Journal of Cellular Biochemistry 85:689–702 (2002)

Evidence That Protein Disulfide Isomerase (PDI) Is Involved in DNA–Nuclear Matrix Anchoring

Robert P. VanderWaal,[1] Douglas R. Spitz,[2] Cara L. Griffith,[1] Ryuji Higashikubo,[1] and Joseph L. Roti Roti[1]*

[1]Washington University School of Medicine, Division of Radiation and Cancer Biology, Department of Radiation Oncology, St. Louis, Missouri
[2]Free Radical and Radiation Biology Program, Department of Radiation Oncology, University of Iowa, Iowa City, Iowa

Abstract DNA–nuclear matrix (NM) anchoring plays a critical role in the organization of DNA within the nucleus and in functional access to DNA for transcription, replication, and DNA repair. The cellular response to oxidative stress involves both gene expression and DNA repair. We, therefore, determined if changes in the oxidative–reductive environment can affect DNA–NM anchoring. The present study used two approaches to study the effect of the reducing agent DTT on DNA–NM anchoring. First, the relative stringency of the DNA–NM attachment was determined by measuring the ability of NM attached DNA loops to undergo supercoiling changes. Second, the effects of DTT on the association of nuclear proteins with DNA were determined by cisplatin crosslinking. When nucleoids (nuclear matrices with attached DNA loops) were prepared from HeLa cells with 1 mM dithiothreitol (DTT), supercoiled DNA loops unwound more efficiently compared with control in the presence of increasing propidium iodide (PI) concentrations. In addition, the rewinding of DNA supercoils in nucleoids treated with DTT was inhibited. Both effects on DNA supercoiling ability were reversed by diamide suggesting that they are dependent on the oxidation state of the protein thiols. When DTT treated nucleoids were isolated from γ-irradiated cells, the inhibition of DNA supercoil rewinding was equal to the sum of the inhibition due to DTT and γ-rays alone. Nucleoids isolated from heat-shocked cells with DTT, showed no inhibition of DNA rewinding, except a small inhibition at high PI concentrations. Nuclear DNA in DTT-treated nuclei was digested faster by DNase I than in untreated nuclei. These results suggest that DTT is altering DNA–NM anchoring by affecting the protein component(s) of the anchoring complex. Extracting NM with increasing concentrations of DTT did not solubilize any protein to a significant extent until measurable NM disintegration occurred. Therefore, we determined if 1 mM DTT affected the ability of 1 mM cisplatin to crosslink proteins to DNA. Isolated nuclei were treated with 1 mM DTT for 30 min or left untreated prior to crosslinking with 1 mM cisplatin for 2 h at 4°C. The ability of capsulation to crosslink DNA to proteins per se, did not appear to be affected by 1 mM DTT because relative amounts of at least four proteins, 69, 60, 40, and 35 kDa, were crosslinked to DNA to the same extent in DTT-treated and untreated nuclei. However, protein disulfide isomerase (PDI) crosslinked to DNA in untreated nuclei, but did not crosslink DNA in nuclei that were treated with 1 mM DTT; 1 mM DTT did not affect the intranuclear localization of PDI. Thus, DTT appears to alter the conformation of PDI, as suggested by the DTT-induced change in DNA association, but not its NM association. These results also imply that DNA–NM anchoring involves the redox state of protein sulfhydryl groups. J. Cell. Biochem. 85: 689–702, 2002.

Key words: nuclear matrix; DNA anchoring; HeLa cells; Halo assay; PDI; redox potential

Abbreviations used: DTT, dithiothreitol; MAR, matrix attachment regions; NM, nuclear matrix; PDI, protein disulfide isomerase; PI, propidium iodide; RPA, replication protein A; SAR, scaffold attachment regions.

Grant sponsor: National Cancer Institute; Grant number: 5 P01 CA75556-05; Grant sponsor: NIH (to DRS); Grant number: RO1 HL51469.

Joseph L. Roti Roti is working as Director.

*Correspondence to: Joseph L. Roti Roti, Ph.D., Division of Radiation and Cancer Biology, 4511 Forest Park Blvd., Suite 411, St. Louis, MO 63108.

Received 22 January 2002; Accepted 6 February 2002

DOI 10.1002/jcb.10169

Genomic DNA in eukaryotic cells is compacted approximately 40,000-fold in order to fit within the nucleus [Reeves, 1984; Van Holde, 1988; Pienta et al., 1991]. In addition to the extensive compaction, it is necessary that nuclear DNA be accessible for replication, transcription, and repair. Recent studies have demonstrated that replication and transcription are separated spatially and temporally as cells progress through the cell cycle [Berezney et al., 1995; Wei et al., 1998]. While certain DNA

repair pathways are coupled to transcription, other repair pathways occur independently of transcription and replication [Jackson and Cook, 1995; Tornaletti and Hanawalt, 1999]. Thus, the disparate requirements for compaction and accessibility for multiple functions, which are temporally and spatially separate, mandate a specific and dynamic organization of the eukaryotic genome.

In eukaryotic cells, compaction and organization of nuclear DNA is mediated, in part, by an underlying structure known as the nuclear matrix (NM) [Berezney et al., 1995]. The NM is operationally defined as a sub-nuclear structure, which is insoluble following several different extraction procedures [Nickerson et al., 1995]. The NM is believed to impose a higher order organization to nuclear DNA through periodic attachments that organize it into loop domains ranging from 5 to 200 kb in length [Razin et al., 1995]. The NM is known to provide an underlying structure, which is associated with DNA replication complexes, transcription complexes, and some repair complexes [Jackson and Cook, 1995]. It has been suggested that these associations contribute to the control of DNA replication, transcription, and repair [Jackson and Cook, 1995 and references therein]. If the association between functional NM proteins and DNA were important to biological processes, then one would expect that DNA–NM anchoring would be specific and variable so that regulation of NM-dependent processes would be possible.

In vitro DNA binding assays have been used to identify DNA sequences that are believed to function as matrix–DNA attachment points in vivo [Mirkovitch et al., 1984; Boulikas, 1995]. These attachment sequences, known as matrix attachment regions (MARs) or scaffold attachment regions (SARs), are AT rich [Comings and Wallack, 1978; Berrios et al., 1985; Gasser and Laemmli, 1986]. MARs often contain topoisomerase II consensus sequences [Cockerill and Garrard, 1986; Gasser and Laemmli, 1986] and are prone to unwinding [Bode et al., 1992]. The specificity of the MAR sequences varies with the type of attachment and the specific gene associated with the attachment [Boulikas, 1995]. Thus, this sequence specificity within MARs implies that there is specificity for DNA–NM associations in the DNA component. However, significantly less is known about the protein components of these associations than for the DNA components. Several NM proteins appear to be involved in DNA binding in a non-stringent manner [Boulikas, 1995]. However, a number of transcription factors are enriched or associated with the NM [van Wijnen et al., 1993]. Thus, a combination of proteins appears to contribute to the specificity of MAR binding for specific genes.

The state of knowledge regarding the regulation of MAR binding is even more limited than knowledge of its specificity. It has been suggested that MAR binding is modulated by protein phosphorylation [Boulikas, 1995], which is reasonable in view of the number of signal transduction pathways that work by modulating phosphorylation status. However, there is some evidence that the oxidation/reduction status (redox) state of sulfhydryl groups can also modulate signal transduction [Gupta et al., 2001]. Recent studies have shown that an isoform of protein disulfide isomerase (PDI), ERp60, which has two thioredoxin-like sites, is associated with NM in chicken liver cells [Ferraro et al., 1999]. Further, it was shown that PDI bound poly(dA), poly(dT) in vitro and that this binding was inhibited by the reducing agent, dithiothreitol (DTT) [Ferraro et al., 1999]. Thus, it appears to be possible that redox sensitive factors could be involved in MAR binding. To detect redox sensitive interactions between the NM and DNA, changes in the rewindability of nuclear DNA loops and in the accessibility of nuclear DNA to digestion by exogenous DNase I were measured in situ in the presence or absence of DTT. The solubility of PDI and related proteins in nuclear and NM preparations was measured in the presence and absence of DTT to determine if any changes in DNA–NM anchoring were associated with changes in the extractability of NM proteins. Also, the effect of DTT on the nuclear localization of PDI was determined. In addition, the effects of DTT on the association of PDI (and other nuclear proteins) with DNA was determined using cisplatin crosslinking. Cisplatin can crosslink DNA to proteins, which are within 4 Å of DNA at the time of the crosslinking reaction. Thus, if DTT altered the association of PDI with displaced DNA by displacing it more than 4 Å from PDI, then the cisplatin induced DNA-PDI crosslinking would be reduced relative to any proteins that were not displaced in this manner.

MATERIALS AND METHODS

Cell Culture

HeLa S3 cells (ATCC, Rockville, MD) were maintained as exponentially growing monolayer cultures in Hams F-10 medium (Sigma, St. Louis, MO), supplemented with 10% calf serum and 10 U/ml penicillin G and 10 μg/ml streptomycin. All supplements were obtained from Life Technologies (Grand Island, NY). Cultures were maintained in a humidified, water-jacketed incubator at 37°C and 5% CO_2.

Isotopic Labeling of Proteins and Extraction of HeLa Nuclei With 1 mM DTT

In order to follow the elution of proteins from the nuclei treated with 1 mM DTT, cells were labeled for 24 h with 21 μCi/ml ^{35}S-methionine, a level which causes no cell cycle delays [Laszlo et al., 1992]. Nuclei were prepared by washing HeLa cells with CSK buffer (10 mM PIPES, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 4 mM vanadyl riboside complex, 1.2 mM PMSF) for 10 min on ice, then collection of the resultant nuclei by centrifugation. The nuclei were then re-suspended in CSK buffer, either with 1 mM DTT or without (control) and incubated on ice. After 30 min, the nuclei were collected by centrifugation and prepared for SDS–PAGE. The supernatant was dialyzed overnight against MilliQ water plus 1 mM PMSF, lyophilized, and prepared for SDS–PAGE. Aliquots were taken for scintillation counting at each step. Alternatively, nuclear matrices were obtained from isolated nuclei by DNase digestion. The DTT treatments and protein recovery were done as described above.

NM Preparation and Extraction With DTT

Nuclear matrices were isolated following the methods of Nickerson et al. [1990]. Briefly, HeLa cells were washed with PBS at 4°C, then extracted with CSK buffer plus protease inhibitors, 2 mM AEBSF, 1.6 μM aprotinin, 42 μM leupeptin, 72 μM bestatin, 3 μM pepstatin A, 28 μM E-64, and 1.2 mM PMSF (Sigma) for 3 min on ice at a cell density of 4×10^6 cells/ml, then centrifuged at 940*g* for 5 min. The pellet was resuspended in the same volume of digestion buffer (same as CSK buffer, except for 50 mM NaCl, plus protease inhibitors) and DNase I was added to a final concentration of 150 μg/ml, and incubated at room temperature. After 30 min, a 1 M stock NH_4SO_4 solution was added to yield a 250 mM final concentration, incubated 10 min at room temperature, and the nuclear matrices were collected by centrifugation. The NM pellet was resuspended in digestion buffer (plus protease inhibitors), and DTT was added to yield final concentrations of 1, 5, 10, 50 mM. The nuclear matrices were incubated for another 30 min on ice, then collected by centrifugation. Nuclear matrices (10^6) were resuspended in 25 μl in SDS–PAGE sample buffer. The supernatant was dialyzed against water overnight, lypholized, and the resultant residue was dissolved in 25 μl SDS sample buffer. SDS–PAGE and immunoblotting were performed as described below.

SDS–PAGE and Immunoblotting

To resolve proteins by molecular weight, the preparations described above were boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 50 mM DTT, 2% SDS). SDS–PAGE (7%) was performed by the method of Laemmli [1970]. The resultant gels were stained with Bio-Safe Coomassie stain (Bio-Rad Labs, Hercules, CA), as per the manufacturer's instructions. The gels were dried onto blotting paper using a Bio-Rad gel dryer (Bio-Rad). The gels were exposed to phosphore screen for 48 h, before being scanned with a Molecular Dynamics Storm Imager (Molecular Dynamics, Sunnyvale, CA).

Colorimetric immunodetection of NM proteins immobilized on PVDF membranes was performed as described previously [Towbin et al., 1979]. Briefly, NM proteins separated by SDS–PAGE (see above) were electrophoretically transferred to PVDF filters (Millipore Corp., Bedford, MA) and probed with the anti-PDI (Affinity Bioreagents, Golden, CO) antibody described above. Immune complexes were detected with alkaline phosphatase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) using BCIP/NBT (Sigma) as a reaction substrate.

Assays for DNA Supercoiling

Irradiated samples were obtained by brief trypsinization (described above) of monolayer cultures, and were adjusted to 2.3×10^5 cells/ml with ice-cold PBS. They were then irradiated in ice in a Shepherd Mark I ^{137}Cs irradiator (JL Shepherd Co., San Fernando, CA) at a dose rate of .33 Gy/min. Cells for heating were

trypsinized, collected by centrifugation at 1,000*g* for 5 min, then resuspended in prewarmed medium and heated in a temperature controlled waterbath with an accuracy of ±0.1°C. After heating, cells were centrifuged again and adjusted to 2.3×10^5 cells/ml with ice-cold PBS. Following treatment as indicated, the cell suspension was placed in each well of a four-well tissue culture chamber slide (Nalge Nunc International, Naperville, IL). An equal volume of each dye/lysis buffer solution (1.95 M NaCl, 10 mM EDTA, 20 mM Tris, 0.5% Tx-100, pH 8) containing 2 × the indicated concentration of propidium iodide (PI) (Sigma) with or without 1 mM DTT is then added and the cells are allowed to lyse for 5 min at room temperature before visualization using an inverted microscope (Olympus, Tokyo, Japan) equipped with epifluorescence optics with 510–560 nm excitation. Fields are selected for image capture based on separation of nucleoids and uniformity of focus, using care not to expose the samples to excess light exposure, since the heat generated by such exposure may cause sample damage. Using a charge-coupled device (CCD) camera (Optronics, Goleta, CA) and a Flashpoint frame grabber (BusLogic, Inc., Santa Clara, CA), the images are captured by PC (Compact, Columbus, OH) using Optimus software (Media Cybernetics, Silver Springs, MD). Digitized images are stored for each PI concentration and particle diameters are subsequently obtained using an "AutoHalo" macroprogram (Computer Imaging Applications, Madison, WI). This program calculates the center of mass of the roughly circular image and then calculates the mean distance from the center of the mass to the image edge. The mean diameter is twice this mean distance.

DNase I Digestion Assay

The DNase I digestion assay was conducted exactly as previously described [Roti Roti et al., 1985]. In brief, HeLa nuclei were prepared by two washes of a 1% Triton X-100 buffer (20 mM EDTA, pH 7.2, 80 mM NaCl, 1% TX-100). The nuclei were then incubated with or without 1 mM DTT in digestion buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM PMSF) for 30 min on ice, followed by a wash with the digestion buffer. The nuclei were then resuspended in the digestion buffer at a density of 2×10^6 nuclei/ml. DNase I (Worthington, Newark, NJ) was added to a final concentration of 225 K units per ml and incubated at 20°C. At 10 min intervals, 0.9 ml aliquots were removed and added to 0.1 ml staining solution (600 μg PI per ml and 2 mM EDTA). Samples were immediately analyzed on a FACS with 588 nm excitation beam.

DNA–Protein Crosslinking

Cisplatin cross-linking of proteins to DNA was performed according to a modification of the method Spencer et al. [1998]. In brief, whole cells or nuclei were washed three times with modified Hank's Balanced Salts (Na acetate is substituted for NaCl at the same concentration), then incubated for 2 h with 1 mM cisplatin (Sigma) in modified Hank's Balanced Salts at 37°C. Nuclei were prepared for crosslinking experiments by incubating cells with 0.5% Triton X-100 in modified Hank's Balanced Salts for 5 min. In some experiments, nuclei were isolated with all buffers supplemented with 1 mM DTT. Cells or nuclei were then collected by centrifugation, lysed with (5 M urea, 2 M guanidine-HCl, 2 M NaCl, 200 mM potassium phosphate, pH 7.5). Hydroxylapatite (Bio-Rad) (2.5 mg per 10^6 cells) were then added and incubated for 1 h. Following incubation, the hydroxylapatite was collected by centrifugation and washed 3 × with lysis buffer. The DNA–protein crosslinks were then reversed with an overnight incubation with thiourea buffer (1 M thiourea, 2 M guanidine-HCl, 2 M NaCl, 200 mM potassium phosphate, pH 7.5) at 4°C. The hydroxylapatite was then removed by centrifugation. The resulting supernatant was dialyzed against MilliQ water, and then lyophilized to a dry powder. The powder was solubilized in SDS sample buffer. Resolution of the protein by SDS–PAGE and analysis by immunoblot are described above.

In Situ Immunofluorescence Staining

HeLa cells were grown on glass coverslips under culture conditions described above. Nuclei and NMs were prepared attached to these coverslips using methods described in detail for NM preparation. In brief, cells growing on the coverslips were first washed with PBS, and then incubated for 30 min with CSK buffer to prepare nuclei. NMs were prepared by digesting the nuclei with DNase I followed by the NH_4SO_4 extraction (see above). One millimolar DTT was added to the CSK buffer used to extract cells on some coverslips. The resultant

nuclei or NMs were then fixed with Histochoice Tissue Fixative (Ameresco, Solon, Ohio) supplemented with 20% ethanol for 15 min. Following another PBS wash, the nuclei or NMs were blocked with 5% BSA and 10% goat serum in PBS for 15 min, followed by 1 h incubation with anti-PDI antibody diluted 1:100 in blocking buffer. Following PBS washes, the coverslips were incubated 30 min with Alexa 546 conjugated goat anti-mouse antibodies (Molecular Probes, Eugene, OR) diluted 1:500 in blocking buffer, and then washed again in PBS. Images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) fitted on an Olympus fluorescent microscope using Optimus 6.2 software (Media Cybernetics).

RESULTS

To determine if DTT alters the stringency of DNA–NM attachment, we used two methods. First, we measured the supercoiling ability of DNA loops that are attached to the NM. We assayed for changes in both unwindability and rewindability of DNA loops because a change in stringency of DNA–NM anchoring would affect either or both. In addition, we would be able to detect the actual loss of DNA–NM anchor points (as opposed to a change in the stringency of the anchor point) as an increase in DNA loop size. Such changes are seen in metal depleted nuclei [Lebkowski and Laemmli, 1982]. Second, we measured the digestibility of nuclear DNA by DNase I. If the stringency of the DNA–NM DNA anchor point is increased, then the DNase I digestibility of nuclear DNA is decreased [Roti Roti et al., 1985]. Conversely, if the stringency is relaxed, then the digestibility will increase.

To study the supercoiling ability of nuclear DNA in situ, we used the method of Vogelstein et al. [1980] as modified by us [Roti Roti and Wright, 1987]. In this assay, cells are lysed in 1 M NaCl with a low concentration of a non-ionic detergent, Triton X-100, in the presence of increasing concentrations (0.5–50 μg/ml) of the fluorescent intercalating dye, PI. Following cell lysis and histone DNA dissociation (due to the detergent and salt, respectively), the DNA loops that remain attached to the NM undergo an unwinding response as a function of increasing intercalator concentration. Since the intercalating agent is fluorescent, we can detect the extended DNA loops using fluorescence microscopy and image analysis. Typical images (Fig. 1) show the DNA loop extension, seen as a fluorescent "halo" about a central core, which is the NM. The extent of DNA loop unwinding can be quantified by measuring the diameter of the resulting fluorescent image [Roti Roti and Wright, 1987]. Because the DNA loops remain attached to the NM; the DNA loops will rewind as the PI concentration increases above 7.5 μg/ml, as seen by the subsequent reduction in image diameter (Fig. 1). This method has been used to investigate agents that alter the ability of nuclear DNA [Roti Roti and Wright, 1987; Malyapa et al., 1994] to undergo supercoiling changes by damaging the DNA [Malyapa et al., 1994] or by altering the anchoring [Kampinga et al., 1989a,b].

To determine if DNA anchoring could be altered in a reducing environment, we added 1 mM DTT to the lysis buffer. When nucleoids were prepared in the presence of 1 mM DTT, two effects on the DNA unwinding/rewinding curve were observed (Fig. 2). First, the nucleoid diameters measured at 2 and 5 μg/ml PI were larger than those measured in the absence of DTT, implying that DNA-supercoil unwinding is enhanced by DTT. Second, nucleoid

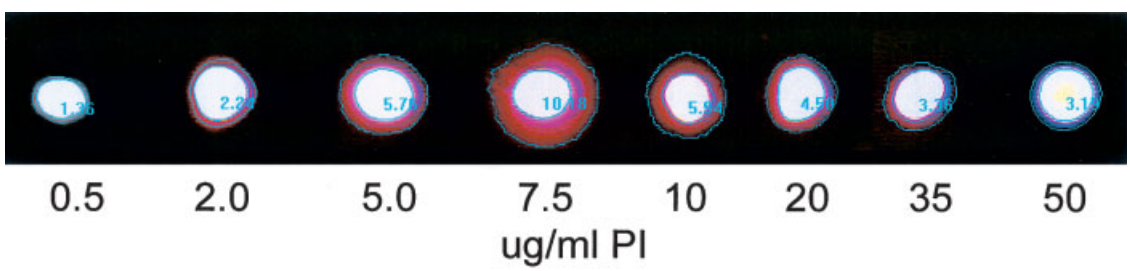

Fig. 1. Typical images of nucleoids at various PI concentrations. The unwinding & rewinding of DNA supercoils in nucleoids can be seen as an increase in nucleoid diameter as a function of increasing PI concentration up to 7.5 μg/ml (the relaxation point) followed by a decrease in nucleoid diameter as PI concentration continues to increase.

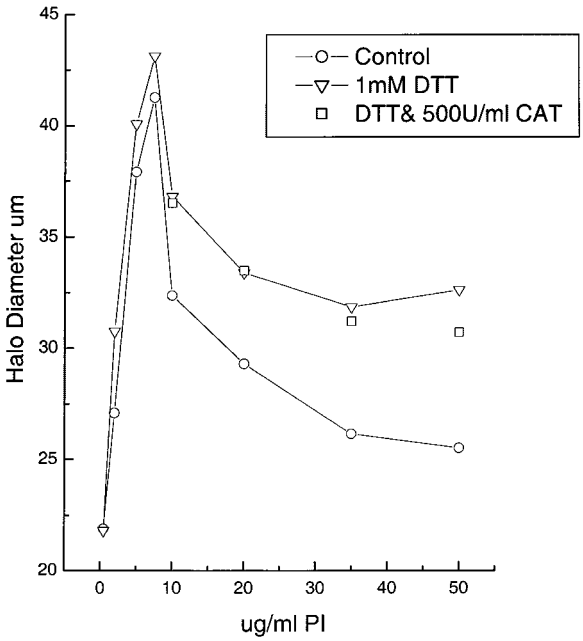

Fig. 2. DNA loop extension, measured as a fluorescent halo of PI-stained DNA, as a function of PI concentration. Nucleoids were prepared from HeLa cells in the presence of increasing PI concentration (abscissa) as described in the Materials and Methods section. One millimolar DTT was added to half the samples (down triangles) during lysis, while the remaining half was lysed normally as a control (circles). In two experiments, 500 U/ml catalase was added along with 1 mM DTT (squares) and the results averaged. After 20 min, the average diameters of the resulting nucleoids were measured by fluorescent image analysis. For each experiment, between 30 and 50 nucleoids were analyzed and a population-mean diameter was obtained. The plotted values (ordinate) represent the average of the population means from five repeated experiments. Standard errors of the mean and 95% confidence limits were calculated for this data, but omitted for clarity. The differences between the curves were tested on a "point by point" basis using the Student's *t*-test (see text for details).

diameters measured at 10, 20, 35, and 50 μg/ml PI were larger than those measured in the absence of DTT, implying that DTT inhibited DNA-supercoil rewinding. This experiment was repeated five times and the differences at each PI concentration tested for statistical significance using the one-tailed, paired Student's *t*-test. Nucleoid diameters measured at all PI concentrations except at 0.5 and 7.5 μg/ml were significantly larger in the presence of DTT than in its absence. The enhanced unwinding and inhibited rewinding of the DNA loops shows that 1 mM DTT reduced the stringency of DNA loop-NM anchoring complexes. Further, the lack of an effect on DNA loop size suggests that the anchoring complexes are altered, but the DNA is still attached.

The effects of 1 mM DTT on DNA supercoil unwinding and rewinding were completely reversed when an oxidizing agent, diamide (10 mM), was added to the lysis buffer (Fig. 3), suggesting that the above effects are due to the reducing ability of DTT. It is known that DTT in low concentrations can auto-oxidize and produce H_2O_2 that can damage DNA [Held et al., 1996]. Since this effect is prevented by catalase, a specific scavenger of H_2O_2, we added 500 U/ml of catalase to the nucleoid preparation with

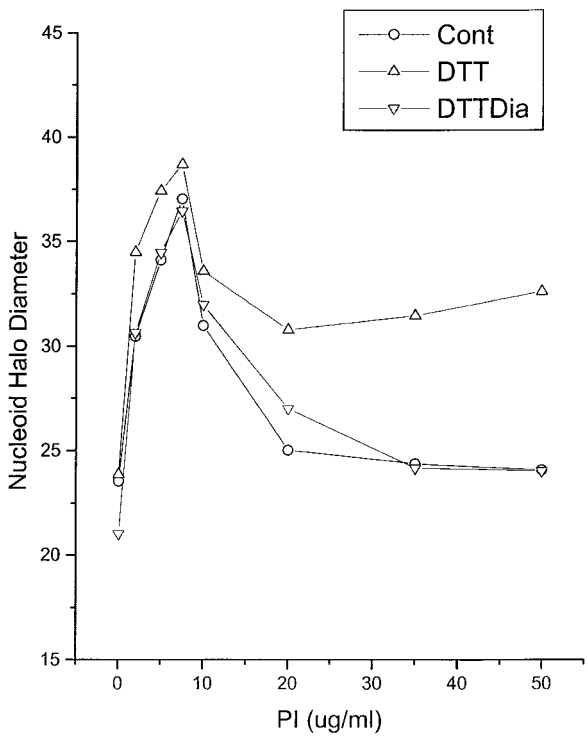

Fig. 3. DNA loop extension, measured as a fluorescent halo of PI stained DNA, as a function of PI concentration: Nucleoids were prepared from HeLa cells in the presence of increasing PI concentration (abscissa) as described in the Materials and Methods section. One millimolar DTT was added to $\frac{1}{4}$ of the samples (triangles) during lysis, $\frac{1}{4}$ was lysed normally as a control (circles), $\frac{1}{4}$ was lysed in the presence of 1 mM DTT plus 10 mM diamide (down triangles), and $\frac{1}{4}$ was lysed in the presence of 10 mM diamide (not shown). After 20 min, the average diameters of the resulting nucleoids were measured by fluorescent image analysis. For each experiment, between 30 and 50 nucleoids were analyzed and a population-mean diameter was obtained. The plotted values (ordinate) represent the average of the population means from three repeated experiments. Standard errors of the mean and 95% confidence limits were calculated for this data, but omitted for clarity. The differences between the curves were tested on a "point by point" basis using the Student's *t*-test (see text for details). Since the data from the samples treated with diamide alone was not different from control or diamide plus DTT, then the data was not plotted for clarity.

DTT. The added catalase retained greater than 90% of its activity for more than 30 min in the lysis buffer (data not shown). The presence of catalase did not alter the DTT effect on DNA supercoil rewinding, showing that the DTT effect is not due to oxidative DNA damage by H_2O_2 produced by DTT auto-oxidation (Fig. 2). Thus, the DTT-induced reduction in the stringency of the DNA–NM attachment appears to be due to an effect on the protein components of the anchoring complex.

Previous studies [Roti Roti and Wright, 1987; Malyapa et al., 1994, 1995, 1996], showed that DNA damage induced by ionizing radiation inhibited DNA-supercoil rewinding. We, therefore, determined if DTT and γ-irradiation would affect the rewinding of DNA supercoils in an additive manner. The unwinding and rewinding of DNA supercoils in nucleoids isolated from cells irradiated with 5 Gy of γ-rays in the presence or absence of DTT was measured as described above. Consistent with previous reports, the DNA loops in nucleoids from irradiated cells showed reduced rewindability, as evidenced by larger nucleoid diameters at PI concentrations above 10 μg/ml (Fig. 4). Nucleoid diameters measured at 10, 20, 35, and 50 μg/ml PI were larger in nucleoids from irradiated cells than those from control cells by the one-tailed, paired Student's *t*-test. At PI concentrations of 7.5 μg/ml and below there was no statistically significant difference in the diameters of nucleoids from irradiated and control cells. When 1 mM DTT was added to the lysis buffer of irradiated cells, nucleoid diameters at 10, 20, 35, and 50 μg/ml PI were significantly larger than those measured in the presence of DTT alone, implying that DTT- and radiation-induced DNA damage inhibited DNA-supercoil rewinding in an additive manner. To illustrate the additive nature of these two effects, the closed squares (Fig. 4) show a plot of the sum of nucleoid diameters for 5 Gy and DTT alone. These values at 20, 35, and 50 μg/ml PI add almost exactly to the observed values following the combined treatment. In contrast, nucleoid diameters measured at 2 and 5 μg/ml PI were the same following the combined treatment as those measured in the presence of DTT alone, implying that the enhancement of DNA-supercoil unwinding by DTT is not affected by DNA damage.

The additive nature of irradiation with 5 Gy and 1 mM DTT suggested that two separate components of DNA–NM anchoring were affected, i.e., the DNA and protein components, respectively. If 1 mM DTT caused a loosening of the protein component of the DNA–NM anchor, then an agent known to tighten the anchor via protein binding should inhibit or mask the DTT effect. Previous work has shown that heat shock causes an enhanced binding of proteins to the NM [Roti Roti and Laszlo, 1988; Vanderwaal et al., 2001], and thereby, reduces DNA loop size and alters the ability of nuclear DNA to undergo supercoiling changes [Kampinga et al., 1989a,b].

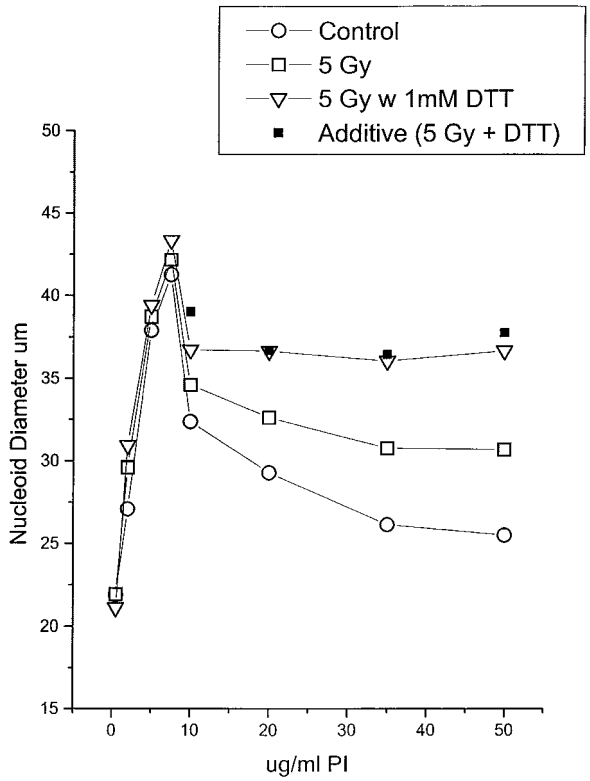

Fig. 4. DNA loop extension measured in nucleoids from irradiated cells. Nucleoids were prepared from HeLa cells irradiated with 5 Gy as described in the Materials and Methods section. One millimolar DTT was added to half the samples (down triangles) during lysis, while the remaining half was lysed normally to show the effects irradiation (open squares) on DNA loop unwinding and rewinding. The sum of the nucleoid diameters for 5 Gy and DTT are plotted as closed squares. Nucleoids from control cells are included for comparison (circles). The average diameters of between 30 and 50 nucleoids were measured by fluorescent image analysis and the population-mean diameter was obtained. The plotted values (ordinate) represent the average of the population means from four repeated experiments are plotted vs. PI concentration (abscissa). Standard errors of the mean and 95% confidence limits were calculated for this data, but omitted for clarity. The differences between the curves were tested on a ''point by point'' basis using the Student's *t*-test (see text for details).

We, therefore, heated cells at 45°C for 30 min prior to preparing nucleoids in the presence or absence of 1 mM DTT. In cells that had been heat shocked prior to nucleoid isolation, the presence of 1 mM DTT did not significantly alter nucleoid diameter measured at PI concentrations of 20 μg/ml and below (Fig. 5). At 35 and 50 μg/ml PI, the nucleoid diameters in the presence of DTT were larger than in its absence. This result shows that for heat-shocked cells, the DTT effect is absent except at high PI concentrations, consistent with the idea that heat-induced protein binding reversed or masked the effect of DTT-induced loosening of protein DNA binding.

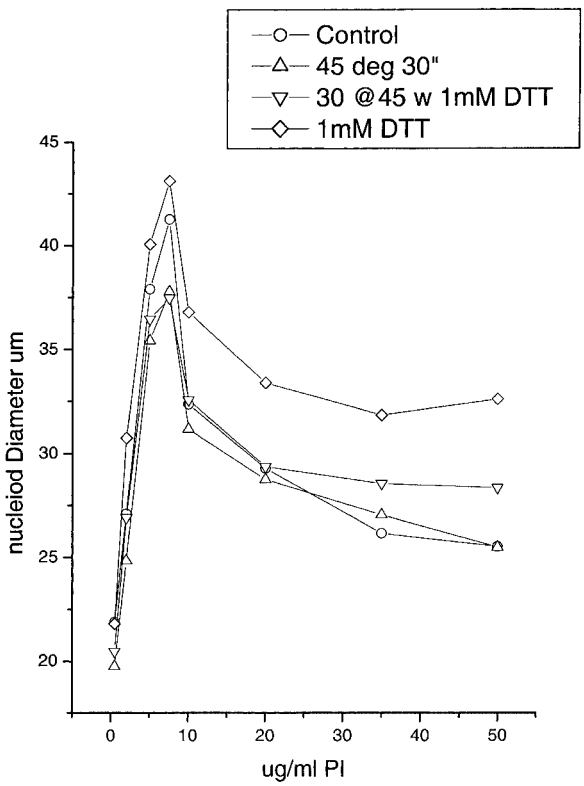

Fig. 5. DNA loop extension measured in nucleoids from heat shocked cells. Nucleoids were prepared from HeLa cells that had been heated at 45°C for 30 min as described in the Materials and Methods section. One millimolar DTT was added to half the samples (down triangles) during lysis, while the remaining half was lysed normally to show the effects of heat shock only (up triangles) on DNA loop unwinding and rewinding. Nucleoids from control cells are included for comparison (circles). The average diameters of between 30 and 50 nucleoids were measured by fluorescent image analysis and the population-mean diameter was obtained. The plotted values (ordinate) representing the average of the population means from four repeated experiments are plotted vs. PI concentration (abscissa). Standard errors of the mean and 95% confidence limits were calculated for this data, but omitted for clarity. The differences between the curves were tested on a ''point by point'' basis using the Student's *t*-test (see text for details).

While heat shock and γ-irradiation experiments support our interpretation of the effects of DTT on DNA loop rewinding, we sought an independent measure of the effects of DTT on DNA loop unwinding, which we attributed to a loosening of the protein DNA anchoring at the base of the loop. We, therefore, decided to test this possibility by measuring the DNase I sensitivity of nuclear DNA following DTT treatment. Sensitivity to DNase I digestion has been used as a probe for chromatin structure [Noll, 1974; Oosterhof et al., 1975]. If DTT is affecting a redox sensitive protein such that the anchoring of the DNA to the NM is loosened, then the relative protection of DNA at the base of the DNA loops from DNase I digestion would be reduced. To test this hypothesis, we measured DNase I digestion kinetics in control HeLa nuclei and in nuclei treated for 30 min with 1 mM DTT prior to digestion. DNA digestion was monitored flow cytometrically by staining aliquots of the reaction system (see Materials and Methods), each 10 min of digestion time. We found a significant increase in DNA digestion during incubation in DTT-treated nuclei, relative to control, as measured by a greater decrease in nuclear PI fluorescence as a function of digestion time (Fig. 6).

Since the results presented in Figures 2 and 6 suggest that DTT alters the protein components of the DNA–NM anchoring complex, a possible explanation for these results is that these components of the NM are solubilized in the presence of DTT during NM isolation. Supporting this idea is evidence that a significant fraction of NM proteins are held together with disulfide bonds [Stuurman et al., 1992]. Further, previous reports showed that PDI, a NM protein in chicken liver cells, bound DNA in vitro and that such binding was absent in the presence of DTT [Ferraro et al., 1999]. We, therefore, determined if PDI was associated with nuclei from HeLa cells, and if so, was its nuclear association altered in the presence of DTT. Nuclei were isolated from HeLa cells in the presence or absence of 1 mM DTT as described in Materials and Methods. Both the nuclear proteins and those solubilized in the cytoplasmic supernatant were resolved by SDS–PAGE, the proteins transferred to a PVDF membrane and probed with an antibody to PDI

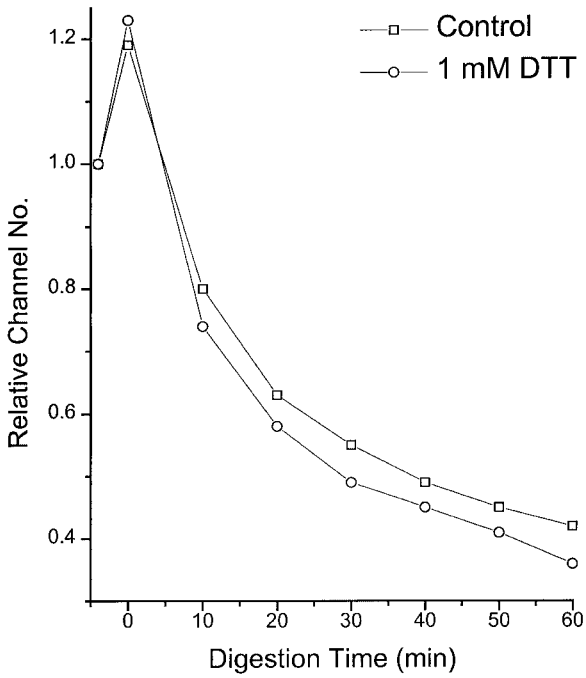

Fig. 6. The digestion of nuclear DNA by DNase I. Isolated nuclei were treated with 1 mM DTT for 30 min prior to DNase I digestion. The DNA remaining was measured flow cytometrically by staining the aliquots nuclei with 50 μg/ml PI at the indicated (abscissa) digestion time. The mean of the DNA content histogram was obtained from each digestion timepoint and plotted (ordinate) relative to the mean of the DNA content histogram obtained in the absence of DNase I plotted at − 5 min [see Roti Roti et al., 1985, for more details on this method]. The plotted data represents the mean of five experiments with error bars omitted for clarity.

(Fig. 7A). A protein cross-reacting with the PDI antibody was found to co-isolate with the nuclei (Fig. 7A) and the NM (Fig. 7A). This immunoreactive PDI was not significantly solubilized in the presence of 1 mM DTT in either the nuclei or NM preparations (Fig. 7A). This result shows that as described by Ferraro et al. [1999], PDI is a component of the NM. However, the association of PDI with the NM is not affected by 1 mM DTT, as measured by solubilization.

To determine if 1 mM DTT solubilized any nuclear proteins, we incubated HeLa cells overnight with ^{35}S methionine. Then, we isolated nuclei, and treated half with 1 mM DTT for 30 min, centrifuged to remove the intact nuclei and analyzed the solubilized proteins by SDS–PAGE, followed by Storm Image analysis. We found similar amounts of protein were solubilized in both control and DTT treated nuclei (Fig. 7B). Furthermore, we did not detect any protein species that was preferentially solubilized following DTT treatment by either silver staining (data not shown) or ^{35}S autoradiography (Fig. 7B). We further tested the ability of DTT to solubilize PDI from the NM. Isolated nuclear matrices were treated with increasing DTT concentrations for 30 min and the number of recovered nuclear matrices, the total protein and the extent of solubilization of PDI were measured (data not shown). For concentrations of up to 10 mM DTT, no PDI was solubilized. No solubilized protein was detectable after 1 and 5 mM DTT. Following 10 mM DTT, 10% of the total protein was solubilized, but 100% of the nuclear matrices was recovered. Following 50 mM DTT for 30 min, 33% of the protein was solubilized, 10% of the nuclear matrices was lost, but very little PDI was solubilized (Fig. 7C). Thus, the best hypothesis to explain data presented herein would be that a redox sensitive component, i.e., PDI of the DNA/nuclear NM anchor relaxes its hold on the DNA in a reducing environment without becoming dissociated from the NM.

To determine if 1 mM DTT altered the ability of 1 mM cisplatin to crosslink PDI (and other proteins) to DNA, HeLa cells and isolated HeLa nuclei were treated with 1 mM cisplatin for 2 h at 37°C in Hank's balanced salts. Following the crosslinking reaction, the cells and nuclear preparations were lysed in 5 M urea (see Materials and Methods). The DNA (plus crosslinked proteins) were trapped with hydroxyapatite, the non-crosslinked proteins were removed by washing the hydroxyl apatite three times. The crosslinked proteins were released from the DNA by 1 M thiourea. The crosslinked proteins were recovered, the thiourea dialyzed out and lyophilized. The recovered proteins were then resolved by 1D PAGE (Fig. 8A). The gels were stained with Coomassie blue and probed the PDI antibody. If no cisplatin is used, no proteins are recovered with this procedure (Fig. 8A shown for whole cells only). PDI is crosslinked to DNA in both nuclei and whole cells. When nuclei were treated with 1 mM DTT for 30 min prior and during cisplatin crosslinking, at least three proteins, 69, 60, 40, and 35 kDa (Fig. 8B) were recovered to the same extent as in nuclei that were not treated with DTT. This result suggests that 1 mM DTT did not significantly inhibit the ability of cisplatin to crosslink proteins to DNA per se. However, the ability of cisplatin to cross-link PDI to DNA was greatly reduced in nuclei treated with 1 mM DTT

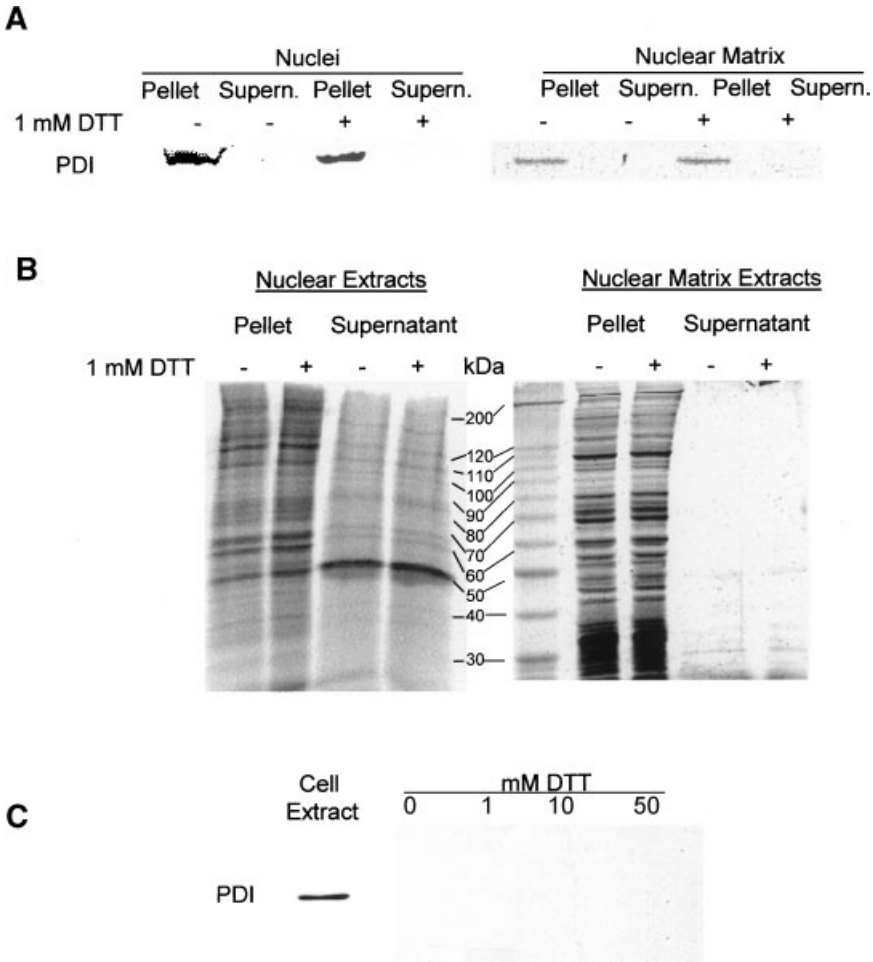

Fig. 7. **A**: Immunoblots of proteins binding the PDI antibody that remain with the NM (P) and those solubilized following DNase I digestion (S) in the presence and absence of 1 mM DTT as indicated. Nuclei were prepared and nuclear matrices isolated from HeLa cells as described in the Materials and Methods section. The supernatants were lyophilized and suspended in the same volume of sample buffer as were the NM proteins. The lane marked nuclei shows the results for nuclei treated with 1 mM DTT. Typical results are shown. **B**: Autoradiogram of ^{35}S-labeled protein in the nuclear pellets and supernatants in the presence and absence of DTT. Migration of molecular weight standards (in kDa) are as indicated. A total of 10^7 nuclei were extracted with or without 1 mM DTT, and the resulting supernatants were dialyzed and lypholized. The resulting material was solubilized in SDS sample buffer and loaded in each lane. Thus, the soluble material is the entire amount of protein recovered from the wash of 10^7 nuclei. The nuclear pellets were solubilized in SDS sample buffer and 10^4 nuclear equivalents were loaded into each lane. **C**: Recovery of PDI with nuclear matrices treated with increasing concentration of DTT for 30 min.

relative to untreated nuclei (Fig. 8), suggesting that DTT altered the association of PDI with DNA.

Given that 1 mM DTT did not solubilize detectable levels of PDI from either nuclei or nuclear matrices (Fig. 7), but did alter the ability of cisplatin to crosslink PDI to DNA (Fig. 8B), the possibility existed that DTT caused a rearrangement of the PDI within the nucleus. To test this possibility, we used in situ immunofluorescence staining. Nuclei were isolated from cells grown on coverslips and treated with 1 mM DTT or left untreated. The localization of PDI was then detected by immunofluorescence staining using anti-PDI as the primary and a fluorescently tagged secondary antibody. The localization of PDI in HeLa nuclei or nuclear matrices was not significantly altered by treatment with 1 mM DTT (Fig. 9). This result is consistent with the idea that the association of PDI with the nucleus or NM is not affected by DTT (as evidenced by the lack of a solubility change and the unchanged localization of PDI within the nucleus).

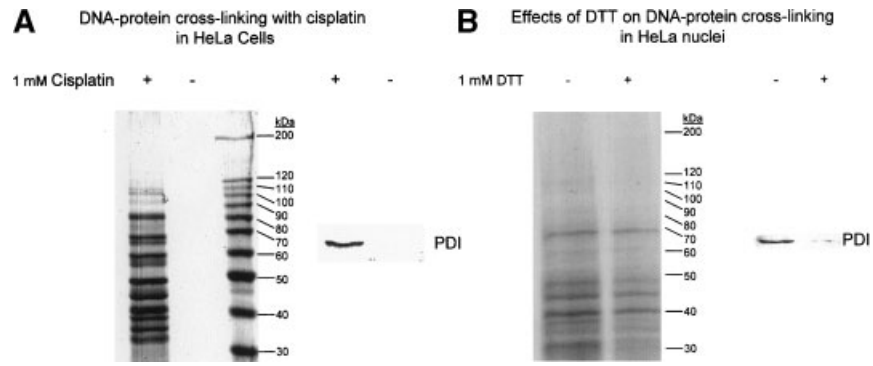

Fig. 8. Proteins crosslinked to DNA by 1 mM cisplatin in whole HeLa cells (**A**, left gel and Western blot) and in isolated HeLa nuclei (**B**, right gel and Western blot). HeLa cells or isolated HeLa nuclei were treated with 1 mM cisplatin for 2 h at 37°C. The proteins that were crosslinked to DNA were recovered as described in the text. At least 15 proteins (including PDI) were recovered if the cisplatin step was omitted. In HeLa nuclei, at least 10 proteins, including PDI were recovered with DNA. The majority of these were also recovered with DNA in nuclei treated with 1 mM DTT for 30 min prior to and during cisplatin crosslinking. However, PDI was not recovered with the DNA fraction in nuclei treated with 1 mM DTT. Typical results are shown.

DISCUSSION

The observation that PDI co-isolates with the NM and crosslinks to DNA (data not shown) is consistent with earlier reports [Ferraro et al., 1992, 1996] that an isoform of PDI, ERp60, is part of the internal NM. Herein, we show that the presence of 1 mM DTT, presumably through its affects on NM-associated proteins containing redox sensitive disulfide linkages, e.g., PDI, altered the anchoring of DNA loops such that the unwinding of supercoils was enhanced and the rewinding of them was inhibited. Two observations show that DTT affects DNA supercoiling by altering the protein component of the anchoring complex. First, the DTT effect was additive with that by ionizing radiation, which is known to inhibit DNA supercoil rewinding by damaging DNA [Roti Roti and Wright, 1987; Taylor et al., 1991; Malyapa et al., 1994, 1995, 1996]. Additive effects usually occur when two agents affect separate targets [Hall, 2000]. Further, the loosening of the DNA–NM anchoring by 1 mM DTT could be seen as greater sensitivity of nuclear DNA to DNase1 digestion. Second, heat shock, which induces enhanced protein binding to the NM [Laszlo et al., 1992; VanderWaal et al., 1996] masked the DTT induced inhibition of DNA supercoil rewinding except at high PI concentrations of 10 and 20 μg/ml. In contrast, heat shock only partially masked the ionizing radiation-induced inhibition of DNA-supercoil rewinding at PI concentrations of 10 and 20 μg/ml [Wynstra et al., 1990]. Finally, the effect of DTT was inhibited by the inclusion of a thiol oxidizing agent, diamide that presumably reversed the DTT mediated reduction of disulfide bonds. The integration of these results provides reasonable evidence that proteins containing disulfide bonds are involved in DNA–NM anchoring and can contribute to the supercoiling status of DNA loops.

The reduction in the stringency of DNA–NM anchoring in the presence of DTT, as measured in vitro by DNase I sensitivity and the fluorescent halo assay, as well as the reversal of the DTT effect on DNA supercoiling rewinding by diamide, suggests that redox sensitive structures are involved in the stability of the DNA–NM association structures. These results suggest that changes in redox status can affect the anchoring of DNA loops in vivo, and thereby, provide an additional mechanism for the regulation of gene expression. PDI appears to play a key role in this response because it can directly bind DNA fragments and has an affinity for the sequences found in MARs [Ferraro et al., 1999]. In fact, this binding was shown to be sensitive to redox state in vitro [Ferraro et al., 1999]. Further, 1 mM DTT changed the association of PDI with DNA such that PDI and DNA were not crosslinkable by cisplatin without solubilizing PDI from the nucleus or NM or altering its intranuclear localization. Since PDI is able to change the status of disulfide bonds, it appears that disulfide bonds play a critical role in DNA–NM anchoring. Whether these bonds are impor-

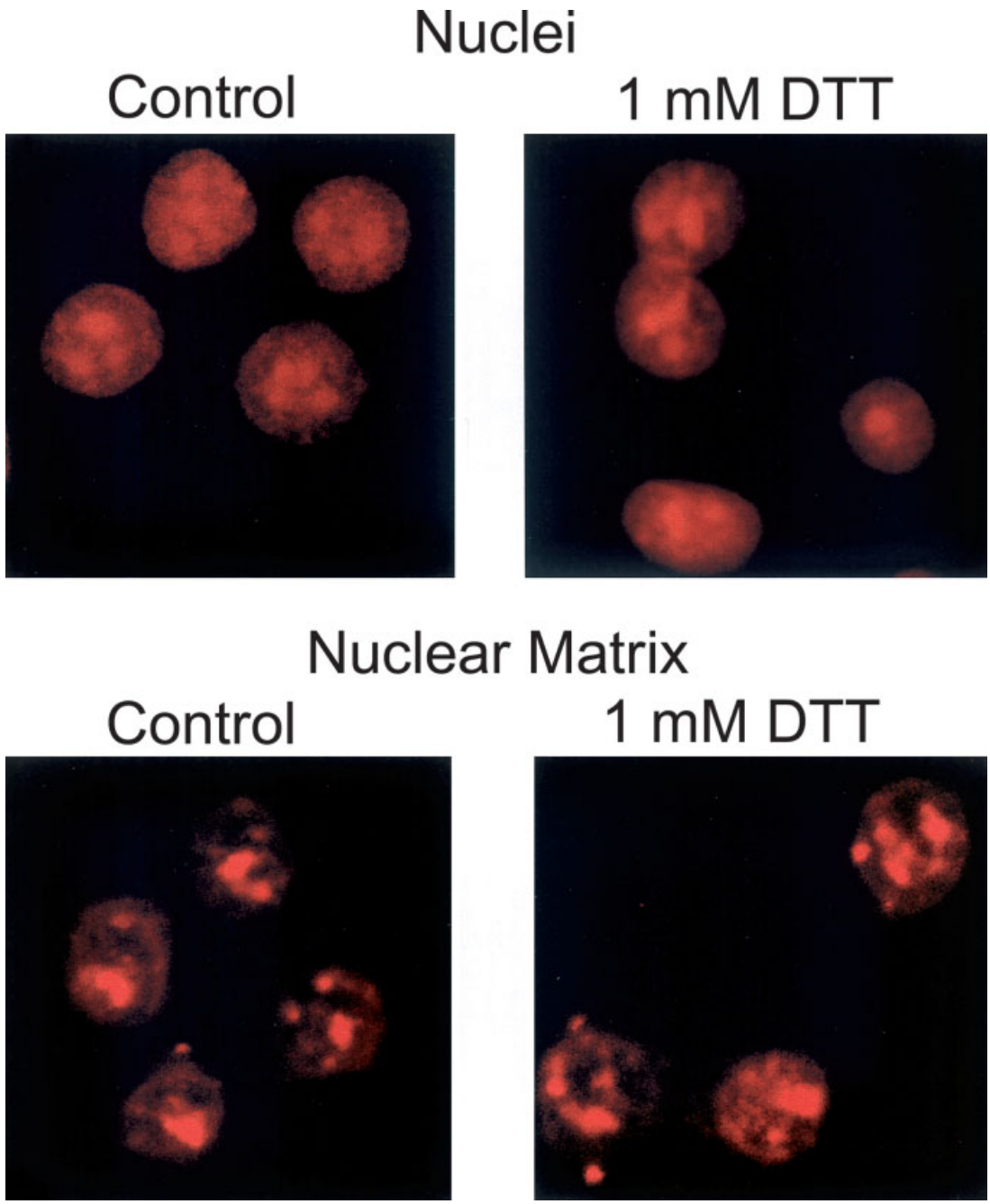

Fig. 9. Localization of PDI within the nucleus (**top**) and NM (**bottom**) isolated from HeLa cells in the presence or absence of 1 mM DTT.

tant for the anchoring of DNA, per se, or are critical for the formation of the protein complex that anchors it, remains open. However, the ability of heat shock to mask the DTT effect implies that PDI is acting as a part of a complex. While heat shock has been shown to cause an increase in the amount of PDI that co-isolates with the NM, it is a significantly smaller change than the heat-induced NM binding of other proteins such as Ku 86 or RPA [Vanderwaal et al., 2001]. This result implies that the heat-induced binding of PDI to the NM may be secondary to the binding of other proteins to the anchoring complex. This notion is consistent with the observation that heat-induced binding of proteins to the NM masks the DTT induced

loosening of the DNA–NM anchor complex. Although the exact structure of the complex remains elusive, these results and those of others [Altieri et al., 1993; Ferraro et al., 1992, 1996], provide significant new insight into DNA–NM anchoring.

The stress responses are universal, characteristic of both prokaryotic and eukaryotic cells [Jolly and Morimoto, 2000]. One of the key steps in all stress responses is changes in gene expression, which produce the proteins that confer resistance to the given stress [Jolly and Morimoto, 2000]. The ability of low levels of stress-induced damage to change gene expression is dependent on signal transduction. However, because of the role played by the NM in gene expression, changes in gene expression should involve changes in the interaction between the NM and the genes to be expressed. Thus, a critical question becomes how the signal transduction pathway contributes to a change in the association of DNA and the NM. The results of the present study suggest a potential scenario for such regulation via changes in redox potential. While the majority of pathways characterized so far proceed via phosphorylation changes [Schmitz et al., 2001], there is evidence that some components of signal transduction occur via changes in redox potential [Kunsch and Medford, 1999; Balon and Yerneni, 2001]. These may be important in the responses to environmental stress because oxidative stress, heat shock, and ionizing radiation have all been proposed to affect cellular oxidative metabolism [Freeman et al., 1990]. Thus, the ability for PDI-mediated changes in DNA–NM anchoring to occur via changes in redox potential may provide a key unifying step in the stress response pathways.

Goswami et al. [2000] showed that an apparent fluctuation in cellular redox potential occurs as HeLa cells progress through the cell cycle. The data presented here, as well as from Ferraro et al. [1999] suggest that redox sensitive protein interactions are involved in anchoring DNA to the NM. Thus, alterations in redox potential could be involved in changes in DNA–NM association required for cell-cycle progression related transcription and DNA replication. Thus, the potential role of PDI in DNA–NM anchoring could provide an oxidation sensitive regulatory site, and thereby, play a significant role in the regulation of gene expression, the responses to stress and progression through the cell cycle.

ACKNOWLEDGMENTS

The authors thank Ms. Kathy Bles for word processing and Beth A. Pierburg for technical assistance.

REFERENCES

Altieri F, Maras B, Eufemi M, Ferraro A, Turano C. 1993. Purification of a 57 kDa nuclear matrix protein associated with thiol:protein-disulfide oxidoreductase and phospholipase C activities. Biochem Biophys Res Commun 194:992–1000.

Balon TW, Yerneni KK. 2001. Redox regulation of skeletal muscle glucose transport. Med Sci Sports Exerc 33:382–385.

Berezney R, Mortillaro MJ, Ma H, Wei X, Samarabandu J. 1995. The nuclear matrix: a structural milieu for nuclear genomic function. In: Berezney R, Jeon K, editors. Nuclear matrix: structural and functional organization. California: Academic Press, pp 1–65.

Berrios M, Osheroff N, Fisher PA. 1985. In situ localization of DNA topoisomerase II, a major polypeptide component of the Drosophila nuclear matrix fraction. Proc Natl Acad Sci USA 82:4142–4146.

Bode J, Kohwi Y, Dickinson L, Joh T, Klehr D, Mielke C, Kohwi-Shigematsu T. 1992. Biological significance of unwinding capability of nuclear matrix-associating DNAs. Science 255:195–197.

Boulikas T. 1995. Chromatin domains and prediction of MAR sequences. Int Rev Cytol 162:279–388.

Cockerill PN, Garrard WT. 1986. Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. Cell 44:273–282.

Comings DE, Wallack AS. 1978. DNA-binding properties of nuclear matrix proteins. J Cell Sci 34:233–246.

Ferraro A, Grandi P, Eufemi M, Altieri F, Turano C. 1992. Crosslinking of nuclear proteins to DNA by *cis*-diammminedichloroplatinum in intact cells. Involvement of nuclear matrix proteins. FEBS 307:282–285.

Ferraro A, Cervoni L, Eufemi M, Altieri F, Turano C. 1996. Comparison of DNA–protein interactions in intact nuclei from avian liver and erythrocytes: a cross-lining study. J Cell Biochem 62:495–505.

Ferraro A, Altieri F, Coppari S, Eufemi M, Chichiarelli S, Turano C. 1999. Binding of the protein disulfide isomerase isoform Ep60 to the nuclear matrix-associated regions of DNA. J Cell Biochem 72:528–539.

Freeman ML, Spitz DR, Meredith MJ. 1990. Does heat shock enhance oxidative stress? Studies with ferrous and ferric iron. Radiat Res 124:288–293.

Gasser SM, Laemmli UK. 1986. The organization of chromatin loops: Characterization of a scaffold attachment site. EMBO J 5:511–518.

Goswami PC, Sheren J, Albee LD, Parsian A, Sim JE, Ridnour LA, Higashikubo R, Gius D, Hunt CR, Spitz DR. 2000. Cell cycle-coupled variation in Topoisomerase II α mRNA is regulated by the 3′-untranslated region. J Biol Chem 275:38384–38392.

Gupta BB, Spessert R, Rimoldi S, Vollrath L. 2001. Sulfhydryl G proteins and phospholipase a(2)-associated G proteins are involved in adrenergic signal transduction in the rat pineal gland. Gen Comp Endocrinol 122:320–328.

Hall EJ. 2000. Radiobiology for the radiologist. Philadelphia: Lippincott Williams and Wilkins, p 521.

Held KD, Sylvester FC, Hopcia KL, Biaglow JE. 1996. Role of Fenton chemistry in thiol-induced toxicity and apoptosis. Radiat Res 145:542–553.

Jackson DA, Cook PR. 1995. The structural basis of nuclear function. In: Berezney R, Jeon K, editors. Nuclear matrix: structural and functional organization. California: Academic Press, pp 125–150.

Jolly C, Morimoto RI. 2000. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst 92:1564–1572.

Kampinga HH, Wright WD, Konings AW, Roti Roti JL. 1989a. Changes in the structure of nucleoids isolated from heat-shocked HeLa cells. Int J Radiat Biol 56:369–382.

Kampinga HH, van den Kruk G, Konings AW. 1989b. Reduced DNA break formation and cytotoxicity of the topoisomerase II drug 4′-(9′-acridinylamino)methanesulfon-m-anisidide when combined with hyperthermia in human and rodent cell lines. Cancer Res 49:1712–1717.

Kunsch C, Medford RM. 1999. Oxidative stress as a regulator of gene expression in the vasculature. Circ Res 85:753–766.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Laszlo A, Wright W, Roti Roti JL. 1992. Initial characterization of heat-induced excess nuclear proteins in HeLa cells. J Cell Physiol 151:519–532.

Lebkowski JS, Laemmli UK. 1982. Evidence for two levels of DNA folding in histone-depleted HeLa interphase nuclei. J Mol Biol 156:309–324.

Malyapa RS, Wright WD, Roti Roti JL. 1994. Radiation sensitivity correlates with changes in DNA supercoiling and nucleoid protein content in cells of three Chinese hamster cell lines. Radiat Res 140:312–320.

Malyapa RS, Wright WD, Roti Roti JL. 1995. Possible role(s) of nuclear matrix and DNA loop organization in fixation or repair of DNA double-strand breaks. In: Fuciarelli AF, Zimbrick JD, editors. Radiation damage in DNA: structure/function relationships at early times. Ohio: Battelle Press.

Malyapa RS, Wright WD, Roti Roti JL. 1996. DNA supercoiling changes and nucleoid protein composition in a group of L5178Y cells of varying radiosensitivity. Radiat Res 145:239–242.

Mirkovitch J, Mirault ME, Laemmli UK. 1984. Organization of the higher order chromatin loop: specific DNA attachment sites on nuclear scaffold. Cell 39:223–232.

Nickerson JA, Krockmalnic G, He D, Penman S. 1990. Immunolocalization in three dimensions: immunogold staining of cytoskeletal and nuclear matrix proteins in resinless electron microscopy sections. Proc Natl Acad Sci USA 87:2259–2263.

Nickerson JA, Blencowe BJ, Penman S. 1995. The architectural organization of nuclear metabolism. In: Berezney R, Jeon KW, editors. Nuclear matrix: structural and functional organization. California: Academic Press, pp 67–124.

Noll M. 1974. Subunit structure of chromatin. Nature 251:249–251.

Oosterhof D, Hozier J, Rill R. 1975. Nuclease action on chromatin: evidence for discrete, repeated nucleoprotein units along chromatin fibrils. Proc Natl Acad Sci USA 72:633–637.

Pienta KJ, Getzenberg RH, Coffey DS. 1991. Cell structure and DNA organization. Crit Rev Eukaryot Gene Expr 1:355–385.

Razin SV, Gromova II, Iarovaia OV. 1995. Specificity and functional significance of DNA interaction with the nuclear matrix: new approaches to clarify the old questions. In: Berezney R, Jeon KW, editors. Nuclear matrix: structural and functional organization. California: Academic Press, pp 405–448.

Reeves R. 1984. Transcriptionally active chromatin. Biochim Biophys Acta 782:343–393.

Roti Roti JL, Laszlo A. 1988. The effects of hyperthermia on cellular macromolecules. Urano M, Douple R, editors. Hyperthermia and Oncology. Utrecht: VSP, pp 13–56.

Roti Roti JL, Wright WD. 1987. Visualization of DNA loops in nucleoids from HeLa cells: assays for DNA damage and repair. Cytometry 8:461–467.

Roti Roti JL, Wright WD, Higashikubo R, Dethlefsen LA. 1985. DNase I sensitivity of nuclear DNA measured by flow cytometry. Cytometry 6:101–108.

Schmitz ML, Bacher S, Kracht M. 2001. I kappa B-independent control of NF-kappa B activity by modulatory phosphorylations. Trends Biochem Sci 26:186–190.

Spencer VA, Coutts AS, Samuel SK, Murphy LC, Davie JR. 1998. Estrogen regulates the association of intermediate filament proteins with nuclear DNA in human breast cancer cells. J Biol Chem 273:29093–29097.

Stuurman N, Floore A, Colen A, DeJong L, Van Driel R. 1992. Stabilization of the nuclear matrix by disulfide bridges: identification of matrix polypeptides that form disulfides. Exp Cell Res 200:285–294.

Taylor YC, Zhang XF, Parsian AJ, Duncan PG. 1991. Image analysis-based measurement of DNA supercoiling changes in transformed and nontransformed human cell lines. Exp Cell Res 197:222–228.

Tornaletti S, Hanawalt PC. 1999. Effect of DNA lesions on transcription elongation. Biochimie 81:139–146.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Van Holde KE. 1988. Chromatin. New York: Springer-Verlag.

van Wijnen AJ, Bidwell JP, Fey EG, Penman S, Lian JP, Stein JL, Stein GS. 1993. Nuclear matrix association of multiple sequence-specific DNA binding activities related to SP-1, ATF, CCAAT, C/EBO, OCT-1 and AP-1. Biochem 32:8397–8402.

VanderWaal R, Thampy G, Wright WD, Roti Roti JL. 1996. Heat-induced modifications in the association of specific proteins with the nuclear matrix. Radiat Res 145:746–753.

Vanderwaal RP, Griffith CL, Wright WD, Borrelli MJ, Roti Roti JL. 2001. Delaying S-phase progression rescues cells from heat-induced S-phase hypertoxicity. J Cell Physiol 187:236–243.

Vogelstein B, Pardoll DM, Coffey BS. 1980. Supercoiled loops and eucaryotic DNA replication. Cell 22:79–85.

Wei X, Samarabandu J, Devdhar RS, Siegel AJ, Acharya R, Berezney R. 1998. Segregation of transcription and replication sites into higher order domains. Science 281:1502–1506.

Wynstra JH, Wright WD, Roti Roti JL. 1990. Repair of radiation-induced DNA damage in thermotolerant and nonthermotolerant HeLa cells. Radiat Res 124:85–89.