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Deoxyribonucleic Acid Excision Repair in Chromatin after Ultraviolet Irradiation of Human Fibroblasts in Culture†

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ABSTRACT: We have exposed confluent normal human fibroblasts to ultraviolet (UV) fluences of 5, 14, or 40 J/m^2 and monitored the specific activity of post-UV repair synthesis in chromatin with [^{3}H]thymidine pulses. We have shown that under conditions where no semiconservative deoxyribonucleic acid (DNA) synthesis is detectable, the specific activity of repair label in micrococcal nuclease resistant (core particle) DNA is about one-fifth that in bulk DNA at all three UV fluences. On the other hand, the distribution of thymine-containing pyrimidine dimers in bulk and nuclease-resistant regions measured either immediately after irradiation or at later times showed no significant differences; preferential labeling of linker (nuclease-sensitive) DNA during repair synthesis is thus apparently not due to a predominance of UV-induced photoproducts in linker relative to core particle DNA in the nucleosome. Pulse and pulse–chase experiments at 14 or 40 J/m^2 with normal human or repair-deficient xeroderma pigmentosum (XP) cells showed that at most 30% of repair label in all these cells shifts from nuclease-sensitive (linker) DNA to nuclease-resistant (core particle) DNA.

Recent work on the structural organization of histones and DNA into repeating monomeric units (nucleosomes) raises interesting questions concerning both the susceptibility of the cellular genome to physical and chemical damage and the accessibility of the genome to enzymes and other possible factors required for the repair of such damage. With respect to the former issue, DNA damage produced by a variety of chemical agents, including trimethylpsoralen plus near-UV light, benzo[*a*]pyrene, dimethylnitrosamine, *N*-acetoxy-2-(acetylamino)fluorene, *N*-hydroxy-2-(acetylamino)fluorene, and bleomycin, has been shown to occur selectively in nuclease-sensitive (linker) regions of nucleosomes (Cooper et al., 1975; Ramanathan et al., 1976a,b; Metzger et al., 1976, 1977; Jahn & Litman, 1977; Cech & Pardue, 1977; Kuo & Hsu, 1978). This distribution of damage is consistent with observations in related studies where DNA repair of certain forms of chemical damage occurs preferentially in linker regions (Ramanathan et al., 1976b; Bodell, 1977; Tlsty & Lieberman, 1978).

It has also been reported (Cleaver, 1977; Smerdon et al., 1978; Smerdon & Lieberman, 1978) that following UV irradiation of human cells, DNA repair as measured by repair synthesis occurs preferentially in linker regions of nucleosomes. However, it has not been established whether or not UV photoproducts such as pyrimidine dimers are similarly preferentially distributed in linker regions of chromatin in intact irradiated cells. The ultimate fate of UV-induced repair synthesis label detected initially in linker regions is also unclear. One group (Cleaver, 1977; J. E. Cleaver and H. Weinbraub, personal communication) has found that repair synthesis label

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in micrococcal nuclease sensitive regions of human chromatin (linker DNA) does not detectably chase into nuclease-resistant regions (core particle DNA), while a second study (Smerdon & Lieberman, 1978) shows almost complete randomization of label between these two regions as a function of time after irradiation.

We have addressed our studies in human cells to these issues since a detailed understanding of the role of nucleosome conformation in DNA repair in human cells has potential relevance to furthering insights into the pathogenesis of certain human diseases characterized by defects in DNA repair. Previous studies from this laboratory (Mortelmans et al., 1976; Friedberg et al., 1977) have suggested that in cells from some of the numerous XP complementation groups the defect in excision repair may involve limited access of repair enzymes to sites of DNA damage in chromatin. In the present study we have confirmed the observations of others that following UV irradiation of normal human fibroblasts, repair synthesis of DNA is preferentially detectable in linker regions of the nucleosome. We show here that this result cannot be interpreted simply in terms of the distribution of pyrimidine dimers in chromatin. We have also followed the fate of repair synthesis label in chromatin and find that contrary to both previously reported pulse–chase studies (Smerdon & Lieberman, 1978; J. E. Cleaver and H. Weintraub, personal communication), in our hands a significant but *limited* (not more than 30%) fraction of repair label initially sensitive to micrococcal nuclease becomes resistant to digestion with this enzyme probe. Finally, we report the results of experiments on two XP cell lines with respect to the distribution of repair label in nucleosomes. Preliminary data from this study have been previously reported (Williams & Friedberg, 1978).

Materials and Methods

Materials. Monolayer cultures of normal human GM38 or GM316 fibroblasts (Institute for Medical Research, Camden, NJ), XP2RO (complementation group E) (CRL1259, American Type Culture Collection), or XP4BE (XP variant) (CRL1162, American Type Culture Collection) fibroblasts were maintained in roller bottles in minimum essential medium (Gibco) supplemented with 10% calf serum (Gibco). Cultures were periodically checked for mycoplasma contamination by the method of Peden (1975) and discarded when $^{14}C/^{3}H$ ratios in excess of 0.01 were found. Micrococcal nuclease (15000 units/mg) from *Staphylococcus aureus* was purchased from Worthington Biochemicals (Freehold, NJ). [*methyl*-^{3}H]Thymidine (50 Ci/mmol), [*methyl*-^{14}C]thymidine (48 mCi/mmol), [5-^{3}H]uridine (20 Ci/mmol), and [2-^{14}C]uracil (55 mCi/mmol) were purchased from New England Nuclear (Boston, MA), and unlabeled nucleosides (thymidine, 5-fluoro-2′-deoxyuridine, 5-bromo-2′-deoxyuridine, and 2′-deoxycytidine) as well as phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Polygram Sil G/UV (Macherey-Nagel and Co.) chromatography plates were obtained from Brinkmann Instruments, Inc. (Westbury, NY). Proteinase K was purchased from Boehringer Mannheim (Indianapolis, IN).

Cell Growth, Labeling, and UV Irradiation Conditions. About 10^5 fibroblasts were seeded in 10 mL of medium in 100-mm diameter plastic petri dishes and incubated at 37 °C until they reached early confluence. At this stage one of two isotope-labeling protocols was used. For repair synthesis experiments, the medium was replaced with that containing [^{14}C]thymidine (0.01 or 0.05 μCi/mL, 48 mCi/mmol) and the cells were incubated for 3–4 days. The radioactive medium was then replaced with fresh unlabeled medium, and the cultures were incubated for at least 1 week prior to irradiation, at which point they were at total confluence. For experiments in which the thymine dimer content of DNA was to be determined, the medium was replaced with that containing [^{3}H]thymidine (2.0 μCi/mL, 50 Ci/mmol), and growth of the cells continued for 3 days. The labeling medium was replaced with nonradioactive medium for at least 16 h prior to irradiation. UV irradiation of cells in the monolayer was carried out as described by Rudé & Friedberg (1977). All cultures were rinsed twice in phosphate buffered saline prior to irradiation. The UV fluence rate was 0.9–1.2 J/(m^2 s).

Isolation of Nuclei. Petri plates were placed on ice, the medium was removed by aspiration, and the cells were rinsed once in phosphate buffered saline before being harvested with a rubber policeman into 2.0 mL of ice-cold 0.15 M NaCl per petri plate. Replicate cultures were pooled for repair synthesis experiments but not for dimer excision experiments. Cells were harvested by centrifugation in an SS-34 Sorvall rotor for 10 min at 1000*g* at 4 °C. The cells were resuspended in cold 50 mM Tris-HCl, pH 7.8, and 5 mM $NaHSO_3$ and harvested again under the same centrifugation conditions. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.9, 5 mM $NaHSO_3$, 3 mM $MgCl_2$, 0.1% Triton X-100, and 100 mM sucrose), placed on ice for 5 min, and then dounced 10–15 times in a tight-fitting glass homogenizer. The homogenate was centrifuged at 3000*g* for 10 min at 4 °C, and the pellet of cell nuclei was washed once in 1.0 mL of nuclease buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM $CaCl_2$, and 50 mM sucrose) with subsequent centrifugation at 3000*g* for 10 min at 4 °C. The washed nuclei were resuspended in nuclease buffer at a concentration of (2–10) × 10^6 nuclei/mL and maintained at 4 °C.

Micrococcal Nuclease Digestion. Micrococcal nuclease (1.0 mg/mL) was diluted 1:100 in nuclease buffer just prior to use. Aliquots of isolated nuclei were brought to a volume of 150 μL with the buffer and preincubated for 3–4 min in a 37 °C water bath before addition of 50 μL of diluted nuclease or nuclease buffer. Incubation was at 37 °C for 0–60 min. Reactions were terminated by the addition of 5 μL of 100 mM Na_2EDTA. Tubes were vortexed vigorously and placed in an ice–water bath. The addition of 1 mM PMSF to the nuclear isolation buffers did not affect the kinetics or extent of DNA degradation by micrococcal nuclease and thus was not routinely done.

Gel Electrophoresis. DNA products of micrococcal nuclease digestion were analyzed by electrophoresis as described by Maniatis et al. (1975) in 5% native polyacrylamide slab gels (10 × 15 × 0.15 cm) using TBE buffer (90 mM Tris-HCl, 90 mM borate, and 2.5 mM Na_2EDTA, pH 8.4) or in 2.8% agarose gels as described by Smerdon et al. (1978). DNA was purified from digested nuclear samples by two extractions with chloroform–isoamyl alcohol (24:1, v/v), one extraction with phenol–chloroform–isoamyl alcohol (24:24:1, v/v), and overnight precipitation at −20 °C with an equal volume of 100% ethanol after raising the salt concentration of the aqueous layer to 0.3 M NaCl. The precipitate was spun down at 10000*g* for 30 min at 4 °C and resuspended in 30 μL of 50 mM Tris-HCl, pH 7.7. Equal volumes of DNA and dye buffer (2× TBE buffer, 40% glycerol, 0.05% bromphenol blue, and 0.05% xylene cyanol) were mixed for electrophoresis. Polyacrylamide gel electrophoresis was carried out at constant voltage (85 V, 17–23 mA) until the bromphenol blue dye marker was near the bottom of the gel, while agarose gel electrophoresis was at 30 V (70 mA) for 9 h. *Hpa*I digests of λDNA of known length were run in polyacrylamide gels, and *Alu*I digests of

pBR322 plasmid DNA were run in agarose gels as DNA size standards. Gels were stained for 30 min in 1 μg/mL ethidium bromide, destained for 60 min in distilled water with one bath change, and photographed with Polaroid film under 313-nm light illumination. Photographic negatives of each polyacrylamide gel channel were scanned with a Joyce-Loebl microdensitometer. Agarose gel slices 4 mm thick were solubilized in 1.0 mL of 0.1 N HCl by autoclaving and then counted by liquid scintillation spectrometry in water plus 0.27% Omnifluor in toluene–Triton X-100 (2:1, v/v).

Repair Synthesis. DNA repair synthesis was measured by a modification of a previously reported procedure (Williams & Cleaver, 1978). Totally confluent cultures prelabeled with [^{14}C]thymidine were treated as follows. All cultures were exposed for 1 h either prior to irradiation or at various times following irradiation to fresh medium containing 10 μM bromodeoxyuridine and 2 μM fluorodeoxyuridine. Control cultures not exposed to UV radiation were similarly treated. The inclusion of bromodeoxyuridine in the incubation medium allowed the monitoring of any semiconservative DNA synthesis in either irradiated or unirradiated cultures by isopycnic sedimentation and did not adversely influence the experimental results when present prior to UV irradiation (see Results). The subsequent pulse-labeling protocol consisted of the exposure of both irradiated and unirradiated cells to medium containing 10 μM bromodeoxyuridine, 2 μM fluorodeoxyuridine, 3 mM hydroxyurea, and [^{3}H]thymidine [10 μCi/mL, 50 Ci/mmol, for 1 h (5 or 40 J/m^2, 0–3 h post-UV), 2 h (14 J/m^2), or 4.5 h (5 or 40 J/m^2, 17–18 h post-UV)].

Following labeling with [^{3}H]thymidine, all petri plates were placed on ice and cells from replicate cultures (3–8 cultures/data point) were harvested. All subsequent steps were carried out at 4 °C unless otherwise stated. In some experiments cultures were rinsed twice in phosphate buffered saline and incubated for varying lengths of time in medium containing 10 μM thymidine and 1.5 μM deoxycytidine prior to harvesting. Nuclei were isolated as described above and were incubated with micrococcal nuclease or nuclease buffer for 30 min. Sodium dodecyl sulfate ($NaDodSO_4$) was added to a final concentration of 0.5% and proteinase K to a final concentration of 200 μg/mL, and samples were incubated overnight at 37 °C. A small portion of each nuclear sample incubated only with nuclease buffer was subjected to alkaline CsCl–$CsSO_4$ isopycnic sedimentation (Cleaver, 1975), and all remaining nuclear lysates were divided into thirds and each third was spotted directly onto a Whatman 3 MM paper filter (100 μL/filter). The filters were dried, washed in 5% trichloroacetic acid (Cl_3AcOH), and rinsed with 70, 95, and 100% ethanol, respectively. The filters were redried and acid-insoluble ^{3}H and ^{14}C radioactivity was measured by liquid scintillation spectrometry.

Measurement of Thymine Dimers in DNA. Petri plates containing confluent GM38 cells were prelabeled with [^{3}H]thymidine (2 μCi/mL, 50 Ci/mmol), exposed to UV radiation, and then incubated at 37 °C for varying periods of time. Cells were harvested in cold 0.15 N NaCl by scraping with a rubber policeman, and the nuclei were isolated and resuspended in 400 μL of nuclease buffer. Aliquots (150 μL) of nuclei were incubated for 30 min at 37 °C with 50 μL of micrococcal nuclease (final concentration 2.5 μg/mL) or with 50 μL of nuclease buffer. Reactions were terminated by the addition of 5 μL of 100 mM Na_2EDTA, the tubes were placed in an ice bath, and 200 μL of calf thymus DNA (1.0 mg/mL) and 0.50 mL of 10% Cl_3AcOH were added. After 10 min at 4 °C, the tubes were centrifuged at 1000*g* for 15 min and the supernatants discarded. The precipitates were hydrolyzed in 95% formic acid and analyzed by thin-layer chromatography (Cook & Friedberg, 1976). Thymine dimer contents were expressed as the percent of total ^{3}H radioactivity present in the dimer-containing region of the chromatogram. Total ^{3}H radioactivity recovered from the chromatograms ranged from 5.4×10^4 to 29.1×10^4 cpm for samples incubated without micrococcal nuclease and 1.1×10^4 to 11.9×10^4 cpm for samples digested with micrococcal nuclease; all samples were counted until at least 10^3 total counts had accumulated at the dimer peak position.

Results

Characterization of Micrococcal Nuclease Digestion Products. Incubation of ^{14}C-labeled GM38 cell nuclei with micrococcal nuclease for increasing periods of time progressively reduced the amount of radioactivity retained on filter papers washed in 5% Cl_3AcOH (data not shown). This observation has been extensively discussed by other authors (Hewish & Burgoyne, 1973; Axel et al., 1974; Kornberg & Thomas, 1974), who have pointed out that the digestion kinetics are not simple first order, suggesting the existence of relatively resistant regions of DNA in chromatin. Detailed characterization of the products of digestion of fibroblast nuclei with micrococcal nuclease was carried out by polyacrylamide gel electrophoresis. We extracted DNA from samples digested for varying periods of time at two different enzyme–substrate ratios and analyzed the ethanol-precipitable fragments by using DNA *Hpa*I restriction fragments of known lengths as size markers. We observed the appearance of DNA fragments less than 200 base pairs long soon after initiating incubation with micrococcal nuclease (Figure 1). Further incubation yielded DNA peaks of 125 ± 10 base pairs and 140 ± 10 base pairs with a prominent shoulder of DNA fragments of smaller size (Figure 1). Digestion for 30 min or longer [$\alpha \geq 75$ (μg min)/mL; see Figure 1] reproducibly yielded DNA gel patterns very similar to those reported by others following limit digestion of nuclei or chromatin with micrococcal nuclease (Sollner-Webb & Felsenfeld, 1975; Greil et al., 1976; Lohr et al., 1977). The DNA gel patterns thus provided direct evidence that the acid-insoluble DNA fraction at longer times of digestion is representative of only core particle and subcore particle DNA. Since our objective in examining parameters of DNA repair in micrococcal nuclease resistant fractions was to specifically eliminate all linker DNA, we routinely digested nuclei for 30 or 60 min [$\alpha \geq 75$ (μg min)/mL].

Repair Synthesis in Bulk and Core Particle DNA. Repair synthesis can be distinguished from semiconservative DNA synthesis by isopycnic sedimentation (Pettijohn & Hanawalt, 1964). The sedimentation profiles of bulk GM38 DNA in alkaline CsCl–$CsSO_4$ gradients from a typical experiment are shown in Figure 2. A 1-h pulse with [^{3}H]thymidine after exposure of cells to 40 J/m^2 of UV radiation revealed all ^{3}H radioactivity at the position of normal density DNA ($\rho = 1.67$ g/cm^3) and coincident with the ^{14}C prelabel peak. ^{3}H radioactivity was detected only in normal density DNA in control experiments in which ^{14}C prelabeling of the DNA was omitted. Although hydroxyurea was routinely added to cultures, this did not occur until 1 h following exposure to bromodeoxyuridine (see Materials and Methods). Thus, any significant semiconservative DNA synthesis occurring during this time should have resulted in sufficient bromodeoxyuridine incorporation to shift the density of the DNA. The results shown in Figure 2 indicate that the use of cultures maintained in total confluence together with the effects of UV radiation completely inhibits detectable semiconservative DNA synthesis. The

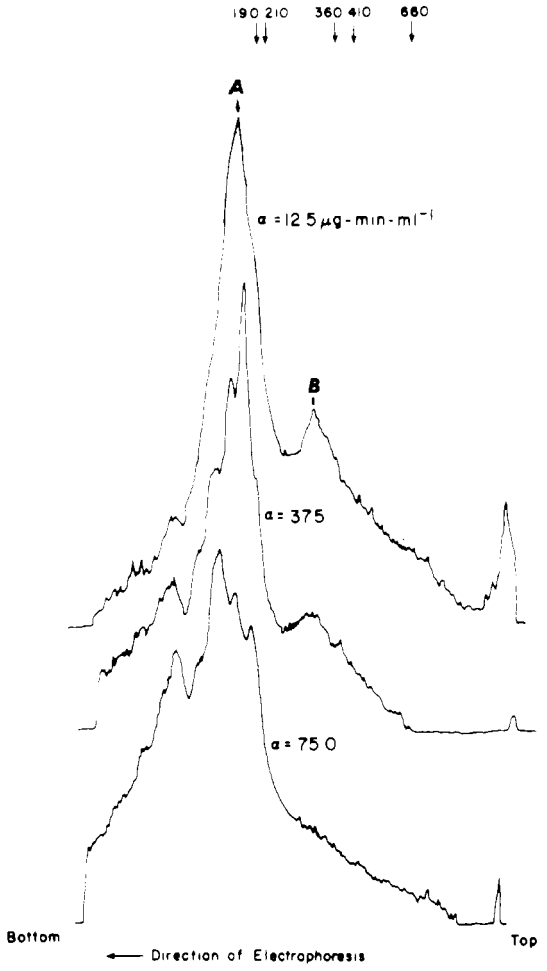

FIGURE 1: Native 5% polyacrylamide gel profiles of GM38 DNA extracted from 2×10^5 nuclei digested with micrococcal nuclease for increasing lengths of time. The product of final nuclease concentration (micrograms per milliliter) and time of digestion (minutes) is denoted, and *Hpa*I restriction digest fragments of λDNA included in each gel are indicated at the top of the figure. A prominent early monomer core particle peak appears at 160 base pairs (A) and is replaced with new peaks at 140 and 125 base pairs with further nuclease digestion. A small dimer peak at ~340 base pairs (B) appears very early after digestion begins and gradually diminishes at later times [$\alpha \geq 12.5$ (μg min)/mL].

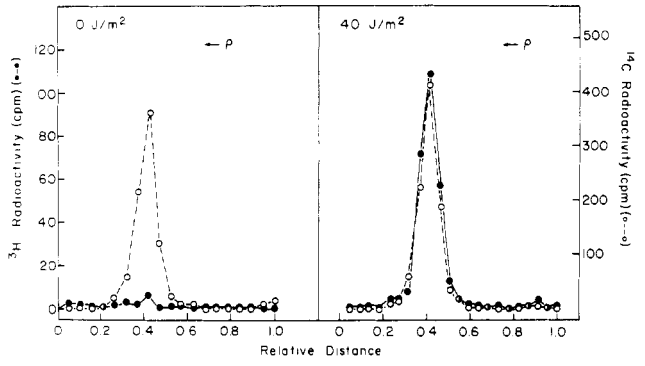

FIGURE 2: Alkaline cesium chloride–cesium sulfate isopycnic gradient profiles of bulk GM38 DNA. Cultures were exposed to 0 J/m² (left panel) or 40 J/m² (right panel) of UV light, and repair synthesis was detected with [³H]thymidine (●—●) as described under Materials and Methods. Positive control experiments revealed DNA synthesized during normal semiconservative replication bands at a density coincident with a relative distance up the gradient of 0.1–0.2, while DNA bases inserted during repair synthesis band at the same position as the [¹⁴C]thymidine (O---O) peak (relative distance 0.3–0.5).

exclusive incorporation of ^3H into normal density DNA (repair synthesis) was also observed in cultures pulse labeled at other

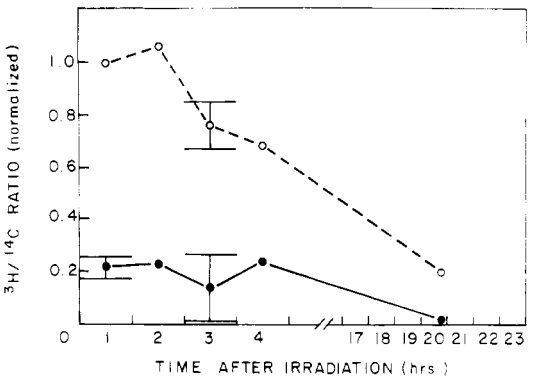

FIGURE 3: Normalized specific activities of repair synthesis label in bulk (O) and core particle (●) GM38 DNA after 40 J/m² of UV light as determined with 60-μL aliquots of lysed nuclei spotted directly on Whatman 3 MM paper filters. The pulse-labeling interval in these experiments was 1 h. All specific activities (expressed as $^3H/^{14}C$ ratios) are normalized such that the specific activity of bulk DNA during the first hour after irradiation is taken as 1.0. Core particle DNA was analyzed after a 30-min micrococcal nuclease digestion (2.5 μg/mL). Error bars represent standard errors of the mean and are omitted by definition (bulk DNA, first hour) or when only one sample was analyzed. The range of acid-insoluble ^3H counts per incubation tube was bulk DNA = 500–960 cpm and core particle DNA = 20–73 cpm (based on a minimum of 10-min scintillation counting per sample).

times after UV irradiation (data not shown). The absence of detectable semiconservative DNA synthesis in unirradiated or irradiated cultures under our experimental conditions obviates potential artifacts due to the presence of bromodeoxyuridine in cells irradiated with UV (e.g., a different spectrum of photoproducts) since bromodeoxyuridine will not have been incorporated into DNA in these cultures.

The lack of detectable semiconservative DNA synthesis also indicated that the routine separation of normal density DNA by isopycnic sedimentation was not necessary. For the quantitation of repair synthesis we therefore measured acid-insoluble ^3H and ^{14}C radioactivity by precipitation of samples directly onto Whatman paper filters. The fraction of acid-insoluble ^{14}C radioactivity after nuclease digestion was found to be very reproducible within any given experiment but to vary slightly between experiments. This is most likely due to slight fluctuations in nuclease concentration and enzyme–substrate ratio. The normalized results (Figure 3) showed a decreased $^3H/^{14}C$ ratio in core particle DNA relative to bulk DNA. In experiments in which repair synthesis was measured at very late times after UV irradiation (17.5–22 h), a longer [^3H]thymidine pulse time was used because of the decreased overall rate of repair synthesis at these times. Nonetheless, the $^3H/^{14}C$ ratios remained about one-fifth of the $^3H/^{14}C$ ratios found in bulk DNA (Figure 3).

The use of hydroxyurea during post-UV labeling has been reported to increase repair synthesis (Clarkson, 1978). We preferred including hydroxyurea during our method of analysis to suppress any residual semiconservative DNA synthesis. We tested the influence hydroxyurea had on our results by comparing the normalized $^3H/^{14}C$ ratio (cf. Figure 3) in core particle DNA from GM38 cultures exposed to 14 J/m² and ^3H labeled in the absence or presence of hydroxyurea. The normalized values found were 0.46 ± 0.27 without hydroxyurea and 0.53 ± 0.05 with hydroxyurea, indicating that hydroxyurea does not substantially affect the preferential insertion of nucleotides into linker DNA during repair synthesis. This result was verified by examining ^3H and ^{14}C agarose gel profiles after a 2-min micrococcal nuclease digestion of ^{14}C-prelabeled GM38 nuclei exposed to 14 J/m² and pulse labeled with ^3H during repair synthesis in the

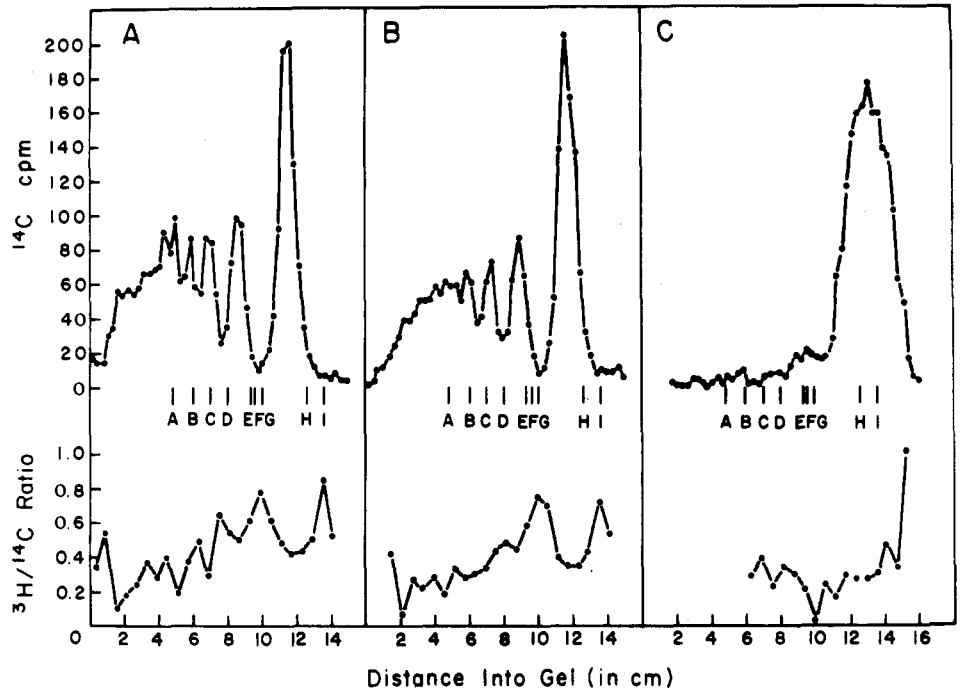

FIGURE 4: Agarose gel profiles of repair synthesis label in GM38 DNA. Nuclei isolated from GM38 cultures exposed to 14 J/m^2 of UV light and pulse-labeled with [^{3}H]thymidine for 2 h in the absence (panel A) or presence (panels B and C) of hydroxyurea were digested for 2 min (panels A and B) or 30 min (panel C) with micrococcal nuclease. Ethanol-precipitated DNA was electrophoresed in a 2.8% agarose gel until the bromphenol blue dye marker had run more than 13 cm (>250 V h). *Alu*I digestion fragments of pBR322 plasmid DNA were run in parallel wells as DNA size standards. Fragment sizes (Sutcliffe, 1978): A = 910; B (doublet) = 655, 659; C = 521; D = 403; E = 281; F = 257; G = 226; H = 136; I = 100 base pairs. The ^{3}H/^{14}C profiles were generated by using mean ^{3}H/^{14}C values for every two gel slices to reduce fluctuations due to gel slice cutting errors. The ^{3}H/^{14}C profile reflects the distribution of repair synthesis label, and the ^{14}C profile reflects the distribution of total DNA. Total recovered counts per well: ^{3}H = 799–1116 cpm; ^{14}C = 1990–2735 cpm. Percent acid-insoluble DNA: (panel A) 88%; (panel B) 89%; (panel C) 18%.

absence or presence of hydroxyurea. The profiles were similar in both cases (parts A and B of Figure 4), as expected if hydroxyurea does not alter the general pattern of repair synthesis labeling within the nucleosome.

The preferential digestion of ^{3}H repair label by micrococcal nuclease suggests strongly that repair synthesis occurs primarily in linker regions of DNA. In order to demonstrate this more directly and simultaneously consider the theoretical possibility that ^{3}H label was incorporated into nonnucleosomal DNA which is nuclease sensitive, we carried out the following experiment. Cells were prelabeled with [^{14}C]thymidine, exposed to UV light, and then pulsed with [^{3}H]thymidine as described above and in the legend to Figure 4. The isolated nuclei were incubated with micrococcal nuclease for short times (2 min) (parts A and B of Figure 4) or longer times (30 min) (Figure 4C). DNA was extracted and subjected to agarose gel electrophoresis, and the distributions of both isotopic labels were determined. As indicated in Figure 4B, after a brief digestion with nuclease the maximal ^{3}H/^{14}C ratios occurred at the sites of ^{14}C minima, i.e., the expected positions of linker DNA in polynucleosomes of increasing unit size. This is well documented in mono-, di-, and trinucleosomes since these are best resolved in the gels relative to nucleosomes of higher order. After 30 min of incubation with micrococcal nuclease, the ^{3}H/^{14}C ratio is essentially uniform throughout all gel regions except the region corresponding to the lowest molecular weight DNA (Figure 4C). This result suggests that after the longer time of digestion most ^{3}H radioactivity is coincident with the single peak of ^{14}C radioactivity expected in the position of monomeric core particle DNA. Thus, we conclude that with increasing times of incubation, ^{3}H was preferentially lost from linker regions of DNA. In addition, the periodicity in the distribution of ^{3}H label (parts A and B of Figure 4) as distinct from a continuous smear in the gel

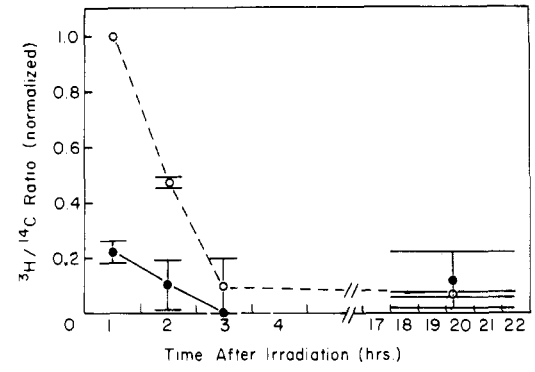

FIGURE 5: Normalized specific activities of repair synthesis in GM38 bulk (○) and core particle (●) DNA after 5 J/m^2 of UV light as determined from acid-insoluble ^{3}H and ^{14}C radioactivity in aliquots of lysed nuclei spotted directly on Whatman paper filters after 0- or 30-min incubations with micrococcal nuclease. Error bars and the pulse-labeling interval are as described in the legend to Figure 3. Percent acid-insoluble ^{14}C counts in 30-min digestion samples = 4–11%.

indicates that most ^{3}H label was associated with nucleosomal rather than with nonnucleosomal DNA.

The repair synthesis results obtained by the method of analysis employed in these experiments were not a function of the relatively high UV dose (40 J/m^2) used. Qualitatively similar results were obtained in experiments in which the cells were irradiated at a dose of 5 J/m^2 (Figure 5). The rate of repair replication in bulk DNA after 5 J/m^2 decreased more rapidly than at 40 J/m^2, presumably reflecting the fact that at the lower UV dose a greater fraction of lesions were repaired per unit time. No differences in the specific activity of repair label in bulk and core particle DNA could be detected at late times after irradiation of cells with 5 J/m^2 of UV light.

Most XP cells are highly defective in nucleotide excision

Table I: Summary of Pulse–Chase Experiments with Normal or Xeroderma Pigmentosum Cells[a]

cell line	UV dose (J/m^2)	incubn after [^{3}H]-thymidine labeling (chase time) (h)	normalized sp act.[b] of repair synthesis ($^3H/^{14}C$) (% ± SEM)		sp act. ratio: $\frac{\text{with mn}}{\text{no mn}} \times 100\%$
			total DNA (no mn)[c]	nuclease-resistant DNA (with mn)	
GM38	40	0	100	22 ± 13	22
		3	125 ± 7	44 ± 6	35
		22	69 ± 14	31 ± 15	45
	40	0	100	17 ± 6	17
		3	113 ± 2	27 ± 9	24
		22	113 ± 2	30 ± 8	27
	14	0	100	22 ± 3[d]	22
		4	82 ± 2	25 ± 4[d]	30
	14	0	100	16 ± 2	16
		4	82 ± 2	19 ± 1	23
GM316	14	0	100	26 ± 1[d]	26
		4	112 ± 10	36 ± 7[d]	32
	14	0	100	21 ± 5	21
		4	112 ± 10	29 ± 9	26
XP2RO (group E)	14	0	100	15 ± 20[d]	15
		4	94 ± 5	25 ± 16[d]	27
	14	0	100	7 ± 3	7
		4	94 ± 5	16 ± 1	17
	14	0	100	20 ± 6	20
		4	101 ± 4	24 ± 6	24
XP4BE (variant)	14	0	100	17 ± 16[d]	17
		4	138 ± 14	36 ± 12[d]	26
	14	0	100	15 ± 14	15
		4	138 ± 14	34 ± 25	25

[a] Acid-insoluble ^{3}H and ^{14}C radioactivities were determined for nuclei isolated in the absence (40 J/m^2) or presence (14 J/m^2) of 1 mM PMSF and incubated for 60 min (unless otherwise noted) with or without micrococcal nuclease (2.5 μg/mL). All $^3H/^{14}C$ ratios were calculated with correction for $^3H/^{14}C$ values observed with unirradiated control cultures. Error values quoted for normalized $^3H/^{14}C$ ratios are standard errors of the mean for 2–3 replicate incubations per data value. Percent acid-insoluble ^{14}C counts, digested samples = 7–29%. [b] Calculated relative to $^3H/^{14}C$ ratio of total DNA pulse labeled immediately after irradiation. [c] mn = micrococcal nuclease digestion. [d] 30-min digestion as described in procedure under Materials and Methods.

repair following UV irradiation, and their levels of residual repair synthesis are too low to be detected by the experimental approach used in these studies. However, XP cells of complementation group E carry out 40–70% of the level of unscheduled DNA synthesis shown by normal cells (Kraemer et al., 1975; Takebe et al., 1977), while XP variant cells show no detectable quantitative defect in repair synthesis (Cleaver, 1972). An examination of the distribution of repair label in nuclease-resistant regions of chromatin in these cells showed no significant differences from normal cells (Table I, 0-h chase time).

The observed differences in the $^3H/^{14}C$ ratios in bulk vs. core particle DNA are open to a number of possible interpretations which are elaborated under Discussion. The simplest is that the absolute rate of repair synthesis is faster in linker than in core particle regions of the nucleosome. Such an explanation, however, assumes that there was little or no redistribution of histones during the course of repair synthesis. In order to examine this question directly, we carried out pulse–chase experiments with normal or XP cultures irradiated at 14 or 40 J/m^2, pulsed for 1 h (40 J/m^2) or 2 h (14 J/m^2) with [^{3}H]thymidine, and then chased for 0, 3, or 22 h (40 J/m^2) or for 0 or 4 h (14 J/m^2) in unlabeled medium containing thymidine and deoxycytidine. The levels of acid-insoluble ^{3}H and ^{14}C were determined by spotting samples directly onto filters as described above. As indicated in Table I, in some experiments the $^3H/^{14}C$ ratio in bulk (total) DNA during pulse–chase experiments was higher than the ratio obtained in pulse experiments without the chase. This increase was not due to residual semiconservative DNA synthesis since in all these experiments this parameter was specifically monitored by isopycnic sedimentation. It is thus most likely that an increased $^3H/^{14}C$ ratio in bulk DNA reflects some continued repair synthesis during the chase, while a decreased ratio probably reflects slight fluctuations in ^{14}C uptake. When the normalized $^3H/^{14}C$ ratios in bulk and core particle DNA (Table I, columns 4 and 5) for the pulse–chase experiments were compared, we reproducibly observed that during the first 3–4 h after pulse labeling the specific activity ratio (Table I, column 6) increased slightly in core particle DNA relative to bulk DNA, with an additional small increase during the next 19 h (40 J/m^2) of chase. These results suggest that a *limited* reassortment of nucleosomes does occur in vivo, resulting in a shift of repair label from micrococcal nuclease sensitive to resistant regions.

Thymine Dimer Excision from Bulk and Core Particle DNA. The differences in the specific activity of repair synthesis in bulk and core particle DNA described above suggested that the extent of induction or removal of thymine dimers might be substantially lower in core particle DNA than in bulk DNA. We examined these parameters directly by incubating samples of nuclei isolated from GM38 and XP4BE (variant) cultures at 0, 3–7, or 21–24 h post-UV in the presence or absence of micrococcal nuclease and measuring the thymine dimer content of the acid-insoluble fractions. Our analysis revealed an initial thymine dimer content of 0.239% (GM38, 40 J/m^2) or 0.109% (XP4BE, 14 J/m^2) for bulk DNA and a value of 0.224% (GM38, 40 J/m^2) or 0.102% (XP4BE, 14 J/m^2) for core particle DNA (Table II). The difference between the bulk and core DNA values is not significant, indicating that thymine-containing pyrimidine dimers are not underrepresented in the core particle region of the nucleosome immediately after UV irradiation. A comparison of the thymine dimer contents of bulk and core particle DNA in both cell lines at 3–7 or 21–24 h post-UV incubation showed an eventual decrease in thymine dimer

Table II: Thymine Dimer Content in Bulk or Core Particle DNA as a Function of Post-UV Incubation Time in Human Cells after 14 (XP4BE) or 40 (GM38) J/m²

cell line	thymine dimer content (% ± SEM)		
	immediately post-UV irradn	3–7 h post-UV irradn	21–24 h post-UV irradn
GM38	0.239 ± 0.022 (N = 11)	0.245 ± 0.044 (N = 8)	0.124 ± 0.019 (N = 6) (bulk DNA)
	0.224 ± 0.035 (N = 10)	0.221 ± 0.047 (N = 8)	0.123 ± 0.014 (N = 5) (core DNA)
XP4BE (variant)	0.109 ± 0.012 (N = 2)	0.103 ± 0.010 (N = 2)	0.050 ± 0.008 (N = 2) (bulk DNA)
	0.102 ± 0.030 (N = 2)	0.080 ± 0.012 (N = 2)	0.046 ± 0.015 (N = 2) (core DNA)

content in both of these fractions, but again did not reveal any significant differences in the extent of dimer removal (Table II).

Discussion

The use of nonspecific nucleases such as micrococcal nuclease and DNase I to define the nucleosome as a basic unit of chromatin secondary structure has stimulated a new approach to fractionating DNA for repair studies. Several investigators have reported a reduced specific activity of DNA repair synthesis within nuclease-resistant DNA following treatment of mammalian cells with alkylating agents (Bodell, 1977; Tlsty & Lieberman, 1978) or UV light (Cleaver, 1977; Smerdon et al., 1978; Smerdon & Lieberman, 1978; J. E. Cleaver and H. Weintraub, personal communication). The reduced repair synthesis in nuclease-resistant DNA following chemical treatment could be due to preferential modification of noncore particle DNA by the agent used (Ramanathan et al., 1976a; Metzger et al., 1976, 1977; Jahn & Litman, 1977; Cech & Pardue, 1977; Kuo & Hsu, 1978), thus precluding any significant conclusions concerning the site specificity of chemically induced DNA repair.

In the present studies we have confirmed the results of others that repair synthesis after UV irradiation occurs primarily in noncore particle DNA. Assuming that ~70% of the DNA in chromatin is in nuclease-resistant core particles, we calculate that there is about a 15-fold greater probability per unit length of DNA that an [^{3}H]thymine base will be inserted in linker DNA than in core particle DNA during UV-induced repair synthesis. This distribution of repair synthesis label cannot be accounted for solely on the basis of the distribution of damage since our measurements of thymine dimer contents immediately following irradiation of cells and at various times of post-UV incubation (Table II) show no significant differences between total chromatin and core particles. The statistical variations in the measurement of thymine dimer content preclude a definitive statement that these lesions are totally randomly distributed in core and linker regions of the nucleosome. However, if there were 15 times as many dimers in linker regions as in core particles, this would have been readily detectable experimentally.

What then is the basis for the observed differences in the distribution of repair label? A number of possible interpretations are tenable. The most interesting in our view is that DNA repair synthesis occurs in both linker and core DNA, but occurs preferentially in the former due to specific structural and/or regulatory phenomena associated with chromatin structure. The observation that repair synthesis occurs preferentially in noncore particle DNA but that thymine dimer removal does not may seem contradictory at first glance. However, previous studies from our laboratory (Ehmann et al., 1978) as well as studies by Williams & Cleaver (1978) have supported a "patch-and-cut" mechanism (Haynes, 1966) of nucleotide excision repair in mammalian cells; i.e., endonucleolytic incision of DNA adjacent to dimers and repair synthesis are early and possibly rate-limiting events, while actual thymine dimer removal from the DNA is a later event that may occur after any necessary pertubations in chromatin structure are completed. There are several other possible explanations for our results on the distribution of repair label in chromatin. One is that repair patches are significantly smaller in core particle regions than in linker regions, a hypothesis that is difficult to test directly with present technical methods for patch size measurements in DNA less than 200 base pairs long. Another possible explanation is that the position of nucleosome shifts during the course of DNA repair. Thus, for instance, repair label could be inserted preferentially into core particles and then shift rapidly into nuclease-sensitive linker regions. Alternatively, the observation of a low level of repair synthesis in core particle DNA may be a reflection of the shift of some repair label from nuclease-sensitive to nuclease-resistant regions. Other workers have investigated the question of nucleosome shift or rearrangement during DNA repair and have reached conflicting conclusions. J. E. Cleaver and H. Weintraub (personal communication) have found that in primary human as well as in chick and hamster fibroblasts repair label initially detected in nuclease-sensitive sites remained so for at least 3 days in confluent cultures and for at least one round of DNA replication in growing cultures. However, in viral-transformed cells in which confluence was not associated with arrest of semiconservative DNA synthesis, label in intracellular pools continued to enter DNA during a chase in unlabeled medium, resulting in a uniform distribution in both nuclease-sensitive and -resistant DNA and giving a false impression of rearrangement of linker and nucleosomal DNA. On the other hand, Smerdon & Lieberman (1978) found that in human diploid fibroblasts nucleotides inserted during repair synthesis became progressively more nuclease resistant with increasing chase times. They reported a rapid rearrangement (within 4–5 h) of greater than 85% of the repair synthesis sites in these cells.

The potential for artifacts in the interpretation of pulse–chase experiments as a result of semiconservative DNA synthesis is an important consideration in the studies referred to above as well as in our own experiments. For this reason, we specifically monitored this parameter in all the experiments shown in Table I. We observed a small but reproducible increase in the specific activity ratio of ^{3}H label in nuclease-resistant DNA relative to that in bulk DNA in the absence of semiconservative DNA synthesis in all cell lines tested (Table I). At UV doses of both 14 and 40 J/m² this increase was ~10–15% during the first 3–4 h of chase, with a further increase of up to 10% over the course of the next 19 h. On the basis of the known relative size of core particle and linker DNA, this increase represents a calculated net shift of ~15% of repair label from a nuclease-sensitive to a nuclease-resistant state. Total randomization of label between linker and core DNA would involve a shift of ~55% of the label from the former to the latter fraction. Thus, a net shift of 15% calculated from our data represents a randomization of ~30% of the label originally present in linker DNA. Qualitatively, our results are intermediate with those of

Smerdon & Lieberman (1978) and those of J. E. Cleaver and H. Weintraub (personal communication); although we detected some reassortment of repair synthesis label, we did not observe the same magnitude of label shift as that reported by the former authors. A possibly crucial difference between our experiments and those of Smerdon & Lieberman (1978) is that their results apply to nuclease-sensitive repair label degraded during the *initial* stages of micrococcal nuclease digestion while our data were collected following much longer times of enzyme digestion. It is an intriguing possibility that different regions of chromatin are attacked by micrococcal nuclease at different stages of nuclease digestion, the more easily digested chromatin displaying enhanced randomization of nucleosomes and hence of repair label. This idea is supported by observations of unique structural and functional properties in nucleosomes released at early stages of nuclease digestion (Bloom & Anderson, 1978; Sanders, 1978).

A specific and exclusive interpretation of our results in terms of the molecular events occurring in the chromatin of UV-irradiated cells is still not possible. However, our data are not inconsistent with our original hypothesis, i.e., that early events in nucleotide excision repair (perhaps specific endonucleolytic incision as well as repair synthesis) require unfolding and/or dissociation of histones from DNA. The specific mechanism and kinetics of this process may facilitate more rapid repair events in linker than in core particle DNA. Following dimer excision (as a late event) and DNA ligation, we hypothesize that histone–DNA reassociation or folding occurs and may be associated with some shift (perhaps as much as 30%) in the position of nucleosomes relative to the prerepair state.

Finally, it should be noted that if the DNA repair events measured in normal cells reflect specific perturbations in chromatin conformation, no defects in these perturbations are detectable in XP cells of complementation group E or in XP variant cells (Table I). Unfortunately, other XP complementation groups are not amenable to this type of experimental analysis since they suffer too extensive a defect in repair synthesis.

Acknowledgments

We gratefully acknowledge Drs. Rich Reynolds, Tom Bonura, and Cliff Selsky and Eric Radany for discussions and criticism which have aided us in this work. We also thank Dr. Linda Robinson for a generous gift of *Hpa*I digest fragments of unlabeled bacteriophage λDNA.

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