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Distribution of Repair-Incorporated Nucleotides and Nucleosome Rearrangement in the Chromatin of Normal and Xeroderma Pigmentosum Human Fibroblasts†

Michael J. Smerdon, Michael B. Kastan,‡ and Michael W. Lieberman*

ABSTRACT: The distribution of UV-induced DNA repair synthesis in chromatin was measured in normal human fibroblasts and in xeroderma pigmentosum (XP) fibroblasts which are partially deficient in excision repair. With normal cells we investigated the effects of hydroxyurea and UV dose on the initial distribution of nucleotides incorporated during repair synthesis and the subsequent changes in distribution during nucleosome rearrangement [Smerdon, M. J., & Lieberman, M. W. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4238]. These cells were pulse-labeled with [^{3}H]dThd for 30 min immediately after irradiation and chased for varying times with unlabeled dThd. The initial distribution (0 chase time) of repair-incorporated nucleotides in chromatin indicated that most of these nucleotides are initially staphylococcal nuclease sensitive, and this distribution was unaffected by either the presence of 10 mM hydroxyurea or the amount of damage (3–40 J/m^2). The rate at which these repair-incorporated nucleotides became increasingly nuclease resistant was also unaffected by hydroxyurea or UV dose. These data lead to two conclusions: (1) under our conditions hydroxyurea has no measurable effect on either the initial distribution of repair-incorporated nucleotides or the subsequent rate of redistribution of these nucleotides during nucleosome rearrangement; (2) if nucleosome rearrangement is *induced* by damage and/or the repair process, then this induction is a "local event". Cells from XP complementation groups C and D were irradiated with 3 J/m^2 UV, pulse-labeled for 60 min, and chased for varying times. In both groups, the initial distribution (0 chase time) indicated that most of the repair-incorporated nucleotides are initially staphylococcal nuclease sensitive. Electrophoretic data demonstrated a concurrent underrepresentation of repair synthesis in core DNA. With increasing chase time, the distribution of repair-incorporated nucleotides became more uniform in both groups of XP cells. Thus, both the initial enhanced sensitivity of repair-incorporated nucleotides to staphylococcal nuclease and the subsequent changes in sensitivity of these nucleotides during nucleosome rearrangement reported for normal cells occur in at least two complementation groups of XP cells.

It is rapidly becoming apparent that the constraints placed upon DNA in chromatin (Kornberg, 1977; Felsenfeld, 1978) play a significant role in the distribution of excision repair synthesis in mammalian cells. Many of the nucleotides incorporated by repair synthesis, induced by either chemical damage (Bodell, 1977; Tlsty & Lieberman, 1978) or UV[1] (Cleaver, 1977; Smerdon et al., 1978; Smerdon & Lieberman, 1978a,b), are rapidly digested by both staphylococcal nuclease and DNase I. Results from our laboratory indicate that, in fact, almost all repair-incorporated nucleotides are initially sensitive to staphylococcal nuclease and are present at only very low levels in core DNA. Furthermore, our results from pulse-chase experiments indicate that the repair-incorporated nucleotides become more staphylococcal nuclease resistant during the chase period and suggest that nucleosome rearrangement occurs in the regions of repaired DNA[2] (Smerdon

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[1] Abbreviations used: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; XP, xeroderma pigmentosum; UV, ultraviolet radiation.

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& Lieberman, 1978a; Tlsty & Lieberman, 1978).

In the present study we extend these observations in a number of significant ways. (1) To date, all reported studies on the distribution of repair synthesis in chromatin have used hydroxyurea to suppress replicative synthesis and increase the fraction of label incorporated by repair synthesis. However, the validity of the assumption that hydroxyurea does not alter the distribution of repair synthesis in chromatin or nucleosome rearrangement has not been formally demonstrated. Furthermore, although several investigators have reported that neither the amount of repair synthesis (Evans & Norman, 1968; Robbins & Kraemer, 1972; Painter et al., 1973; Cleaver, 1974; Smith & Hanawalt, 1976; Ikenaga & Kakunaga, 1977; Smerdon et al., 1978) nor the amount of damage removed (Cleaver, 1974; Ikenaga & Kakunaga, 1977; Ikenaga et al., 1977) is greatly affected by hydroxyurea, some investigators have observed partial inhibition of DNA repair by the drug (Ben-Hur & Ben-Ishai, 1971; Collins et al., 1977; Cornelis, 1978; Erixon & Ahnström, 1978). Thus, we have studied the effects of 10 mM hydroxyurea on the distribution of repair-incorporated nucleotides in chromatin and the subsequent changes in distribution during nucleosome rearrangement and report our results here. (2) We have previously shown that the initial distribution (after a 90-min pulse) of repair synthesis in chromatin is essentially the same after low or moderate UV doses (1.5–12 J/m^2; Smerdon & Lieberman, 1978b). In the present communication, we examine the effect of different levels of damage on the *kinetics of redistribution* of nucleotides incorporated during repair synthesis. (3) It is of interest to analyze the distribution of repair-incorporated nucleotides and the subsequent nucleosome rearrangement in cells which are partially deficient in DNA repair. There is now convincing evidence that some xeroderma pigmentosum (XP) cells are in this category [see reviews by Robbins et al. (1974), Cleaver & Bootsma (1975), and Bootsma (1978)]. Seven different complementation groups of XP cells have been reported and are, in general, associated with different levels of excision repair of UV damage (Bootsma, 1978). All groups, however, are less competent at repairing UV damage than normal cells. Although there are many possible explanations for the inability of XP cells to repair DNA as effectively as normal cells, one possibility is that chromatin structure may play a significant role in these differences (Mortelmans et al., 1976). An indication of the potential importance of chromatin structure might come from differences between XP and normal cells in the distribution of repair synthesis in chromatin or the rearrangement of nucleosomes in repaired regions. As an initial step in addressing this question, we have investigated the initial distribution of repair-incorporated nucleotides and their subsequent redistribution in XP cells (complementation groups C and D) and report our results here.

Materials and Methods

Cell Culture and Labeling. Normal human diploid fibroblasts from foreskin (line AG1518) and fetal lung (line IMR-90) were purchased from the Human Genetic Mutant Cell Repository, Camden, NJ. Xeroderma pigmentosum skin fibroblasts, complementation groups C (XP10BE; line CRL 1204) and D (XP6BE; line CRL 1157), were purchased from the American Type Culture Collection, Rockville, MD. Cells were prelabeled with 0.001–0.025 μCi/mL [^{14}C]dThd (>50 mCi/mmol; Amersham) during the logarithmic phase of growth, grown to confluence, and treated with 10 mM hydroxyurea as previously described (Smerdon et al., 1978). After removing the medium, we irradiated confluent cells (predominantly 254 nm) with a flux of 1 W/m^2 for doses less than 6 J/m^2 and 2 W/m^2 for all higher doses. The flux was determined for each experiment with a UV monitor (Ultraviolet Products, Inc.). After irradiation the medium was replaced, and cells were pulse-labeled with 10 μCi/mL [^{3}H]dThd (40–60 Ci/mmol; Amersham). Following the pulse period, cells were either harvested or chased for varying times with conditioned medium containing 50 μM dThd (unlabeled) and 10 mM hydroxyurea. Experiments without hydroxyurea were performed in a manner identical with those described above except no hydroxyurea was added at any time to the cells. In some experiments confluent, or growing, prelabeled cells were treated with 5×10^{-5} M BrdUrd (Sigma) and varying concentrations of hydroxyurea (see text) 1 h prior to irradiation and labeled with 10 μCi/mL [^{3}H]dThd as described above.

Preparation of Nuclei. Nuclei were prepared by a modification of the procedure outlined in Smerdon et al. (1978). Following centrifugation of cells in 0.15 M NaCl, the cell pellet was washed in ice-cold B1 buffer (10 mM Tris, pH 8, 1 mM $CaCl_2$, and 0.25 M sucrose) and repelleted at 600g for 10 min at 2 °C. Cells were lysed, and nuclei were recovered by either vortexing or dounce homogenizing (5–10 strokes with a loose-fitting pestle; Kontes) the cell pellet in ice-cold B2 buffer (B1 plus 0.5% Triton X-100), followed by centrifugation at 4000g for 5 min at 2 °C. After the lysing step was repeated two more times, the nuclei were washed once with B1 buffer and then twice with ice-cold B3 buffer (10 mM Tris, pH 8, 0.1 mM $CaCl_2$, and 0.25 M sucrose). The nuclear pellet was resuspended in B3 buffer by dounce homogenization (five strokes with a tight-fitting pestle). This modified procedure is similar to that of Sollner-Webb & Felsenfeld (1975).

Nuclease Digestions and Electrophoresis. Staphylococcal nuclease (Worthington) digestions of suspended nuclei in B3 buffer, agarose gel electrophoresis, and data analysis were all carried out as described elsewhere (Smerdon et al., 1978). The CsCl preparation of DNA for the determination of M_{NAK} (Smerdon et al., 1978) was eliminated by assuming $H_{100\%}/C_{100\%} = M_{NAK}$, where $H_{100\%}$ and $C_{100\%}$ are the ^{3}H cpm and ^{14}C cpm, respectively, obtained from the 100% digestion (see Results). The sizes of digestion products were determined by coelectrophoresis with *Hae*III (New England Biolabs) restriction fragments of ϕX174 RF DNA which has been completely sequenced (Sanger et al., 1978). ϕX174 RF DNA was prepared by using the method described by Barnes (1978).

Preparation and Isopycnic Centrifugation of BrdUrd-Labeled DNA. DNA was prepared by resuspending the nuclear pellet in 10 mM Tris (pH 8), 10 mM EDTA, 10 mM NaCl, and 0.5% $NaDodSO_4$ and incubating for 4 h at 47 °C with 120–250 μg/mL proteinase K (EM Laboratories). The solution was brought to a density of 1.80 g/cm^3, a final concentration of 0.1 M K_2HPO_4, and a final volume of 7.5 mL with the addition of CsCl, 1 M K_2HPO_4 (pH 12.5), and H_2O. This solution was centrifuged at 8000g for 10 min. An aliquot (7 mL) of the clear phase was transferred to polyallomer tubes, overlayered with paraffin oil, and centrifuged (Beckman 50 Ti rotor) at 40000 rpm at 20 °C for 36 h. The gradients were fractionated and the radioactivity was measured as described elsewhere (Smerdon et al., 1978).

Results

Effect of Hydroxyurea on the Distribution of Repair-Incorporated Nucleotides in Chromatin. In previous studies,

[2] In this paper the term *nucleosome rearrangement* refers only to those nucleosomes located in or near regions in which DNA repair synthesis has occurred.

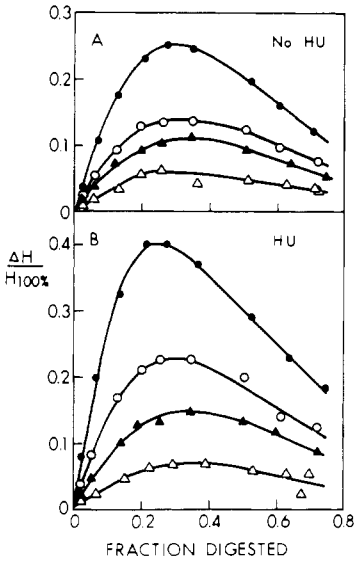

FIGURE 1: Normalized difference curves [see Results and Smerdon et al. (1978)] for normal human fibroblasts (IMR-90 cells) irradiated with 12 J/m^2 UV, pulse-labeled for 30 min, and chased for 0 (●), 0.5 (○), 3.5 (▲), and 24.5 (△) h. (A) Control cells (no hydroxyurea). (B) Cells labeled and chased in the presence of 10 mM hydroxyurea. Nuclei and DNA were prepared and digested with staphylococcal nuclease (see Materials and Methods).

we have used a confluent cell–hydroxyurea suppression system to obtain nuclei in which nearly all of the label is incorporated by repair synthesis (Smerdon et al., 1978; Smerdon & Lieberman, 1978a,b; Tlsty & Lieberman, 1978). In this report we have measured the distribution of repair synthesis in nuclei from cells not treated with hydroxyurea to determine if the presence of the drug affected our previous results. In order to obtain nuclei with most of the label incorporated by repair synthesis, we used a UV dose of 12 J/m^2. This relatively high dose stimulates a near-maximal amount of repair synthesis under our conditions (Smerdon & Lieberman, 1978b) and also suppresses replicative synthesis (see last section of Results and Figure 7). Thus, cells were prelabeled with [^{14}C]dThd (replicative synthesis) and allowed to come to confluence. After UV irradiation (12 J/m^2), they were labeled for 30 min with [^{3}H]dThd in the presence or absence of 10 mM hydroxyurea. Some cells were harvested immediately after the 30-min pulse, while others were chased for varying times with 50 μM unlabeled dThd. The nuclei from all these cells were prepared and digested with staphylococcal nuclease (Materials and Methods). Figure 1 shows normalized difference curves (Smerdon et al., 1978) obtained from cells harvested after 0, 0.5, 3.5, or 24.5 h of chase. Each curve is calculated from the expression

$$\frac{\Delta H}{H_{100\%}} = \left[H(t) - \left(\frac{H_{100\%}}{C_{100\%}} \right) C(t) \right] (H_{100\%})^{-1} \qquad (1)$$

where $H(t)$ and $C(t)$ are the ^{3}H cpm and ^{14}C cpm, respectively, of the DNA rendered acid soluble from nuclei by staphylococcal nuclease after time t and $H_{100\%}$ and $C_{100\%}$ are the total ^{3}H cpm and ^{14}C cpm, respectively, obtained from complete staphylococcal nuclease digestion of DNA in proteinase K treated nuclei (Smerdon et al., 1978). We note that in our original report (Smerdon et al., 1978) the ratio $H_{100\%}/C_{100\%}$ was not used in this expression. Instead, the slope (M_{NAK}) of the $H(t)$ vs. $C(t)$ plot for the digestion of the corresponding CsCl-purified DNA was used. We have found, however, that the ratio $H_{100\%}/C_{100\%}$ is a very good estimate of M_{NAK} for repair-synthesis experiments [$(H_{100\%}/C_{100\%})/M_{NAK}$ = 1.018 ± 0.022 (mean ± SD); 29 determinations]. Thus, we have eliminated the CsCl purification step for most of our determinations.

Since each curve in Figure 1 represents the difference between the staphylococcal nuclease digestion data for nuclei and the corresponding "naked" DNA, a constant value of $\Delta H/H_{100\%} = 0$ as a function of the fraction digested would indicate a uniform distribution in chromatin. In agreement with our previous results (Smerdon et al., 1978; Smerdon & Lieberman, 1978a,b), nucleotides incorporated by repair synthesis occurring immediately after UV and in the presence of hydroxyurea are initially staphylococcal nuclease sensitive; also, these repair-incorporated nucleotides become more uniformly distributed with increasing chase times (Figure 1B). The same pattern is seen for repair synthesis occurring in the absence of hydroxyurea (Figure 1A). The magnitudes of the differences seen are not as great as those for cells treated with the drug. As discussed below, this probably arises from the fact that in the absence of hydroxyurea more label is incorporated by replicative synthesis, which, at least for these time periods, is distributed uniformly in chromatin [Seale, 1975; Worcel et al., 1978; Murphy et al., 1978; see also Smerdon et al. (1978)].

The data in Figure 1 were analyzed by using the method of Smerdon et al. (1978). In essence, this method utilizes the initial (M_i) and final (M_f) slopes of each normalized difference curve, as well as the fractional value (ξ) obtained from the intersection of the two lines defined by these slopes. When $\Delta H/H_{100\%}$ is calculated by eq 1, the fraction of repair-incorporated nucleotides that is nuclease sensitive (f_S) is given by

$$f_S = \left[\frac{(M_i + 1)\sigma - 1}{\sigma - 1} \right] \xi \qquad (2)$$

where

$$\sigma = \frac{H_{100\%}/C_{100\%} \text{ for nuclei from irradiated cells}}{H_{100\%}/C_{100\%} \text{ for nuclei from control (unirradiated cells)}}$$

Similarly, the fraction of repair-incorporated nucleotides that is nuclease resistant (f_R) is given by

$$f_R = \left[\frac{(M_f + 1)\sigma - 1}{\sigma - 1} \right] (1 - \xi)$$

A "test" of the assumptions of the method is then made by calculating $f_S + f_R$ which theoretically should be 1.

The values for σ, f_S, f_R, ξ, and $f_S + f_R$ for the curves shown in Figure 1 are given in Table I. We have included (in parentheses) determinations using a correction for σ based on the suppression of replicative synthesis by UV (see last section of Results and Figure 7).

The values for f_S/ξ for each chase time are shown in Figure 2 along with results from other experiments. (For convenience, we refer to this ratio as the fraction of repair-incorporated nucleotides per unit of DNA that is nuclease sensitive.) We have included the values of f_S/ξ for comparison since this parameter is dependent only on the *initial slope* of the normalized difference curve (see eq 2) and, therefore, is less subject to error than f_S. As can be seen, the values for f_S, f_R, ξ, and f_S/ξ are similar for both hydroxyurea-treated and untreated cells. Furthermore, in each case a sum test close to 1 was obtained ($f_S + f_R$; Table I).

As expected, the proportions of label incorporated by replicative synthesis during the 30-min pulse period are different. In the absence of hydroxyurea, the amount of label

Table I: Effect of Hydroxyurea on Chromatin Distribution and Rearrangement[a]

chase time (h) after 0.5-h pulse	HU[b] (10 mM)	σ	sp act.[c]	f_S	f_R	ξ	$f_S + f_R$
0	−	3.7 (18)	0.69	0.65 (0.54)	0.35 (0.46)	0.19	1.00 (1.00)
0	+	27.3 (136)	0.76	0.65 (0.63)	0.41 (0.42)	0.17	1.06 (1.05)
0.5	−	3.5 (17)	1.04	0.47 (0.40)	0.56 (0.62)	0.21	1.03 (1.02)
0.5	+	25.6 (128)	1.15	0.48 (0.47)	0.56 (0.57)	0.20	1.04 (1.04)
3.5	−	3.1 (16)	0.85	0.42 (0.37)	0.60 (0.64)	0.22	1.02 (1.01)
3.5	+	21.6 (108)	0.96	0.45 (0.44)	0.58 (0.59)	0.24	1.03 (1.03)
24.5	−	3.4 (17)	0.98	0.32 (0.30)	0.70 (0.72)	0.22	1.02 (1.02)
24.5	+	23.6 (118)	1.05	0.36 (0.35)	0.66 (0.66)	0.27	1.02 (1.01)

[a] The data in Figure 1 were analyzed as described in Smerdon et al. (1978) and are reported as the ratio of the specific activities of repaired DNA and control (unirradiated) DNA (σ), fraction of repair-incorporated nucleotides that is nuclease sensitive (f_S), fraction of repair-incorporated nucleotides that is nuclease resistant (f_R), and fraction of DNA bases that is nuclease sensitive (ξ). Values in parentheses were obtained by using a correction for UV suppression of replicative synthesis (see last section of Results and Figure 7). [b] HU: hydroxyurea. [c] Data are reported as the difference between the ^3H cpm/^{14}C cpm ratio for repaired DNA and the ^3H cpm/^{14}C cpm ratio for control DNA in each case.

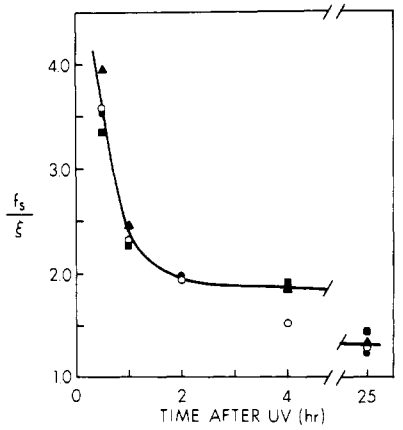

FIGURE 2: Fraction of repair-incorporated nucleotides per unit of DNA that is staphylococcal nuclease sensitive (f_S/ξ; see Results) as a function of time after UV irradiation. Normal human fibroblasts (IMR-90 cells) were irradiated with 3 (○) or 12 (●, ■, ▲) J/m^2 UV, pulse-labeled for 30 min, and chased for the times shown. Analyses of the data in Figure 1 are included: (■) control cells (no hydroxyurea) and (▲) hydroxyurea-treated cells. Curve shown is fit to 12 J/m^2 data.

incorporated by control cells (unirradiated) was 27–32% of that of irradiated cells (σ = 3.1–3.7). In the presence of the drug, control cells incorporated only 3.6–4.6% of the amount of label incorporated by irradiated cells (σ = 21.6–27.3). Thus, since our method of analysis makes a correction for the amount of label incorporated by replicative synthesis in any given experiment, it is apparent from Table I and Figure 2 that the differences in magnitude seen in Figure 1 between hydroxyurea-treated and untreated cells are probably the result of different relative amounts of repair synthesis and replicative synthesis.

We note that in each case the DNA from chased cells has a 20–30% higher specific activity than DNA from cells harvested immediately after the pulse (Table I). Thus, even with the presence of 50 μM unlabeled dThd in the chase medium, some label is incorporated during the chase period. The increase in specific activity occurs only during the first 10–15 min of chase time (data not shown) and represents label incorporated before complete dilution of the isotope has occurred. However, the *relative amounts* of label incorporated by repair synthesis and replicative synthesis (σ) remain constant. Also, we did not observe this increase in experiments with AG1518 or XP cells or when IMR-90 cells were pulsed for longer periods (≥60 min). Since nucleosome rearrangement in repaired regions was observed under all of these conditions (see Figure 5) (Smerdon & Lieberman, 1978a and unpublished experiments), the change in distribution of label in chromatin during the chase period cannot be explained by residual incorporation of label.

Also, we note that with increasing chase times we find a slight increase in the fraction of DNA bases in nuclease-sensitive regions (ξ). For short pulse times (15–30 min) we routinely obtain a value of ξ = 0.15–0.20. This value increases to 0.20–0.30 after 24 h of chase. However, the error in determining ξ also increases as the difference between the nuclei digestion data and DNA digestion data becomes small (Smerdon et al., 1978). Therefore, whether the slight increase in ξ represents a consistent error in our analysis of data following long chase times or an actual increase in the fraction of nuclease-sensitive DNA is at present unknown.

Effect of UV Dose on the Distribution of Repair-Incorporated Nucleotides in Chromatin. To examine the effect of UV dose on the changes in distribution of repair-incorporated nucleotides, we measured the distribution of label at several different chase times following two UV doses (3 and 12 J/m^2). These two doses were chosen since they represent doses below (3 J/m^2) and above (12 J/m^2) saturation of repair synthesis under our conditions (Smerdon & Lieberman, 1978b). Cells were pulse-labeled for 30 min immediately after irradiation and chased for 0, 0.5, 1.5, 3.5, and 24.5 h. Nuclei were prepared and digested with staphylococcal nuclease, and the data were analyzed as described above. The fraction of repair-incorporated nucleotides per unit of DNA that is nuclease sensitive (f_S/ξ) for both UV doses was determined for each chase time (Figure 2). The kinetics of redistribution are similar for the two UV doses. In order to examine a wider range of UV doses, we measured the distribution at only two different chase times (0 and 3.5 h). A direct comparison of the data (normalized difference plots) obtained for three UV doses (6, 20, and 40 J/m^2) is presented in Figure 3. Since the value of σ was similar in each case, the data can be compared directly and indicate that there is little difference in the distribution among these doses at both chase times. The results of analyses of these and other data (Figure 4) indicate that the kinetics of redistribution of repair-incorporated nucleotides during the 3.5-h chase time are essentially unaffected by the amount of UV damage over the dose range of 3–40 J/m^2.

Distribution of Repair-Incorporated Nucleotides in the Chromatin of AG1518 Cells and XP Cells. We have performed pulse-chase experiments on AG1518 cells to insure that our previous results with IMR-90 cells are representative of normal cells. AG1518 cells were irradiated with 12 J/m^2 UV, pulse-labeled for 30 min, and chased for 0, 0.5, 1.5, 3.5, and 24 h. Nuclei were prepared and digested with staphylococcal nuclease, and the data were analyzed as described above. The

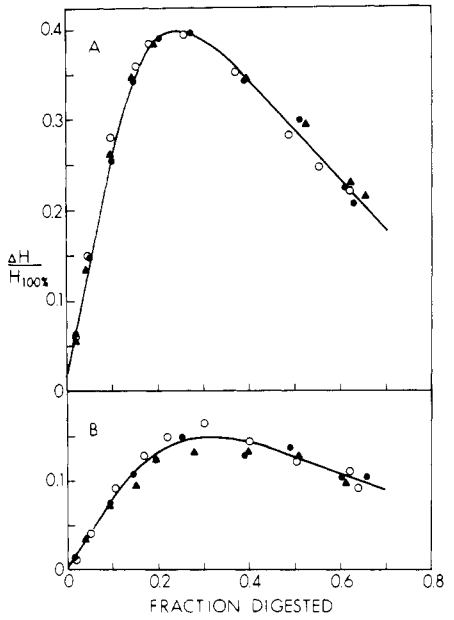

FIGURE 3: Normalized difference curves for normal human fibroblasts (IMR-90 cells) irradiated with 6 (●), 20 (○), and 40 (▲) J/m^2. Cells were pulse-labeled for 30 min and chased for (A) 0 and (B) 3.5 h. Curves shown are fitted to 6 J/m^2 data. Values obtained for σ were 23, 26, and 28 for 6, 20, and 40 J/m^2, respectively.

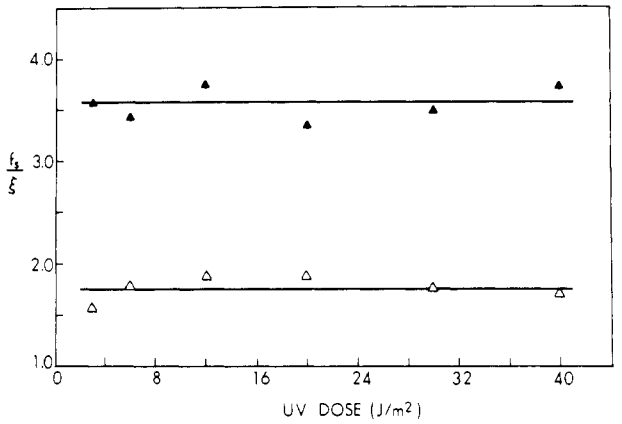

FIGURE 4: Fraction of repair-incorporated nucleotides per unit of DNA that is staphylococcal nuclease sensitive (f_S/ξ) as a function of UV dose. IMR-90 cells were pulse-labeled for 30 min and chased for 0 (▲) and 3.5 (△) h.

distribution of repair-incorporated nucleotides measured at each chase time was similar to those found for IMR-90 cells (compare Figures 5A and 2). These data confirm our previous findings: initially, almost all repair-incorporated nucleotides are staphylococcal nuclease sensitive; subsequently, many of these nucleotides become staphylococcal nuclease resistant and yield a more uniform distribution; the kinetics of this redistribution appear to be biphasic (Smerdon & Lieberman, 1978a; Tlsty & Lieberman, 1978).

We have also performed experiments on XP cells from complementation groups C and D. These two XP cell lines, although deficient in repair synthesis relative to normal cells, do undergo a minimal amount of repair synthesis. Values of 20–25% for group C cells and 30–35% for group D cells were obtained for the amount of repair synthesis relative to IMR-90 cells (data not shown). Because the XP cells yielded a much lower level of repair synthesis, we were concerned about the effect of UV suppression of replicative synthesis [e.g., Painter (1978)] on σ. σ, as defined previously [Table I and Smerdon et al. (1978)], does not take this suppression into account. However, in normal cells treated with hydroxyurea σ is

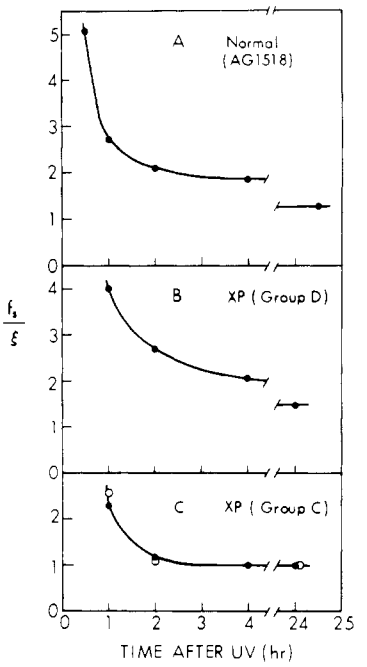

FIGURE 5: Fraction of repair-incorporated nucleotides per unit of DNA that is staphylococcal nuclease sensitive (f_S/ξ) as a function of time after UV irradiation. (A) Normal human fibroblasts (AG1518 cells) irradiated with 12 J/m^2 UV, pulse-labeled for 30 min, and chased for the times shown. (B) XP group D cells and (C) XP group C cells irradiated with 3 J/m^2 UV, pulse-labeled for 60 min, and chased for the times shown. Different symbols represent different experiments.

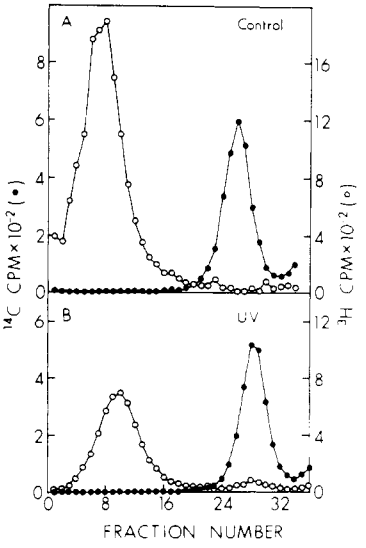

FIGURE 6: Alkaline-CsCl gradient profiles of (A) control and (B) irradiated XP group C cells. Near confluent cells were irradiated with 6 J/m^2 UV and labeled for 8 h in the presence of BrdUrd (see Materials and Methods). No hydroxyurea was added. The densities of the two peaks were 1.85 (○) and 1.76 (●) g/cm^3.

sufficiently high to make any effect by UV suppression negligible. For low values ($\sigma \lesssim 3$) the results of the analysis are much more dependent on σ and, therefore, UV suppression of replicative synthesis could affect our results with XP cells (Smerdon et al., 1978). Therefore, we determined the amount of suppression of replicative synthesis in irradiated cells relative to control cells using the BrdUrd density-shift method. The amount of "heavy" (newly replicated) DNA relative to the amount of "light" (bulk) DNA was determined for both irradiated and unirradiated cells (e.g., Figure 6). The ratio of these two numbers provided a measure of the suppression of replicative synthesis by UV. Suppression values were obtained for different hydroxyurea concentrations, labeling

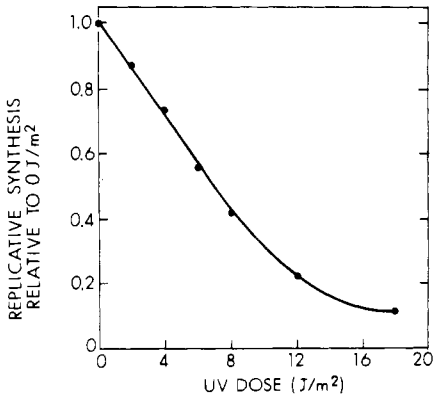

FIGURE 7: Relative suppression of replicative synthesis by UV in XP group C cells. Data shown are for confluent cells labeled for 14 h in the presence of 0.1 mM hydroxyurea. Ordinate values represent the ratio of "heavy" to "light" DNA (from irradiated cells) obtained in alkaline-CsCl gradients (e.g., Figure 6) divided by the control (no UV) value.

times, and UV doses in both confluent and growing cells. Figure 7 shows an example of the suppression of replicative synthesis as a function of UV dose for group C cells. Data like these were used to calculate a corrected σ by dividing the apparent σ by the appropriate suppression value.

To minimize the effect of σ correction on our data we used a UV dose of 3 J/m^2 for the XP experiments (Figure 7). Also, survival studies indicate that XP cells are more sensitive to UV than normal cells (e.g., Robbins et al. (1974)] and, therefore, a comparison of XP cells and normal cells may be more valid at low UV doses. Both groups of XP cells were labeled for 60 min to obtain sufficient label in the DNA. Figures 5B and 5C show the values of f_S/ξ for 0-, 1-, 3-, and 23-h chase times. In both groups of XP cells, repair-incorporated nucleotides are initially staphylococcal nuclease sensitive (0 chase time; $f_S/\xi > 1$), and subsequently many of these nucleotides become staphylococcal nuclease resistant, yielding a more uniform distribution. These results are qualitatively similar to our findings with normal cells. However, there are apparent differences in the rates and extents of redistribution among these cell types. Whether or not these differences are biologically significant remains to be investigated.

The staphylococcal nuclease digestion products obtained from these cells after various chase times were examined by electrophoresis. Figure 8 shows the profiles of bulk DNA (^{14}C counts per minute), repaired DNA (^{3}H), and RS (the relative amount of repair synthesis) for group D cells after 0-, 1-, and 23-h chase periods. The fraction of DNA digested at each chase time is approximately the same; therefore, these profiles can be compared directly (Smerdon and Lieberman, unpublished experiments). Immediately after the pulse period, the RS has minima at monomer and dimer positions in the gel and maxima at regions between the resistant fragments (Figure 8A), indicating underrepresentation of repair-incorporated nucleotides in core DNA. Also, with increasing chase times the RS values approach unity throughout the gel (Figure 8B,C), indicating a redistribution of repair-incorporated nucleotides into core regions. Both findings agree with the digestion kinetics results (Figure 5B) and are similar to those observed for normal cells (Smerdon & Lieberman, 1978a). In each case, however, the relative changes in RS are not as great as those observed for normal cells (Smerdon & Lieberman, 1978a). This result is expected since a greater fraction of the total label is incorporated by replicative synthesis in XP cells. Similar results were obtained for group

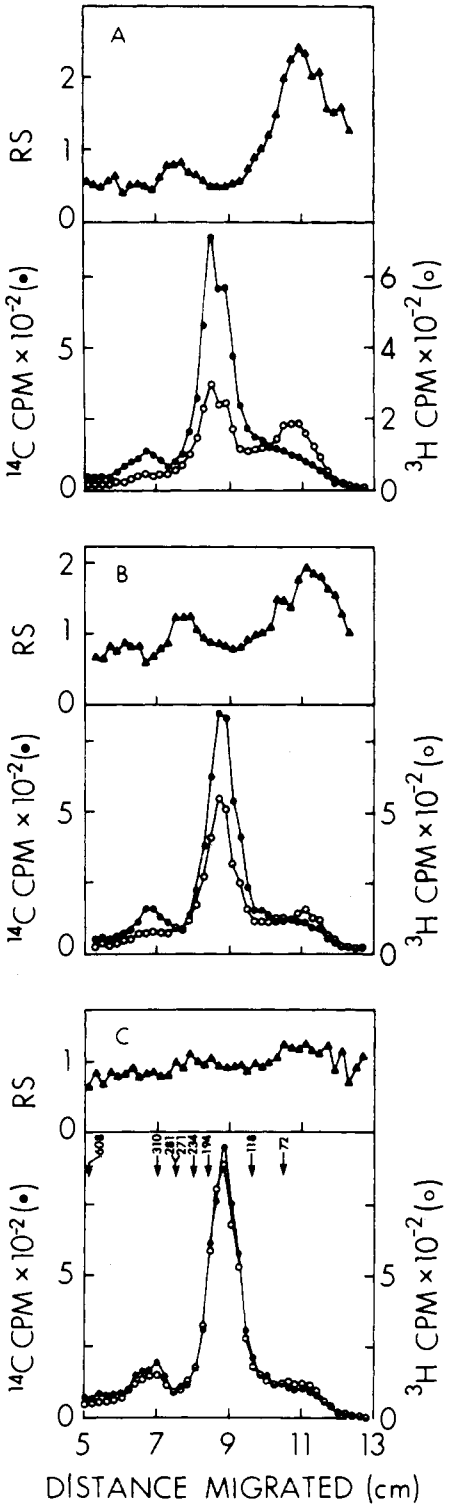

FIGURE 8: Electrophoresis profiles of staphylococcal nuclease resistant DNA from XP group D cells irradiated with 3 J/m^2 UV, pulse-labeled for 60 min, and chased for (A) 0, (B) 1, and (C) 23 h. The following is included in each panel: parental DNA profile (^{14}C cpm) and repaired DNA profile (^{3}H cpm) (lower graph); relative specific activity (RS = ^{3}H cpm/^{14}C cpm of digested DNA divided by ^{3}H cpm/^{14}C cpm of undigested DNA) as a function of migration (upper graph). Arrows indicate the positions of the *Hae*III ϕX174 RF marker fragments and above each arrow is listed the respective size in base pairs. The monomer peak is between 8.5 and 8.9 cm. The dimer peak is between 6.7 and 7.1 cm. The fraction of total ^{14}C cpm rendered acid soluble was 0.13, 0.12, and 0.13 for (A), (B), and (C), respectively. DNA (30–40 μg) was applied to each gel. It is noted that the ^{3}H peak migrating ahead of the monomer (<72 bp) has been found to be comprised of heterogeneous sizes of DNA and is present as a peak due to the nonlinearity of the gel in this size range (Smerdon and Lieberman, unpublished experiments).

C and AG1518 fibroblasts (data not shown).

Discussion

We have measured both the initial distribution of repair-incorporated nucleotides and the subsequent changes in distribution during nucleosome rearrangement in the presence and absence of hydroxyurea. This study was possible since our method of analysis allows for the determination of the distribution of repair synthesis in chromatin even in the presence of relatively high levels of replicative synthesis (Smerdon et al., 1978). Our results indicate that for confluent human diploid fibroblasts, neither the overall distribution of repair synthesis in chromatin nor the kinetics of nucleosome rearrangement in repaired regions is altered when 10 mM hydroxyurea is present. These findings demonstrate the validity of our previous studies (Smerdon et al., 1978; Smerdon & Lieberman, 1978a,b; Tlsty & Lieberman, 1978).

Second, we have found that after different UV doses in the range of 3–40 J/m^2 the kinetics of redistribution of repair-incorporated nucleotides in chromatin are essentially the same. Over this dose range, the number of pyrimidine dimers produced in the DNA follows a linear relationship with UV dose and does not approach saturation until much higher doses [e.g., Williams & Cleaver (1978)]. However, this range spans both nonsaturating and saturating ($\geq$6 J/m^2) doses for repair synthesis (Smerdon & Lieberman, 1978b).

These results allow insight into two general models proposed for the onset of nucleosome rearrangement (Smerdon & Lieberman, 1978a): (1) nucleosome rearrangement may be *induced* by damage and/or repair; (2) nucleosome rearrangement may be *constitutive*, occurring in the presence or absence of damage and repair. One possibility to explain our results is that induction of rearrangement may occur through long-range interactions; i.e., repaired regions may undergo rearrangement as a result of damage or repair synthesis at some other point in the genome. If *damage* induces rearrangement in this way, then, at least under conditions where repair synthesis is saturated, the kinetics of rearrangement would be expected to change with UV dose. We do not observe such changes. Similarly, if rearrangement is induced by *repair synthesis* acting through long-range interactions, then a change in rearrangement kinetics would be expected with a change in UV dose under nonsaturating conditions. Our results indicate that this is not the case. Therefore, if rearrangement is induced, then our results suggest that this induction is a "local event". For example, one possibility is that lesions and/or repair enzymes produce local perturbations in nucleosome structure (perhaps by unfolding some of the nucleosome core) and, following repair synthesis, the nucleosomes return to their original states. We have discussed this model in greater detail elsewhere (Lieberman et al., 1979). Also, we cannot rule out the possibility that nucleosome rearrangement in chromatin is constitutive. In addition, we note that Cleaver did not observe rearrangement and proposed an alternative explanation for the decreased staphylococcal nuclease sensitivity observed in continuous-labeling experiments (Cleaver, 1977).

Third, we have shown that in two different complementation groups of XP cells nucleotides incorporated during UV-induced DNA repair synthesis are initially staphylococcal nuclease sensitive and are underrepresented in core DNA. We have also shown that in these cells the repair-incorporated nucleotides are involved in nucleosome rearrangement. Therefore, with regard to the overall distribution of repair synthesis in chromatin and the occurrence of nucleosome rearrangement, these two groups of XP cells are similar to normal cells. We did, however, observe quantitative differences in the distributions in each cell type, and further experimentation is required to ascertain the significance of these differences.

Finally, we note that in the present communication we have made no attempt to determine if the initial distribution of repair-incorporated nucleotides in chromatin is related to the distribution of DNA damage in chromatin. Recent studies in our laboratory have dealt with this question and indicate that the distribution of repair synthesis in chromatin is not simply the result of preferential damage of nuclease-sensitive DNA or preferential removal of damage (Oleson, Mitchell, Dipple, and Lieberman, unpublished experiments).

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Chromatin Fractionation Procedure That Yields Nucleosomes Containing Near-Stoichiometric Amounts of High Mobility Group Nonhistone Chromosomal Proteins†

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ABSTRACT: Initial results of an approach to the isolation of functionally active chromatin are described. Slight digestion of mouse myeloma nuclei at 0 °C with micrococcal nuclease, followed by dialysis against near-physiological saline solution containing 1 mM Mg^{2+}, caused release of up to 17% of the nuclear DNA as soluble nucleoproteins. This soluble (S) fraction was relatively depleted in H1 histones and methylated DNA (5-methylcytosine) but highly enriched in RNA, single-stranded DNA, and nonhistone chromosomal proteins, particularly two species of the high mobility group identified as HMG 1 and HMG 2. The S fraction released most rapidly (6–8% of the total DNA) consisted mainly of mono- and small oligonucleosomes. The mononucleosomes appeared normal in terms of sedimentation behavior, DNA length, and content of histones H2A, H2B, H3, and H4, but lacked H1, and instead were associated with approximately stoichiometric amounts of HMG 1 and HMG 2. Studies using isolated, fluorescence-labeled, total mouse HMG proteins indicated that added HMG 1 and HMG 2 do not bind strongly to S-fraction nucleoproteins but that two smaller HMG species (probably HMG 14 and HMG 17) do bind preferentially to S-fraction mono- and dinucleosomes. These results argue against artifactual redistribution of HMG 1 and HMG 2 during this fractionation but suggest caution in interpreting the distribution of smaller HMG proteins after digestion of chromatin. The potential relationship of this soluble fraction to transcriptionally active chromatin is discussed.

The nucleosome model of chromatin structure has provided a unifying conceptual basis for understanding molecular aspects of genomic functions, but little is yet known about the structural distinctions between inactive chromatin and chromatin that is active in transcription or other nuclear events [reviewed by Felsenfeld (1978)]. Certain features of transcribing chromatin seem clear, such as its diffuse appearance in microscopic views [e.g., Faken (1978)]. In inactive chromatin, the ~100-Å diameter nucleosome fibers (Olins & Olins, 1974; Oudet et al., 1975) are further folded into thicker fibers of "solenoids" (Finch & Klug, 1976) or "superbeads" (Renz et al., 1977), at least partly through the influence of histone H1 and divalent cations. Thick fibers are not observed in active transcription units (Franke et al., 1978), and even the folded form of nucleosomes may be absent from exceedingly active genes (Franke et al., 1976; Laird et al., 1976; Woodcock et al., 1976). Structural alterations of nucleosomes in active regions are also indicated by their preferential degradation by DNase I and DNase II (Felsenfeld, 1978).

Active and inactive chromatin are expected to differ in both chemical composition (e.g., nonhistone proteins, histone modifications, and DNA modifications) and physical properties such as solubility and accessibility to nucleases. Differences of the latter type have been used with some success as the basis for isolating chromatin enriched in active gene sequences [reviewed by Gottesfeld (1978)].

We have attempted to improve these methods by taking fuller advantage of the natural solubility properties of chromatin, the influence of the native structure on the specificity of micrococcal nuclease cleavage, and the ability of the nuclear substructure to act as a sieving matrix. To preserve the nuclear matrix, thick chromatin fibers, and the distinctions between heterochromatin and euchromatin, we sheared mouse myeloma chromatin in situ by very slight digestion of nuclei at 0 °C under near-physiological ionic conditions (Ris & Kubai, 1970; Olins & Olins, 1972; Pooley et al., 1974). Fractionation on the basis of solubility was performed under similar conditions, and mechanical aggitation was avoided throughout the procedure. Micrococcal nuclease was used because it prefers nucleosome spacers, does not degrade active genes (Sollner-Webb & Felsenfeld, 1975; Garel & Axel, 1976; Felsenfeld, 1978), and may rapidly attack transiently single-stranded DNA in active chromatin (see Discussion). Very recent evidence has shown that this nuclease preferentially excises, but does not degrade, nucleosomes from transcrip-

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