# UV-Induced Pyrimidine Dimers and Trimethylpsoralen Cross-Links Do Not Alter Chromatin Folding in Vitro†

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ABSTRACT: We have examined the ability of intact and histone H1 depleted chromatin fibers to fold into higher ordered structures in vitro following DNA damage by two different agents: UV irradiation at 254 nm and trimethylpsoralen plus near-UV light. Both agents damage DNA specifically, yet cause different degrees of unwinding (and possibly bending) of the DNA helix. In addition, trimethylpsoralen forms interstrand DNA cross-links. The structural transitions of intact and histone H1 depleted chromatin fibers, induced by NaCl, were monitored by analytical ultracentrifugation, light scattering, and circular dichroism. Our results indicate that when chromatin fibers contain even large, nonphysiological amounts of DNA photodamage by either agent, the salt-induced folding of these fibers into higher ordered structures is unaffected. The compact 30-nm fiber must therefore be able to accommodate a large amount of DNA photodamage (greater than one UV-induced photoproduct or trimethylpsoralen interstrand cross-link per nucleosome) with little or no change in the overall size or compaction of this structure.

The primary level of DNA organization in chromatin is the repeating nucleosome unit consisting of 166 base pairs (bp)[1] of DNA wrapped in two complete turns around an octamer of the core histones H2A, H2B, H3, and H4 (chromatosome) and varying lengths of linker DNA connecting these subunits (McGhee & Felsenfeld, 1980). In the extended form, the chromatin fiber resembles "beads-on-a-string" in the electron microscope with a thickness of ~10 nm (Finch et al., 1975; Langmore & Wooley, 1975; Oudet et al., 1975). The next level of packaging involves folding of the nucleofilament into a 30-nm-thick fiber, requiring histone H1 (Thoma et al., 1979, 1983; McGhee et al., 1980; Thoma & Koller, 1981). While the exact arrangement of nucleosomes in the 30-nm fiber remains unsolved, a number of models have been proposed: the original solenoid (or contact helix) model of Finch and Klug (1976), the superbead model (Renz et al., 1977; Kiryanov et al., 1982), and a series of models based on a helical coil arrangement of nucleosomes (Azorin et al., 1980; Woodcock et al., 1984; Worcel & Benyahati, 1977; Fulmer & Bloomfield, 1982; Williams et al., 1986). Of these, the solenoid and the helical coil models have received considerable support. The solenoid model depicts a right-handed helix with 6–7 nucleosomes per turn and a helical pitch of 11 nm with the location of the linker DNA undefined (Finch & Klug, 1976). Alternatively, one helical model, the cross-linker model, suggests a left-handed double helix with 12 nucleosomes per turn whose linker DNA crisscrosses the center of the helix (Williams et al., 1986). The diameter and mass per unit length of the helix are therefore dependent on the linker length.

Increasing amounts of attention have been focused on the structural transitions of chromatin. Chromatin structure is clearly dynamic in vivo, where processing by enzymes during transcription, replication, and repair seems to require a more "open" conformation of chromatin [for reviews, see Cartwright et al. (1982), Reeves (1984) and Lieberman (1982)]. Furthermore, the regulation of genes in domain loops of chromatin may be influenced by factors that alter chromatin structure in these loops [reviewed in Weintraub (1985) and Gasser and Laemmli (1987)]. Regions of chromatin not being actively processed appear to be associated with the compact 30-nm fiber structure (Cartwright et al., 1982; Reeves, 1984). However, while there is extensive literature pertaining to chromatin structure and structural transitions, little is known about the factors that alter chromatin structure in vivo and the effects of bulky DNA lesions on these transitions.

The folding of intact chromatin fibers from the extended 10-nm form to the compact 30-nm fiber can be induced in vitro by increasing the concentration of monovalent or divalent salts (typically from 0 to 100 mM NaCl or 0 to 0.4 mM $MgCl_2$) (Thoma et al., 1979; McGhee et al., 1980, 1983). Thoma and Koller (1981) have characterized the folding of intact chromatin in varying NaCl concentrations using electron microscopy and describe four basic structures: (1) at 1 mM NaCl, an extended beads-on-a-string structure, (2) at 10 mM, a "zigzag" conformation, (3) at 40 mM, a discontinuous compact fiber, and (4) at 100 mM, the compact 30-nm fiber. Chromatin fibers depleted of histone H1 were also shown to undergo some compaction with salt, but not nearly to the extent of intact fibers (Thoma et al., 1979, 1983; Thoma & Koller, 1981).

We have examined these structural transitions in chromatin fibers containing varied amounts of photoadducts induced by UV radiation at 254 nm and 4,5′,8-trimethylpsoralen (TMP) (plus near-UV radiation). These agents were chosen because (1) they induce adducts in DNA specifically (i.e., at much higher levels than in protein) and (2) the adducts formed by each agent are predicted to cause different degrees of DNA helix distortion. The primary adduct formed by 254-nm UV radiation is the cis–syn cyclobutane pyrimidine dimer (PD), whose structure and photochemistry has been studied in detail (Wang, 1976). Combination of computer modeling and X-ray crystallography of small oligonucleotides containing a PD

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[1] Abbreviations: bp, base pairs; UV, ultraviolet; TMP, 4,5′,8-trimethylpsoralen; PD, cis–syn cyclobutane pyrimidine dimers; CMBS, *p*-(chloromercuri)benzenesulfonic acid; CD, circular dichroism.

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predicts that the thymine dimer may unwind the DNA helix by as much as 19.7° and cause a bend (or "kink") of 27° (Pearlman et al., 1985). Experiments examining the unwinding of supercoiled plasmids by PD via mobility changes in agarose gels have indicated that the DNA helix unwinding is 13.7–14.3° per dimer (Ciarrocchi et al., 1982; Ciarrocchi & Pedrini, 1982). Furthermore, PD appear to form uniformly between nucleosome core and linker DNA in irradiated cells (Williams & Friedberg, 1979; Niggli & Cerutti, 1982), although the dimer yield *within* core DNA is clearly modulated by histone binding (Gale et al., 1987).

Trimethylpsoralen is a naturally occurring tricyclic compound that intercalates into the DNA helix [for reviews, see Song and Tapley, Jr. (1979) and Cimino et al. (1985)]. With exposure to near-UV light (320–380 nm) three major covalent adducts are produced, which form preferentially with thymine, primarily at 5′-TA sites (Sinden & Hagerman, 1984; Gamper et al., 1984). Two are monoadducts (pyrone side and furan side) and one is a diadduct, or interstrand cross-link. The ratios of formation of these adducts in double-stranded DNA are approximately 3%, 52%, and 39%, respectively (Kanne et al., 1982). Unlike PD, TMP cross-links form preferentially in linker DNA regions (Wiesehahn et al., 1977; Cech & Pardue, 1977). By use of the same computer modeling technique as for PD, the interstrand cross-link was estimated to unwind the DNA helix by 87.7° and cause a bend of up to 46.5° (Pearlman et al., 1985). Recent 2-D NMR experiments on a 8-bp DNA fragment at 10° containing a single TMP cross-link yielded similar values (Tomic et al., 1987). Such extreme distortions in the DNA helix might cause significant alterations in the structural transitions of chromatin. However, results from experiments examining the unwinding of supercoiled plasmids on agarose gels yield a helix unwinding of only 28° (Wiesehahn & Hearst, 1978). Furthermore, by examining the electrophoretic mobility of TMP-cross-linked DNA in polyacrylamide gels, Sinden and Hagerman (1984) found no evidence for significant DNA bending at cross-linked sites. Thus, the degree of helix distortion at TMP cross-link sites remains controversial.

We have examined the salt-induced folding of chromatin fibers, containing varying levels of these two different classes of photoproducts, using analytical ultracentrifugation, light scattering, and circular dichroism techniques. No change in the folding was detected in intact or histone H1 depleted fibers containing even saturating levels (>1 PD or TMP cross-link/nucleosome) of these adducts. Our results are in agreement with an earlier report that TMP cross-links do not significantly alter the salt-induced compaction of chromatin fibers as visualized by electron microscopy (Conconi et al., 1984).

## Materials and Methods

*Chromatin Preparation.* Nuclei were prepared from bovine thymus as previously described (Lee et al., 1979) with some modification. Briefly, thymus tissue was homogenized in buffer A [0.25 M sucrose, 50 mM triethanolamine, pH 6.5, 25 mM NaCl, 5 mM $MgCl_2$, and 1 mM protease inhibitor *p*-(chloromercuri)benzenesulfonic acid (CMBS, Sigma) (Paulson, 1980)], followed by filtration through cheesecloth and centrifugation through a 2.3 M sucrose cushion to remove cytoplasmid debris (Rill et al., 1978). Nuclei were then washed once in buffer A and once in buffer B (buffer A with 1 mM $CaCl_2$ and 4 mM $MgCl_2$ instead of 5 mM $MgCl_2$) and suspended in buffer B. Nuclei were then incubated with staphylococcal nuclease (Worthington) at 2 units/1 absorbance unit at 260 nm for 30–60 min on ice. Soluble chromatin was released by lysis in 0.5 mM EDTA and 0.1 mM CMBS and fractionated on 15–35% sucrose gradients in a Vti-50 vertical rotor at 35000 rpm for 2 h. The vertical rotor was chosen because large amounts of chromatin (40–100 absorbance units at 260 nm) could be applied to the gradients, and therefore, the individual fractions would contain enough material for sedimentation and circular dichroism experiments without further concentration. The DNA size range in each fraction was determined on 1% agarose gels. Fractionated chromatin samples were dialyzed versus buffer C (2 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, and 0.1 mM CMBS) to remove the sucrose. For histone H1 depleted chromatin, H1 was removed by treatment with CM-Sephadex (30 mg/mL) for 90 min in the presence of 75 mM NaCl on ice (Libertini & Small, 1980). Histone H1 content was determined from 18% polyacrylamide–SDS gels (Thomas & Kornberg, 1975) visualized by silver staining (Wray et al., 1981).

*Photodamage of Chromatin Fibers.* Chromatin fibers at ~1 absorbance unit at 260 nm (after correction for the absorbance of CMBS) in buffer C were irradiated at (predominantly) 254 nm on ice in a 60 × 15 mm Petri dish with a low-pressure mercury lamp (Sylvania Model G 30T8). The chromatin solutions were evenly dispersed across the bottom of the Petri dish with a solution depth of less than 2 mm. Irradiation doses varied between 0 and 20000 J/m$^2$ at fluxes ranging between 2 and 16 W/m$^2$ achieved by changing the distance between the sample and the lamp. Doses were determined with a Blak-Ray UV meter (Ultra-Violet Products). After irradiation, samples were used immediately for folding analysis.

For TMP treatment, chromatin solutions were made 10 μg/mL in TMP (Sigma) by addition of a stock TMP solution (~400 μg/mL) in absolute ethanol (Hyde & Hearst, 1978). The samples were allowed to stand 10 min in the dark and then were irradiated with near-UV light (320–400 nm) on ice with a 450-W medium-pressure mercury lamp (Conrad-Hanovia) filtered by Pyrex glass (to remove radiation below 300 nm). Irradiation times ranged from 0 to 30 min, where saturation of TMP photobinding was observed in our system. Fibers were then dialyzed versus 50 mM Tris, pH 7.4, 0.1 mM EDTA, 1 mM CMBS, and 1 mM $MgCl_2$ to facilitate removal of intercalated, noncovalently bound TMP (Hyde & Hearst, 1978). Samples were then dialyzed extensively against buffer C and used immediately for folding analysis.

*Analytical Ultracentrifugation.* Sedimentation velocity analysis was performed on a Beckman Model E analytical ultracentrifuge equipped with UV scanning optics and RTIC temperature controller as described by Butler and Thomas (1980). Chromatin samples (0.6–1.2 absorbance units at 260 nm) in buffer C and varying salt concentrations were analyzed in a double-sector cell at 4 °C and speeds ranging from 15000 to 24000 rpm. Sedimentation coefficients were calculated from the midpoint of the sedimenting boundary and converted to $s_{20,w}$ by standard equations (Svedberg & Pedersen, 1940). The concentration of salt was raised in each sample by addition of 2.0 M NaCl directly to the centrifugation cell with a Hamilton syringe, followed by vigorous shaking. Samples were thus repeatedly centrifuged in the presence of 0, 10, 25, 50, 75, and 100 mM NaCl.

*Light Scattering.* Light scattering of chromatin fibers was measured at 90° on a SLM 4800 spectrofluorometer (SLM instruments) at 10 °C equipped with a Hewlett-Packard 9825A computer. Measurements were made by setting the excitation and emission monochrometers at the same wavelength, which was beyond the absorbance of the sample. A

wavelength of 340 nm was used for UV-damaged fibers and 420 nm was used for TMP-damaged fibers (due to absorbance of the furan side monoadduct; Cimino et al., 1985). Chromatin samples were diluted to 0.1–0.2 absorbance unit at 260 nm with buffer C. The salt concentration was increased by additions of 2.0 M NaCl directly to the cuvette with rapid mixing in increments of 0, 10, 25, 50, 75, and 100 mM. Scattering by the buffer alone was subtracted from all values and the final light scattering intensity reported as a percent change from the scattering of the sample at 0 mM NaCl:

$$\% \text{ change in light scattering intensity} = \frac{(S_i - B_i) - (S_0 - B_0)}{(S_0 - B_0)} \times 100$$

where $S_0$ and $S_i$ are the scattering intensities of the sample at NaCl concentrations 0 and $i$ mM, respectively, and $B_0$ and $B_i$ are the scattering intensities of the buffer containing NaCl concentrations of 0 and $i$ mM, respectively. The light scattering data are presented in this way to allow direct comparisons of different samples since the absolute scattering intensity of a sample is dependent on the concentration of chromatin fibers. It should be noted that treatment of chromatin fibers with 254-nm UV light or TMP + near-UV radiation had no effect on the initial scattering intensity (i.e., $S_0$) of any of the samples tested.

*Circular Dichroism.* Circular dichroism spectra from 200 to 400 nm were recorded at room temperature on a Jasco ORD/UV-5 spectrophotometer equipped with a CD SS-20 modification. Concentrations of DNA nucleotides in chromatin were determined from the absorbance at 258 nm using $\epsilon_{258} = 6800$ cm$^{-1}$ mol$^{-1}$. In experiments examining the influence of UV radiation on chromatin structure, complete spectra were obtained for chromatin samples (0.6–1.2 absorbance units at 260 nm) in buffer C, exposed to varying doses of 254-nm UV light. In some experiments examining chromatin folding, only the ellipticity at 283 nm was monitored as the salt concentration was raised by adding 2 M NaCl directly to the cuvette followed by rapid mixing. All data were corrected for the dilution by NaCl addition.

*Determination of Pyrimidine Dimers by HPLC.* To quantify the yield of pyrimidine dimers in our system, chromatin fibers were prepared from mouse L 1.4-3 cells (Zaret & Yamamoto, 1984) whose DNA was labeled during a 1-week incubation with 20 nCi/mL [$^3$H]dThd (New England Nuclear). Cells were harvested and nuclei prepared as previously described (Smerdon et al., 1979). Soluble chromatin was prepared from nuclei in the same manner as for bovine thymus nuclei. Chromatin samples were irradiated at 254 nm at doses between 40 and 30 000 J/m$^2$ in the same manner as for the folding experiments. The samples were deproteinized by treatment with proteinase K, followed by phenol extraction and ethanol precipitation. Samples were then hydrolyzed as described by Carrier and Setlow (1971). Prior to HPLC, the samples were filtered through a 2-mL centrifugation filter (Rainen) containing a 0.45-$\mu$m filter (Millipore). HPLC was performed on a 25-cm Partisil-10 ODS-2 column (Whatman) as described by Cadet et al. (1983). Hydrolysis products (DNA bases and photoproducts) were eluted isocratically with water at 1 mL/min on a Beckman dual-pump HPLC system (Beckman 112 solvent delivery modules, 421 controller, and 165 variable-wavelength detector) and collected directly into scintillation vials. The fractions were assayed for radioactivity on a Beckman LS 7500 liquid scintillation counting spectrometer. The elution position of the cyclobutane thymine dimer was determined by comparison with authentic standards prepared by UV-irradiating thymidine in the frozen state (Beukers & Berends, 1960), followed by acid hydrolysis and examination of the absorbance characteristics of the elution peaks.

*Quantitation of TMP Cross-Links by Electron Microscopy.* TMP-cross-linked samples were prepared for electron microscopy by the BAC method (Vollenweider et al., 1975) as described by Sogo et al. (1979, 1984). Closed circular SV-40 chromatin, extensively cross-linked by TMP, was prepared as described (Sogo et al., 1984; Bernardin et al., 1986) and added to the chromatin samples to serve as an internal size reference. The isolated DNA samples were partially denatured by the addition of formamide and BAC solution and the DNA solutions spread over a hypophase of redistilled water. The DNA–BAC film was picked up on carbon-coated grids pretreated with ethidium bromide. The grids were stained with uranyl acetate and rotary shadowed with platinum–carbon. Micrographs were taken with a Siemens Elkiskop 101 electron microscope at a magnification of 5000×. Because TMP cross-links DNA almost exclusively in linker regions of chromatin (Wiesehahn et al., 1977; Cech & Pardue, 1977), the minimum size of the single-strand bubbles seen in the SV-40 cross-linked DNA was assumed to be one nucleosome in length. On the basis of this length, the average distance between cross-links was measured for the chromatin samples cross-linked by TMP. Quantitation of DNA lengths was performed with a Jandel Scientific digitizing tablet and software (Sigma Scan System) and an IBM PS/2 Model 60 computer.

## Results

*Characterization of Undamaged Chromatin Fibers.* For all of the chromatin folding studies in this report, chromatin fibers were isolated from bovine thymus tissue. The fraction of chromatin released by lysis of thymus cell nuclei after staphylococcal nuclease digestion was generally 30–50% of the total nuclear chromatin. Different size classes of chromatin fibers were obtained by fractionation in sucrose gradients. A typical separation of chromatin fragments on these gradients, as well as the histone content of selected fractions, is shown in Figure 1. These results indicate that the chromatin fibers contained a full complement of histones. In experiments where histone H1 depleted fibers were examined, H1 was removed by incubation of the fibers with CM-Sephadex at 4 °C (Libertini & Small, 1980). In order to optimize the removal of H1 in our system the kinetics and salt dependence of this technique were examined (data not shown). On the basis of these results, 90 min in the presence of 75 mM NaCl was chosen to assure complete removal of histone H1. Little (or no) removal of the core histones was observed under these conditions.

The salt-induced folding of chromatin fibers was monitored by sedimentation velocity and light scattering measurements. Sedimentation coefficients were measured on an analytical ultracentrifuge. Light scattering was measured on a spectrofluorometer and was chosen to complement the sedimentation data because of the simplicity, speed, and increased sensitivity of the technique. A comparison between sedimentation velocity and light scattering results for the same sample is shown in Figure 2. Figure 2A shows the sedimentation coefficient at different salt concentrations for a sample of intact chromatin fibers (i.e., containing a full complement of histones) having a mean length of 22 nucleosomes. Figure 2B shows the actual values of relative scattering intensity for the same chromatin sample at the same salt concentrations. The correlation of the data between these two

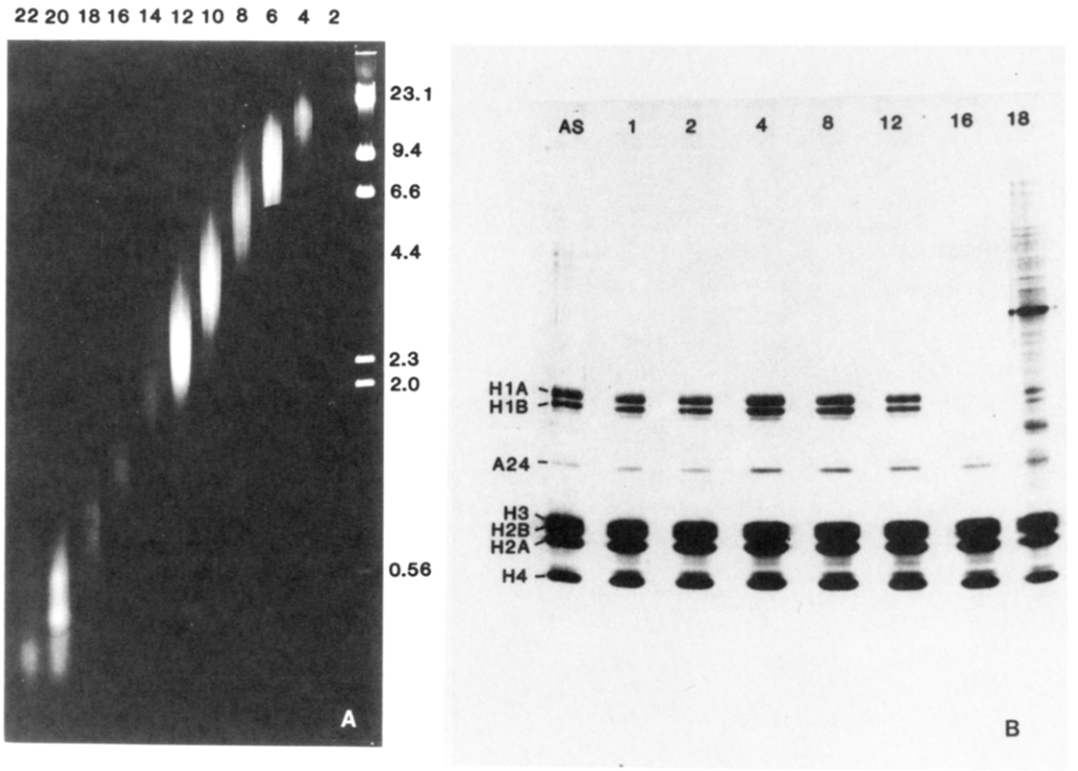


FIGURE 1: (A) 1% agarose gel of DNA from chromatin fragments isolated on a 15–35% sucrose gradient. Chromatin was fractionated in 40-mL gradients in a Vti-50 rotor at 35 000 rpm for 2 h. The corresponding fraction number (24 total fractions) is shown above each gel lane. The size markers (in kb) shown in the far right lane of the figure are a *Hin*dIII digest of λ DNA. (B) SDS–polyacrylamide gel of proteins from chromatin fractions taken as described in (A). Fractions were mixed with an equal volume of sample buffer (containing 0.2% SDS) and electrophoresed on 18% SDS–polyacrylamide with a 6% stacking gel and visualized by silver staining. The corresponding fraction number (19 total fractions) is shown above each gel lane. For comparison, the total acid-soluble (0.4 N $H_2SO_4$) extract of bovine thymus nuclei is shown in the far left lane (AS).

analyses, shown in Figure 2C, shows excellent agreement between the sedimentation and light scattering techniques.

To ensure that the overall change in sedimentation velocity and light scattering that we observe (i.e., from 0 to 100 mM NaCl) does indeed reflect compaction of the chromatin fibers into the 30-nm structure observed in the electron microscope, we compared the sedimentation coefficients for many different size classes of chromatin fibers at 0 and 100 mM NaCl to the values obtained by Butler and Thomas (1980). These authors performed electron microscopy on the same samples used for sedimentation velocity analyses and suggested that chromatin fibers greater than ~6 nucleosomes in length formed 30-nm-thick fibers. As can be seen in Figure 3, the sedimentation coefficients we observe at 100 mM NaCl are in excellent agreement with the values reported by Butler and Thomas (1980), indicating that the fibers do form compact 30-nm structures and are not simply aggregating. [Additional studies on the change in light scattering intensity and sedimentation coefficient after salt addition as a function of time and temperature also eliminated the possibility of aggregation in our samples (data not shown).] Our sedimentation values at 0 mM NaCl are consistently lower than the values reported for 5 mM NaCl by Butler and Thomas (Figure 3). This suggests that at 0 mM NaCl chromatin is in a more extended state than at 5 mM NaCl, and that a moderate amount of compaction is induced even by the addition of 5 mM NaCl. This is consistent with work by Thoma and Koller (1981) showing a considerable amount of compaction in chromatin when the salt is raised from 1 to 10 mM NaCl. Similarly, we examined the ability of different size classes of histone H1 depleted chromatin to form more compact structures in 75 mM NaCl (data not shown). Although the exact structures of histone H1 depleted fibers in varying NaCl concentrations is unknown, the data clearly showed that H1-depleted fibers do form less compact structures than intact fibers.

The ability of different size classes of chromatin to fold into higher ordered structures was further examined by light scattering. In a manner analogous to the sedimentation analyses, the light scattering intensity was measured for intact and histone H1 depleted fibers of many different sizes in the presence of 0 and 100 mM NaCl. Figure 4 shows the total percent change in light scattering upon raising the concentration of NaCl from 0 to 100 mM as a function of the log of chromatin fiber length. An interesting feature of the data in Figure 4 is that both intact and histone H1 depleted fibers show a transition in scattering intensity in the range of 10–14 nucleosomes. This may indicate that a minimum length of 10–14 nucleosomes is required to form "regular" structures. Although this minimum length does not help distinguish between any of the models for the 30-nm structure, since it could represent two turns of a solenoid (6 nucleosomes per turn) or one turn of a double helix coil model (12 nucleosomes per turn), it suggested to us that fibers greater than 12 nucleosomes in length be used for our folding studies.

*Quantitation of 254-nm UV Photodamage in Chromatin Fibers.* To quantitate the amount of PD formed in chromatin fibers under our irradiation conditions, we used chromatin fibers prepared from a cultured mouse L-cell line labeled in the DNA with [$^3$H]dThd. Mouse cell chromatin was UV-irradiated at doses ranging from 40 to 30 000 J/m$^2$, the DNA acid hydrolyzed to individual bases, and the percent of thymine as cyclobutane dimers determined by HPLC (Cadet et al.,

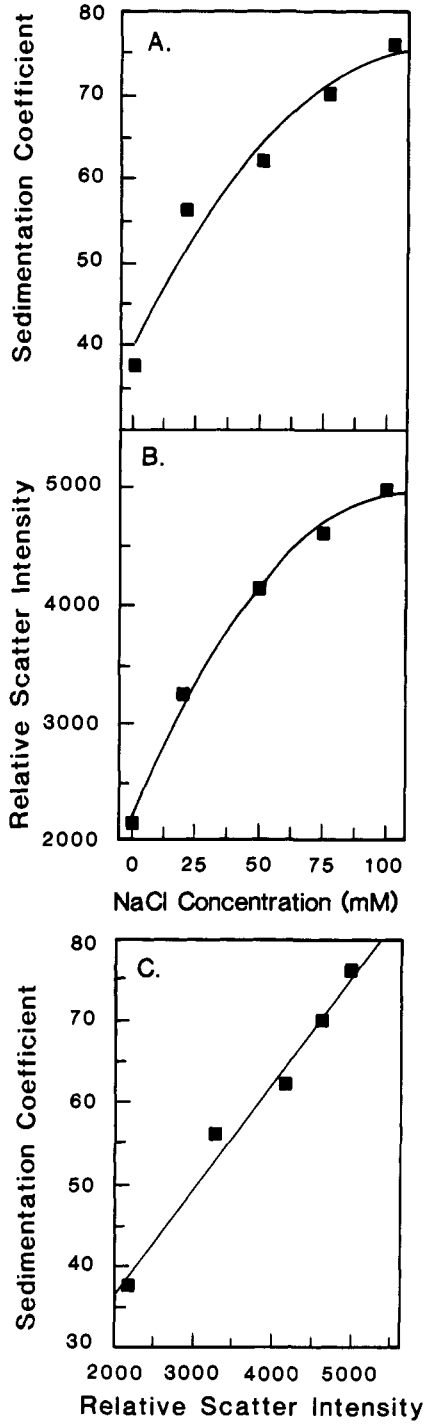


FIGURE 2: Comparison of the salt-induced folding of chromatin measured by sedimentation velocity and light scattering for the same sample. (A) Sedimentation coefficient ($s_{20,w}$) as a function of NaCl concentration for a sample of intact chromatin having an average length of 22 nucleosomes. (B) Relative light scattering intensity as a function of salt concentration for the same chromatin sample. (C) Correlation between the sedimentation and light scattering data shown in panels A and B. The correlation coefficient was calculated by linear regression to be 0.990.

1983). The thymine pyrimidine dimer content for UV-irradiated mouse cell chromatin and naked DNA is shown in Figure 5. At 100 J/m$^2$ the rate of dimer formation for L-cell chromatin is $1.3 \times 10^{-2}$ dimers per 1000 bp per J m$^{-2}$. In comparison, values of $(0.8–1.7) \times 10^{-2}$ dimers per 1000 bp per J m$^{-2}$ have been reported for irradiated whole cells or naked DNA in solution (Ciarrocchi, & Pedrini, 1979; Ahmed & Setlow, 1979; Ganesan, 1973; Van Zeeland et al., 1981; Cadet et al., 1983).

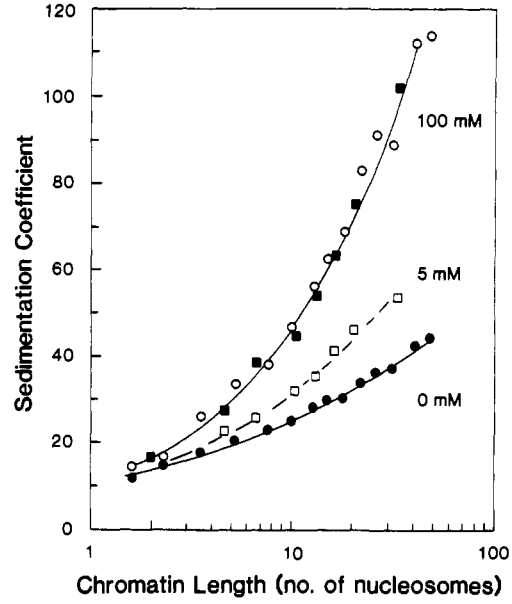


FIGURE 3: Dependence of sedimentation coefficients on the average length of intact chromatin fragments at different salt concentrations. The sedimentation coefficients ($s_{20,w}$) for intact chromatin fractionated on sucrose gradients were measured at 0 (●) and 100 mM (○) NaCl. For comparison, the sedimentation coefficients obtained by Butler and Thomas (1980) for 5 (□) and 100 mM (■) NaCl have also been included in the figure.

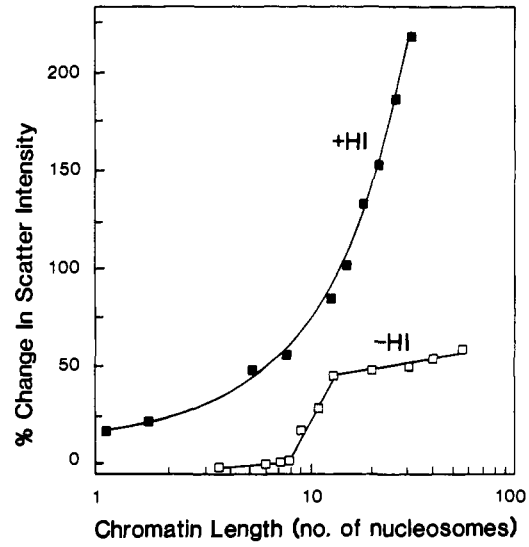


FIGURE 4: Change in light scattering intensity as a function of the average length of chromatin fibers. Light scattering intensity was measured for intact (■) and H1 depleted fibers (□), fractionated on sucrose gradients, at 0 and 100 mM NaCl. The data shown are plotted as the percent increase in light scattering intensity between 0 and 100 mM NaCl and are obtained by taking the difference in scatter intensity between 0 and 100 mM for a given sample and dividing by the scatter intensity at 0 mM (see Materials and Methods).

*Effect of 254-nm UV Photodamage on Chromatin Folding.* Initially, we examined the folding of intact and histone H1 depleted chromatin fibers that were irradiated with 200, 500, or 2000 J/m$^2$. At these doses there is an average of 1 PD induced every 670 bp, 445 bp, and 220 bp, respectively. Both the light scattering and sedimentation data (Figure 6) indicate that the salt-induced folding of intact and histone H1 depleted fibers is unaffected by irradiation at these UV doses.

To determine if a higher level of DNA damage by UV radiation alters chromatin folding, these experiments were repeated with chromatin fibers irradiated with the extremely large dose of 20 000 J/m$^2$. This dose yields an average of 1 PD every 60 bp (see Figure 5). Even at this high level of

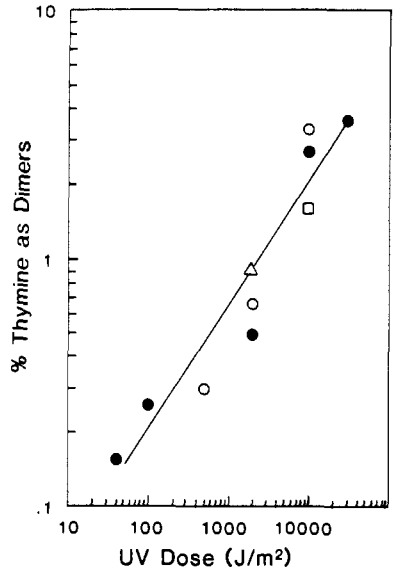


FIGURE 5: Thymine dimer content of mouse L-cell chromatin and DNA as a function of UV dose at 254 nm. Mouse L-cell chromatin (○, □, △) and DNA (●), radiolabeled with [$^3$H]dThd, were irradiated with 40–30 000 J/m$^2$ UV light at 254 nm, and the thymine dimer content was determined by HPLC (Materials and Methods). The amount of radiolabel was determined in the thymine and thymine dimer peaks by integration, and the data were presented as percent of thymine as dimers. Different symbols represent different sample preparations.

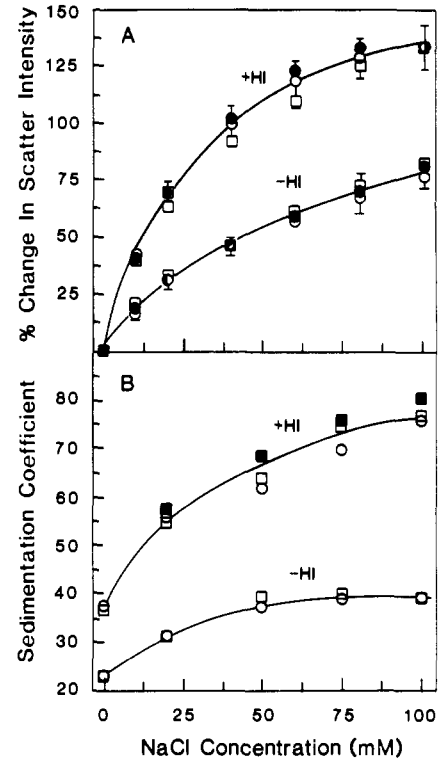


FIGURE 6: (A) Light scattering intensity of chromatin as a function of NaCl concentration following different doses of UV light at 254 nm. The percent change in light scattering intensity from 0 mM NaCl was measured for intact (upper curve) and histone H1 depleted (lower curve) chromatin fibers having an average length of 25 nucleosomes. Samples were irradiated with 0 (○), 200 (●), or 2000 (□) J/m$^2$ UV light. Each point represents the mean of three independent determinations, and the error bars designate one standard deviation from the mean for the 0 J/m$^2$ sample. (B) Sedimentation coefficient of chromatin as a function of NaCl concentration following different doses of UV light at 254 nm. Sedimentation coefficients ($s_{20,w}$) were measured for intact (upper curve) and histone H1 depleted (lower curve) chromatin having an average length of 16 nucleosomes. Samples were irradiated with 0 (○), 500 (■), or 2000 (□) J/m$^2$ UV light at 254 nm.

photodamage, the folding of chromatin into higher ordered structures is not measurably affected (data not shown). We note that in this experiment some aggregation of fibers, damaged by 20 000 J/m$^2$, was observed at 100 mM NaCl. This may reflect protein–protein cross-links that form in a minor fraction of the fibers following such a large UV dose (Gregg & Mansbridge, 1981; Callaway et al., 1985; Peak et al., 1985).

Since it was apparent from these experiments that damage by UV light at 254 nm had little or no effect on salt-induced chromatin folding, even at the extremely large dose of 20 000 J/m$^2$, it was of interest to determine if changes in DNA secondary structure could be detected in UV-irradiated chromatin (Vorlickova et al., 1979). To this end, we performed circular dichroism (CD) measurements on chromatin fibers irradiated with different UV doses. Chromatin fibers were irradiated with 254-nm UV light at 200, 2000, and 20 000 J/m$^2$ and complete CD spectra (230–300 nm) taken in 0 and 100 mM NaCl. As can be seen in Figure 7, an increase in ellipticity at 282 and 270 nm was measured for chromatin fibers exposed to UV radiation. A change in ellipticity at 270 nm was seen for doses above 200 J/m$^2$ and a change at 283 nm detected for doses above 2000 J/m$^2$. This increase in ellipticity is most likely due to unwinding of DNA at PD sites (Pearlman et al., 1985) and indicates that at the higher levels of UV damage used in the chromatin folding experiments the DNA contains measurable structural alterations. Also apparent in Figure 7 is a decrease in ellipticity of chromatin fibers upon folding when the concentration of NaCl is raised from 0 to 100 mM. This decrease in ellipticity, associated with compaction of the fiber, has been reported previously (Lee et al., 1981; Watanabe & Iso, 1981). Experiments using CD spectra to monitor chromatin folding were performed on intact and histone H1 depleted chromatin fibers damaged by 2000 and 10 000 J/m$^2$ and at 0, 10, 25, 50, 75, and 100 mM NaCl. Once again, no effect of UV irradiation was observed in these studies (data not shown).

*Quantitation of TMP Cross-Links in Chromatin Fibers.* The effects of TMP photoadducts on chromatin folding were also investigated, since these adducts include interstrand DNA cross-links and have been predicted to generate larger distortions in the DNA helix than PD (see the introduction). Chromatin fibers were treated with 10 μg/mL TMP, which was then covalently bound to DNA by irradiation with near-UV light (320–380 nm). After irradiation, unbound TMP was removed by dialysis (Materials and Methods).

The amount of TMP interstrand DNA cross-links in intact chromatin fibers was estimated for 1-, 5-, and 30-min irradiation times by electron microscopy. This method was used because it yields information on both quantity and distance between cross-links. Quantitation of cross-links in chromatin DNA was accomplished by fixing the DNA for electron microscopy under denaturing conditions. Under these conditions, single-stranded DNA is joined together only at cross-links and therefore appears as a series of "bubbles" of different lengths in the micrographs (Figure 8). Because TMP cross-links form preferentially in linker DNA (Wiesehahn et al., 1977; Cech & Pardue, 1977), the smallest bubble observed in the micrographs is ~1 nucleosome in length. The distance between cross-links is therefore measured in integral nucleosome lengths directly on the electron micrographs based on the measurement of single nucleosome-sized bubbles seen in heavily TMP-cross-linked SV-40 DNA (which was included in all samples) (Sogo et al., 1986; Bernardin et al., 1986). By this technique, the average distance between TMP cross-links for 1-, 5-, and

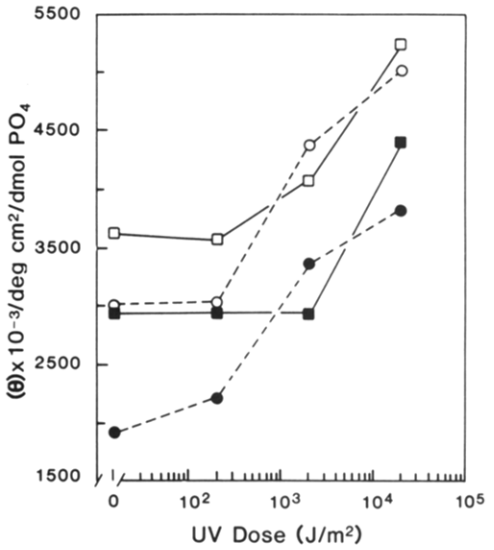


FIGURE 7: Circular dichroism of chromatin as a function of UV dose and salt concentration. Complete circular dichroism spectra were obtained for intact chromatin having an average length of 80 nucleosomes in 0 (open symbols) and 100 mM NaCl (closed symbols). Ellipticity values at 283 (□, ■) and 270 (○, ●) nm represent a single determination, and values at 100 mM NaCl have been corrected for dilution by salt.

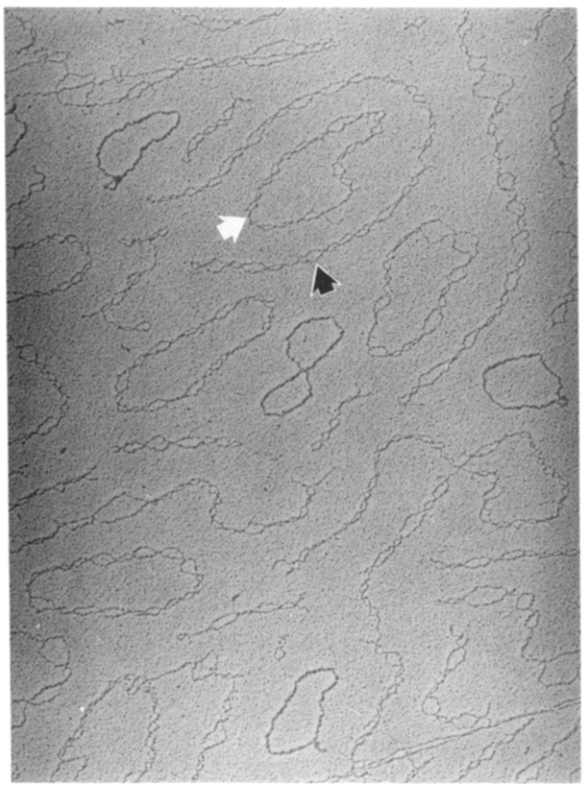

FIGURE 8: Electron micrograph of chromatin cross-linked by trimethylpsoralen. Intact chromatin (30–60 nucleosomes in length) was treated with 10 μg/mL TMP and irradiated for 5 min with near-UV light (320–380 nm) on ice with a 450-W medium-pressure mercury lamp. Electron micrographs were made of the TMP-cross-linked DNA (black arrow) under denaturing conditions so the DNA single strands are joined only at cross-links (creating the appearance of "bubbles"). The closed circular DNA present in the micrographs is from TMP-cross-linked SV-40 chromatin that has been added to the samples as an internal size standard. Some of these SV-40 DNA molecules contain single-strand nicks and, thus, give rise to regularly occurring bubbles (white arrow) with a minimum size that is presumed to be 1 nucleosome in length.

30-min irradiation times was determined to be 3.7 ± 0.7, 1.5 ± 0.8, and 1.2 ± 0.5 nucleosomes, respectively. The average

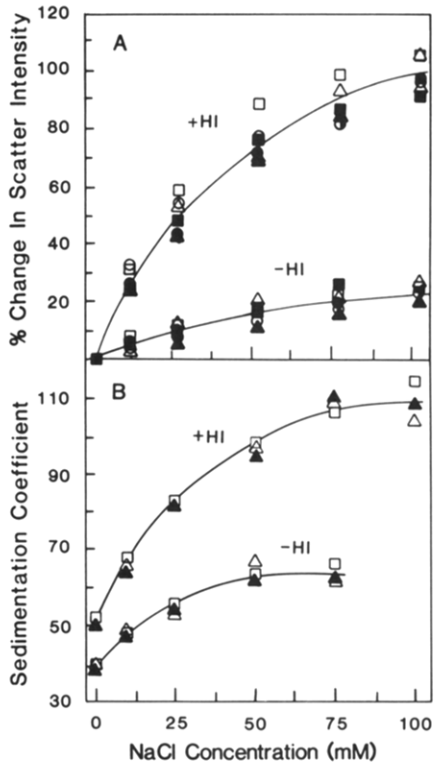


FIGURE 9: (A) Light scattering intensity of chromatin as a function of NaCl concentration following treatment with trimethylpsoralen. The percent change in light scattering from 0 mM NaCl was measured for intact (upper curve) and histone H1 depleted (lower curve) chromatin fibers having an average length of 16 nucleosomes. Samples were treated with 10 μg/mL TMP and were either kept in the dark (■) or irradiated for 10 s (○) or 1 (●), 5 (△), or 30 (▲) min with near-UV light (320–380 nm). As an additional control, a sample not treated with TMP (□) was also examined. Each data point represents the mean of two independent determinations. (B) Sedimentation coefficient of chromatin as a function of NaCl concentration following treatment with TMP. Sedimentation coefficients ($s_{20,w}$) were measured for intact (upper curve) and histone H1 depleted (lower curve) chromatin having an average length of 32 nucleosomes. Samples were treated with 10 μg/mL TMP and irradiated for 0 (□), 5 (△), or 30 (▲) min with near-UV light (320–380 nm). Data points represent a single determination.

distance between cross-links for the 30-min irradiation time is most likely an overestimation, due to the presence of multiple cross-links in the linker DNA at this long photoreaction time. It should also be noted that TMP monoadducts, not detected by this analysis, may also be present in the chromatin fibers at even higher yields than the cross-links (Kanne et al., 1982).

*Effect of Photodamage by TMP and Near-UV Light on Chromatin Folding.* The folding of chromatin fibers treated with TMP and irradiated for varying times with UV light (320–380 nm) was examined in the same manner as described earlier for 254-nm UV-damaged fibers. In addition, two different controls were used: (1) fibers not treated with TMP and irradiated and (2) fibers treated with TMP but not irradiated. The results of these experiments are shown in Figure 9. Both the light scattering and sedimentation velocity data indicate that no significant change in the overall folding equilibrium occurred in intact or H1 depleted fibers at any level of TMP damage examined.

## Discussion

The results of this study demonstrate that the folding of chromatin into the compact 30-nm structure in vitro is not significantly altered by the presence of DNA photodamage induced by either UV light at 254 nm or TMP (plus near-UV radiation). Using both light scattering and sedimentation

velocity measurements to follow the salt-induced folding of intact and histone H1 depleted fibers, no change in the folding characteristics could be detected even at levels of one (or more) TMP cross-link (and presumably one, or more, TMP monoadduct) per nucleosome or ~1 PD per 60 bp. This was the case even though significant changes in the circular dichroism of DNA were detected at the higher UV doses.

It is perhaps surprising that such large, nonphysiological amounts of DNA photodamage had little effect on chromatin folding, especially on the structure of the compact 30-nm fiber. One possible explanation concerns the actual *degree* of compaction of the 30-nm fiber in vitro. It has been suggested that chromatin isolated in low salt, as in these studies, will not form as compact a structure in vitro as chromatin isolated in higher, physiological ionic strengths (Zentgraf & Franke, 1984). If this were the case, a more compact fiber in vivo may place a higher restriction on the level of damage tolerated for complete chromatin folding. However, this view has not been supported by X-ray diffraction data, which yield similar dimensions for chromatin refolded in vitro and in whole chicken erythrocytes (Widom & Klug, 1985) or isolated nuclei (Langmore & Schutt, 1980; Langmore & Paulson, 1983). Furthermore, Butler and Thomas (1980) have compared the folding of chromatin isolated in 65 mM NaCl to the folding of chromatin isolated by hypotonic lysis using sedimentation velocity measurements and found no difference in the 30-nm structure. In contrast, Walker and Sikorska (1987) have recently reported that chromatin isolated in 100 mM NaCl forms more compact fibers than fibers isolated at low ionic strength and then exposed to 100 mM salt. Thus, it is unclear whether chromatin refolded in vitro obtains the same level of compaction as the 30-nm fiber in vivo and whether the potentially higher level of compaction might be more sensitive to DNA photodamage. It should be noted that even the 30-nm fiber in vivo must retain a moderate amount of longitudinal flexibility required for the bending of these fibers into higher ordered structures such as chromosomes (Yabuki et al., 1982). Perhaps it is this inherent nature of chromatin, to form a 30-nm fiber not too compact so as to allow bending, that enables this structure to absorb distortions in the DNA helix by PD and TMP cross-links.

Another consideration is the nature of the DNA distortion caused by UV light at 254 nm and by TMP cross-links. In the case of PD, there is good agreement on the degree of DNA helix unwinding and distortion caused by these adducts (see the introduction). Alterations in DNA structure by PD are also observed on the basis of changes in sedimentation (Marmur et al., 1961), thermal denaturation (Marmur et al., 1961), and CD spectra (Vorlickova et al., 1979; Lang & Luck, 1973). Indeed, the CD data presented in Figure 7 support these findings.

The data concerning the structure of TMP cross-links are more controversial. With the extent of DNA helix unwinding and bending caused by TMP currently unresolved (see the introduction), it is not clear as to the nature of the helix distortion being successfully packaged into the 30-nm fiber. Our results that the DNA helix distortion caused by TMP can be folded into the 30-nm fiber of chromatin with no detectable changes in the overall structure of the fiber might suggest that the actual helix distortion caused by TMP may be less than originally estimated by Pearlman et al. (1985) or that the TMP cross-link may retain considerable flexibility (Tomic et al., 1987). This flexibility may then allow the photoadducts to conform to the geometrical requirements of the 30-nm fiber. Whatever the reason, our results are supported by the report by Conconi et al. (1984) showing that TMP cross-links did not appear to alter the salt-induced compaction of chromatin visualized by electron microscopy.

Finally, an impetus for these studies was the possibility that bulky DNA photoadducts might alter the structural transitions of the nucleofilament, and these alterations may play a roll in the recognition and eventual repair of DNA photodamage. It is apparent from this work that these transitions are not significantly altered by even large amounts of DNA photodamage. Thus, it is unlikely that gross alterations in the 30-nm-fiber structure of chromatin, such as local unwinding of the 30-nm fiber, play a role in the recognition and repair of these adducts. Repair enzymes, therefore, must recognize more subtle structural features of the damaged sites.

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