation assay. Transcription was performed in 25 μl of volume (20 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.2 mM EDTA, 100 mM KCl, 10% (v/v) glycerol, 0.66 mM each (ATP, GTP and CTP), 12.5 μM UTP and 1 μCi of [α-^{32}P] UTP (800 Ci/mol) using standard conditions. After incubation for 1 h at 30 °C proteins were digested by addition of 25 μl Proteinase K-mix (2% SDS, 20 mM EDTA, 0.5 mg/ml tRNA, 0.4 mg/ml Proteinase K) for 30 min at 40 °C. Precipitation of the synthesized RNA was performed with 200 μl of ice-cold 100% ethanol and 25 μl of 7.5 M ammonium acetate. The pelleted RNA was washed with 50 μl of 70% ethanol and resolved on a native 4% TBE/polyacrylamide gel.

The end-to-end transcription of the pUC18 NdeI/EcoRI fragment was performed in the presence of α-amanitin (100 μg/ml) (Roche, Mannheim, Germany).

2.6. Preparation of immobilized template

The biotinylated template was synthesized by PCR using Advantage 2 Polymerase (Clontech Laboratories, Mountain View, CA, USA). The plasmid $pHrP_2$ containing the human rDNA sequence was amplified with sense primers biotinylated at the 5′-end (Panov et al, 2001). One microgram of the template was immobilized on 20 μl (10 mg/ml) of streptavidin-coated paramagnetic beads (M280 Dynabeads, Dynal, Oslo, Norway) according to the manufacturer's instruction.

2.7. Isolation of RNA polymerase I initiation complexes

One microliter of immobilized template-DNA was preincubated with 120 μg of nuclear extracts for 20 min at 30 °C in 80 mM KCl. Subsequently, the beads were washed with buffer containing 20 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.2 mM EDTA, 50 mM KCl, 10% (v/v) glycerol, and transcription was initiated by addition of 0.66 mM each ATP, CTP and GTP, 12.5 μM UTP and 1 μCi of [α-^{32}P]UTP (800 Ci/mmol). Transcription reaction was stopped after 60 min at 30 °C. RNA produced was precipitated and separated on 4% polyacrylamide gels.

2.8. Immunoprecipitation

One milligram of nuclear extract was incubated 2 h with 4 μg of anti-WRN or anti-IgG antibody (both Santa Cruz) with overhead rotation at 4 °C. Immunocomplexes were isolated with 20 μl Protein A–agarose (Roche, Mannheim, Germany) by incubation for 1 h. The beads were sequential washed (5×) with 200 μl of buffer AM containing 100 mM KCl. Proteins were eluted with Laemli buffer and subject to SDS–PAGE.

2.9. Chromatin immunoprecipitation (ChIP)

ChIP assays were done as described (Weinmann et al., 2001). In brief, HEK 293 cells were incubated with 1% formaldehyde for 10 min at room temperature. Crosslinking was stopped by addition of glycine to a final concentration of 0.125 M. The pH of the glycine