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Distribution within Chromatin of Deoxyribonucleic Acid Repair Synthesis Occurring at Different Times after Ultraviolet Radiation†

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ABSTRACT: We have compared the initial distribution and subsequent redistribution within chromatin of nucleotides incorporated during the early ("rapid") phase and the late ("slow") phase of UV-induced DNA repair synthesis. As has been observed for the early repair phase, most or all of the nucleotides incorporated during the late repair phase are initially staphylococcal nuclease and DNase I "sensitive" (i.e., rapidly digested). This initial enhanced sensitivity is accompanied by both an underrepresentation of these nucleotides in the 145-165 base pair (core) DNA produced by staphylococcal nuclease digestion and an absence of these nucleotides in the ~10-base repeat pattern produced by DNase I digestion. Furthermore, nucleotides incorporated at late times after damage are involved in nucleosome rearrangement as reported previously for repair synthesis occurring at early times [Smerdon, M. J., & Lieberman, M. W. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4238-4241]. The kinetics of redistribution, however, appear to be more rapid than those observed for early times. Following redistribution the average nucleosome repeat length of DNA containing repair-incorporated nucleotides is the same as that of bulk DNA regardless of the time after damage that repair occurs; also, many of these nucleotides coelectrophorese with the ~10-base repeat fragments generated by DNase I. These results yield a new interpretation of our previous studies [Smerdon, M. J., Tlsty, T. D., & Lieberman, M. W. (1978) *Biochemistry 17*, 2377-2386] on the distribution of nucleotides incorporated at long times after UV irradiation.

Our understanding of DNA repair in the chromatin of mammalian cells has grown rapidly over the last few years [for recent reviews of chromatin structure, see Kornberg (1977), Chambon (1977), and Felsenfeld (1978)]. When nucleotides are incorporated by DNA repair synthesis occurring immediately after damage by either UV[1] (Cleaver, 1977; Smerdon et al., 1978, 1979; Smerdon & Lieberman, 1978a,b; Williams & Friedberg, 1979) or chemicals (Bodell, 1977; Tlsty & Lieberman, 1978; Bodell & Banerjee, 1979; Oleson et al., 1979), a majority of these nucleotides are located in DNA which is initially staphylococcal nuclease sensitive (i.e., rapidly digested). This enhanced sensitivity is accompanied by an underrepresentation of repair-incorporated nucleotides in the DNA of intact nucleosome cores (i.e., 145-165-bp DNA protected from rapid digestion by staphylococcal nuclease) (Smerdon et al., 1978, 1979; Smerdon & Lieberman, 1978a; Tlsty & Lieberman, 1978; Oleson et al., 1979). These results have been interpreted as evidence that DNA repair synthesis occurring immediately after damage is located mainly in linker DNA (Cleaver, 1977; Smerdon et al., 1978; Bodell & Banerjee, 1979); however, an alternative explanation is that repair synthesis is associated with a perturbation of the nucleosome structure such that some (or all) of the core DNA becomes nuclease sensitive (Lieberman et al., 1979; Oleson et al., 1979).

Two different laboratories have reported that during the first few hours after UV irradiation the distribution of repair-incorporated nucleotides becomes more uniform as the labeling times are increased (Cleaver, 1977; Smerdon & Lieberman, 1978b). Two possible explanations for these results have been proposed: (1) following repair synthesis nucleosome rearrangement occurs, resulting in a more uniform distribution of repair-incorporated nucleotides and/or (2) nuclease-sensitive regions of chromatin are repaired more rapidly than nuclease-resistant regions. Clearly, these two possibilities are not mutually exclusive. In the report by Cleaver (1977), pulse-chase experiments failed to show a change in the distribution of repair-incorporated nucleotides during the chase period. Thus, he concluded that the second possibility was more likely. Pulse-chase experiments performed in our laboratory, however, indicate that repair synthesis occurring immediately after damage by either UV (Smerdon & Lieberman, 1978a; Smerdon et al., 1979), 2-(acetylacetoxyamino)fluorene (Tlsty & Lieberman, 1978), or 7-(bromomethyl)benz[*a*]anthracene (Oleson et al., 1979) is followed by a rapid and

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[1] Abbreviations used: UV, ultraviolet; dThd, thymidine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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extensive redistribution of the repair-incorporated nucleotides to a more uniform distribution. [Recently, these results have been verified by Williams & Friedberg (1979) for UV-induced repair synthesis (see Discussion).] Furthermore, the kinetics of this redistribution during the chase period are similar to those observed in continuous label experiments (Tlsty & Lieberman, 1978). Thus, we believe that nucleotides incorporated by repair synthesis during the first few hours after damage are initially staphylococcal nuclease sensitive and subsequently undergo rearrangement to a more uniform distribution. The second possibility (i.e., that nuclease-sensitive regions of chromatin are repaired most rapidly) can be tested by measuring the distribution and rearrangement properties of repair synthesis occurring at long times after damage.

A study of the distribution within chromatin occurring at late times is also of interest for other reasons. A number of investigators have reported that DNA repair synthesis occurs for many hours after UV irradiation and that the initial rate of nucleotide incorporation (i.e., during the first 1–6 h) is much greater than that at longer times (Spiegler & Norman, 1970; Conner & Norman, 1971; Edenberg & Hanawalt, 1973; Smerdon et al., 1978; Ahmed & Setlow, 1978; Williams & Cleaver, 1978). Furthermore, some investigators have observed both a rapid phase and a slow phase in the removal of pyrimidine dimers (Paterson et al., 1973; Amacher et al., 1977; Cornelis, 1978; Williams & Cleaver, 1978). Thus, repair synthesis occurring during the slow phase may have a different distribution within chromatin than repair synthesis occurring immediately after damage. In a previous report, we observed that, following 12 J/m^2 UV, cells labeled during the slow repair phase yielded a more uniform distribution of repair synthesis in chromatin (Smerdon et al., 1978). However, in order to obtain approximately equal amounts of label in the DNA during each phase, we used a longer labeling time (~20 h) during the slow phase than during the fast phase (3 h). In light of our more recent findings concerning nucleosome rearrangement in the regions of repair-incorporated nucleotides, we have reinvestigated this important question and report our results here.

Materials and Methods

Damage and Labeling of Cells. Human diploid fibroblasts (IMR-90 cells; Nichols et al., 1977) were prelabeled with 0.02–0.025 μCi/mL [^{14}C]dThd (>50 mCi/mmol; Amersham) during the growth phase, grown to confluence, treated with 10 mM hydroxyurea, and irradiated (predominantly 254 nm; 2 W/m^2) with 12 J/m^2 UV as previously described (Smerdon et al., 1978, 1979). Cells were pulse labeled for 0.5 or 1 h with 10 μCi/mL [^{3}H]dThd (40–60 Ci/mmol; Amersham) at different times after irradiation (0–23 h; see text). Following the pulse period, cells were either harvested immediately or subjected to chase periods of varying times prior to harvest with conditioned medium containing 50 μM dThd (unlabeled) and 10 mM hydroxyurea. Approximately 4 × 10^7 cells were used for the generation of each normalized difference curve (see below), and ~7 × 10^6 cells/gel lane were used for electrophoresis experiments.

Enzyme Digestion Methods. Suspended nuclei (35–70 μg of DNA/mL), prepared as described previously (Smerdon et al., 1979), were incubated at 37 °C with either staphylococcal nuclease (0.10–0.13 unit/μg of DNA; Worthington) or DNase I (0.013 μg/μg of DNA; Sigma) in 10 mM Tris (pH 7.8), 0.1 mM $CaCl_2$, and 0.25 M sucrose as described (Smerdon et al., 1978). For the 100% determinations, nuclei were first incubated at 37 °C for ~3 h with 20 μg/mL proteinase K (EM Laboratories) followed by incubation at 37 °C with ~500 units/mL staphylococcal nuclease. Aliquots were taken at 60, 80, 100, and 120 min after the addition of nuclease, and the total radioactivity in acid was determined (Smerdon et al., 1978). [Following this treatment, we observe no difference in the level of radioactivity between the acid-soluble fraction (i.e., following centrifugation) and the combination of soluble and insoluble material (i.e., a suspension of insoluble material in acid prior to centrifugation).] In most experiments, the specific activity of the DNA was 150–1500 ^{3}H cpm/μg (depending on when after damage the cells were pulsed) and 1200–1500 ^{14}C cpm/μg.

Electrophoresis. Electrophoresis on 2.8% agarose slab gels was carried out as described (Smerdon et al., 1978). Electrophoresis on 7 M urea and 8% polyacrylamide cylindrical gels (0.8 × 21 cm) was carried out in TBE (Tris–borate–EDTA) buffer according to Maniatis et al. (1975). Samples were prepared by taking 1-mL aliquots of the digestion mixture, adding 0.1 mL of 0.1 M EDTA, and incubating overnight in a 37 °C shaker bath with 100 μg/mL proteinase K. The DNA was precipitated by adding 0.125 mL of 1 M NaCl and 2.5 mL of 95% ethanol and storing at −20 °C overnight. The samples were then centrifuged at 23500*g* for 20 min at 2 °C, washed once with 70% ethanol (to remove salts), and recentrifuged (23500*g* for 10 min). Samples were air-dried at room temperature for 1 to 2 h. The DNA in each tube was dissolved in 130 μL of 98% formamide [deionized according to Boedtker et al. (1973)], containing 0.1 × TBE buffer, by shaking overnight at room temperature. To ensure complete denaturation of the DNA, we placed the tubes in boiling water for 2–2.5 min and quickly cooled them on ice prior to applying the samples to the gels (Maniatis et al., 1975). Gels were sliced (2 mm) with a Mickle gel slicer (Brinkmann Instruments, Inc.), and the slices were solubilized in 0.5 mL of H_2O_2–NH_4OH (99:1; Albanese & Goodman, 1977) by heating to 70 °C for 3 h. Samples were then cooled to room temperature, and 6 mL of Insta Gel (Packard) was added. The samples were stored overnight in the dark at 4 °C (to reduce chemiluminescence to background levels) and then assayed for radioactivity. Crossover of ^{14}C into the ^{3}H channel was corrected in the standard fashion (Kobayashi & Maudsley, 1974; Horrocks, 1974). The sizes of the DNase I digestion products were determined by coelectrophoresis with *Hae*III restriction fragments of φX 174 RF DNA (Smerdon et al., 1979). The positions of the staphylococcal nuclease digestion products on agarose gels were determined from the location of the bromophenol blue marker dye which migrates at ~135 bp on these gels as determined by coelectrophoresis with the *Hae*III φX 174 RF fragments (unpublished results).

Analysis of Nuclease Digestion Kinetics. Data analyses were carried out as described in Smerdon et al. (1978, 1979). In using this method, one determines the ^{3}H cpm [$H(t)$] and ^{14}C cpm [$C(t)$] rendered acid soluble after various times (t) of digestion of nuclei with nuclease. Also, the total ^{3}H cpm ($H_{100\%}$) and ^{14}C cpm ($C_{100\%}$) are determined by nuclease digestion of proteinase K treated nuclei (see above). It has been our experience that, in most cases, both direct comparisons and analyses of the data are facilitated when "normalized difference curves" are used rather than curves obtained from plotting $H(t)$ vs. $C(t)$ as originally described (Smerdon et al., 1978). Normalized difference curves yield a positive initial slope and a negative final slope (e.g., Figure 1) for nuclease-"sensitive" and -"resistant" regions, respectively, rather than a "more positive" initial slope and a "less positive" final slope as in $H(t)$ vs. $C(t)$ curves [e.g., Figure 4 of Smerdon et al. (1978)]. Therefore, determination of both the initial and

final slopes, as well as their intercept (ξ), is improved by using normalized difference curves.

For repair synthesis experiments, the ordinate values of normalized difference curves can be calculated from the expression

$$\frac{\Delta H}{H_{100\%}} = \frac{H(t) - (H_{100\%}/C_{100\%})C(t)}{H_{100\%}} \quad (1)$$

[In this equation, we assume that $H_{100\%}/C_{100\%}$ = ^{3}H cpm/^{14}C cpm for purified DNA, an assumption that is valid for repair synthesis experiments (Smerdon et al., 1979; see below).] The abscissa values are $C(t)/C_{100\%}$ (or fraction digested; e.g., Figure 1).

The ratio of the fraction of repair-incorporated nucleotides that is nuclease sensitive (f_S) to the fraction of total DNA that is nuclease sensitive (ξ) is given by

$$f_S/\xi = \frac{(M_i + 1)\sigma - 1}{\sigma - 1} \quad (2)$$

where M_i = initial slope of the normalized curve and σ = ($H_{100\%}/C_{100\%}$ for nuclei from irradiated cells)/[$H_{100\%}/C_{100\%}$ for nuclei from control (unirradiated) cells]. We note that eq 2 is the same as eq A6 in our original report (Smerdon et al., 1978) except that the parameter η_s has been replaced by $M_i + 1$. The relationship of the slope M_i to η_s can be derived as follows for early digestion times.

$$M_i = \frac{d(\Delta H/H_{100\%})}{d[C(t)/C_{100\%}]}$$

Therefore, from eq 1

$$M_i + 1 = \frac{C_{100\%}}{H_{100\%}} \frac{dH(t)}{dC(t)}$$

Since $\eta_s = (1/M_{NAK})[dH(t)/dC(t)]$ and M_{NAK} = ^{3}H cpm/^{14}C cpm for purified DNA (Smerdon et al., 1978), it follows from the assumption given above that $M_i + 1 = \eta_s$.

The values for σ obtained for cells pulse labeled immediately after irradiation ranged from 45 to >100. This value decreased to between 10 and 12 for cells pulse labeled during the 23–24-h interval. Thus, even during the latter pulse period, there was at least a ninefold increase in incorporation of label induced by UV irradiation over that of control cells.

We note that, in the past, we have referred to f_S/ξ as the "fraction of repair-incorporated nucleotides per unit DNA that is nuclease sensitive". However, because this phrase is somewhat confusing, we have chosen here to refer to f_S/ξ as the "relative nuclease sensitivity" of repair-incorporated nucleotides.

Results

Analysis of Staphylococcal Nuclease Digestion Kinetics. We have investigated the distribution within chromatin of repair synthesis occurring at different times after damage in confluent, hydroxyurea-suppressed human fibroblasts irradiated with 12 J/m^2 UV. Previous data demonstrated that under these conditions repair synthesis occurs for at least 35 h after irradiation and the nucleotide incorporation rate is characterized by an initial rapid phase (2 to 3 h) and a prolonged, slower phase (Smerdon et al., 1978). In the present study human fibroblasts were prelabeled with [^{14}C]dThd during growth, allowed to come to confluence, treated with hydroxyurea, and pulse labeled with [^{3}H]dThd at different times after UV. Some cells were harvested immediately after the pulse period while others were subjected to chase periods of varying times with unlabeled dThd. Label incorporated by replicative

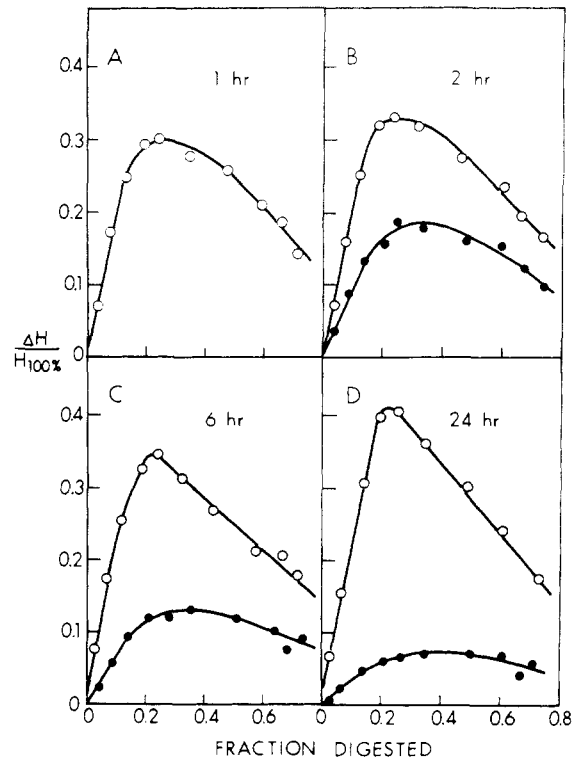

FIGURE 1: Normalized difference curves (Smerdon et al., 1978, 1979) for the staphylococcal nuclease digestion of repair-labeled nuclei. Cells, prelabeled during replication with [^{14}C]dThd, were pulse labeled for 1 h with [^{3}H]dThd starting at 0 (A), 1 (B), 5 (C), or 23 (D) h after UV irradiation and harvested without subsequent chase (○). Alternatively, cells were pulse labeled for 1 h immediately after irradiation and subjected to chase periods of 1 (B), 5 (C), or 23 (D) h prior to harvest (●). Ordinate and abscissa values are defined under Materials and Methods.

synthesis during the growth phase (^{14}C) serves as a marker for bulk DNA (i.e., total DNA in chromatin) and is uniformly distributed in chromatin (Smerdon et al., 1978); label incorporated during the pulse period (^{3}H) serves as a marker for repaired DNA since replicative synthesis is reduced to very low levels by both contact inhibition (confluence) and hydroxyurea.

Nuclei from these double-labeled cells were digested with staphylococcal nuclease, and at various times aliquots were assayed for acid-soluble radioactivity. The data were then analyzed by using a method previously described by us (Smerdon et al., 1978, 1979). The method involves the representation of nuclease digestion data as "normalized difference curves" and the calculation of the "relative nuclease sensitivity" of repair-incorporated nucleotides (f_S/ξ) from these curves (see Materials and Methods for details). Examples of the normalized difference curves that were obtained are shown in Figure 1. These data are for cells which were pulse labeled for 1 h starting at 0, 1, 5, and 23 h after damage with no subsequent chase (open circles) and for cells pulse labeled for 1 h immediately after damage and subjected to chase periods of 1, 5, and 23 h (closed circles). Each set of data represents the difference between the release of ^{3}H by staphylococcal nuclease from nuclei and the DNA from those nuclei. These curves may be interpreted by considering the following. (1) The initial slope is proportional to the fraction of repair-incorporated nucleotides which is released from chromatin at a rate similar to that of linker DNA (referred to by us as nuclease sensitive) (Smerdon et al., 1978 and unpublished results). (2) The final slope is proportional to the fraction of repair-incorporated nucleotides which is released from chro-

matin at a rate similar to that of core DNA (referred to by us as nuclease resistant) (Smerdon et al., 1978 and unpublished results). (3) A uniform distribution of repair-incorporated nucleotides within chromatin is represented by a constant value of $\Delta H/H_{100\%} = 0$ as a function of fraction digested (Smerdon et al., 1978). From these considerations, the curves in Figure 1 (open circles) demonstrate that, at least for times as long as 24 h after damage, many of the newly incorporated nucleotides are located in DNA that is digested at a rate similar to that of linker DNA in chromatin. We note that as the interval between the time of irradiation and the time of the pulse increases, the data more closely approximate two straight lines (see Discussion). Also, nuclei from cells pulse labeled during the first hour after damage and subjected to chase periods of various times (closed circles) yield values of $\Delta H/H_{100\%}$ which are closer to 0 at all values of fraction digested than the nuclei from cells not receiving a chase. These results are in agreement with our previous findings (Smerdon & Lieberman, 1978a) that nucleotides incorporated immediately after irradiation are preferentially digested by staphylococcal nuclease and become more uniformly distributed with increasing chase times.

If one assumes a two-state model for the digestion of DNA in chromatin, the intercept of the initial and final slopes of each of these curves yields the fraction of DNA in staphylococcal nuclease sensitive regions of chromatin (ξ; Smerdon et al., 1978). The values obtained for ξ from the cells receiving no chase were 0.20, 0.19, 0.17, and 0.20 for parts A, B, C, and D of Figure 1, respectively, and are similar to the calculated values for the amount of DNA in linker regions in these cells (Smerdon et al., 1978).

The relative nuclease sensitivity of repair-incorporated nucleotides (f_S/ξ) was determined from each of the curves in Figure 1 (and the corresponding values for σ; Materials and Methods). The values for cells which were pulse labeled for 1 h starting 0, 1, 5, and 23 h after irradiation are shown in Figure 2A (dashed line). For each of these times this ratio is significantly greater than 1.0 (i.e., the value corresponding to a uniform distribution), and it increases slightly with increasing times after damage. Therefore, a large fraction of the repair-incorporated nucleotides is staphylococcal nuclease sensitive following each of the 1-h pulse periods, and this fraction increases slightly with increasing time after damage. A possible explanation for this increase might be that, at late times after UV, the rate of redistribution of label is slower than at early times. This would result in fewer of the repair-incorporated nucleotides becoming nuclease resistant during the 1-h pulse and would increase the measured value of f_S/ξ. This possibility was tested by measuring the distribution of label in cells pulse labeled for 1 h starting at 23 h after damage and subjected to chase periods of varying times. These results are also shown in Figure 2A. For comparison, we have included results from cells pulse labeled during the first hour after damage and then chased. As can be seen, nucleotides incorporated during the 23–24-h interval do undergo a redistribution during the chase period. Furthermore, this redistribution appears to be even more rapid than that seen for nucleotides incorporated during the first hour after damage.

Extrapolation of the initial slope of these data to one-half the pulse interval yields the value of f_S/ξ corresponding to the *initial* distribution of label, if one assumes a constant rate of nucleotide incorporation and redistribution during the pulse period. From this calculation, a value of $f_S/\xi = 5.1 \pm 0.2$ is obtained for the nucleotides incorporated during the 23–24-h interval. Since $\xi \simeq 0.20$, this result suggests that essentially

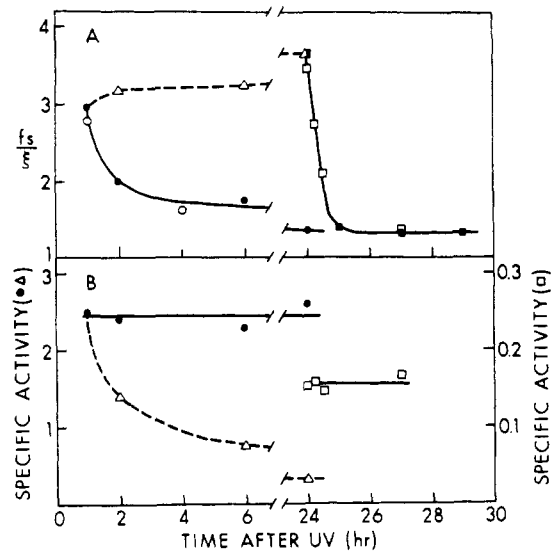

FIGURE 2: (A) Kinetics of redistribution of nucleotides incorporated by repair synthesis at early and late times after damage using staphylococcal nuclease as a probe. Cells were pulse labeled for 1 h starting at 0, 1, 5, or 23 h after damage and harvested without subsequent chase (△). Alternatively, cells were pulse labeled for 1 h either immediately after damage (● and ○) or 23 h after damage (■ and □) and subjected to the chase periods shown. Different symbols represent different experiments. f_S/ξ = (fraction of repair-incorporated nucleotides that is nuclease sensitive)/(fraction of bulk DNA that is nuclease sensitive). (B) Specific activity (^{3}H cpm/^{14}C cpm) of the DNA: cells pulse labeled for 1 h immediately after irradiation and subjected to chase periods shown (●); cells pulse labeled for 1 h starting at 23 h after irradiation and subjected to chase periods shown (□); cells pulse labeled for 1 h and harvested immediately (△). Symbols matching those in (A) are for the same cells.

all of the nucleotides incorporated during the 23–24-h interval are initially staphylococcal nuclease sensitive. We also performed this calculation on data from cells which were pulse labeled for 30 min immediately after damage and chased for varying times (data not shown). The 30-min pulse was used in order to obtain a better estimate of the initial slope than can be obtained from the 1-h pulse data in Figure 2A (circles). In this case a value of $f_S/\xi = 4.4 \pm 0.5$ was obtained, indicating that immediately after damage 78–98% of the repair-incorporated nucleotides are initially staphylococcal nuclease sensitive, in good agreement with results obtained using a different nuclei preparation method (Smerdon & Lieberman, 1978a). Thus, for both early and late times after damage, most (or all) of the repair-incorporated nucleotides are initially located in DNA that is rapidly digested by staphylococcal nuclease.

The specific activity of the DNA was essentially constant for the cells which were pulse labeled and then chased (Figure 2B). These data demonstrate that no further label was incorporated during the chase period. Also, as expected, the specific activity of the DNA from cells which were pulse labeled at different times after damage decreased rapidly over the first few hours and was ~10% of the initial value by 24 h (Figure 2B; open triangles).

Electrophoretic Analysis of Staphylococcal Nuclease Digestion Products. Staphylococcal nuclease digestion products from cells pulse labeled for 1 h immediately after damage were electrophoresed on agarose gels. Figure 3 shows the profiles of each label (parts A–C) and the corresponding profiles of the ratio of the two labels (normalized to the ratio for undigested DNA; RS; parts D–F) for three different extents of digestion. These data represent an intermediate point (i.e., following a 1-h pulse) in the redistribution process where 55–60% of the repair-incorporated nucleotides are nuclease

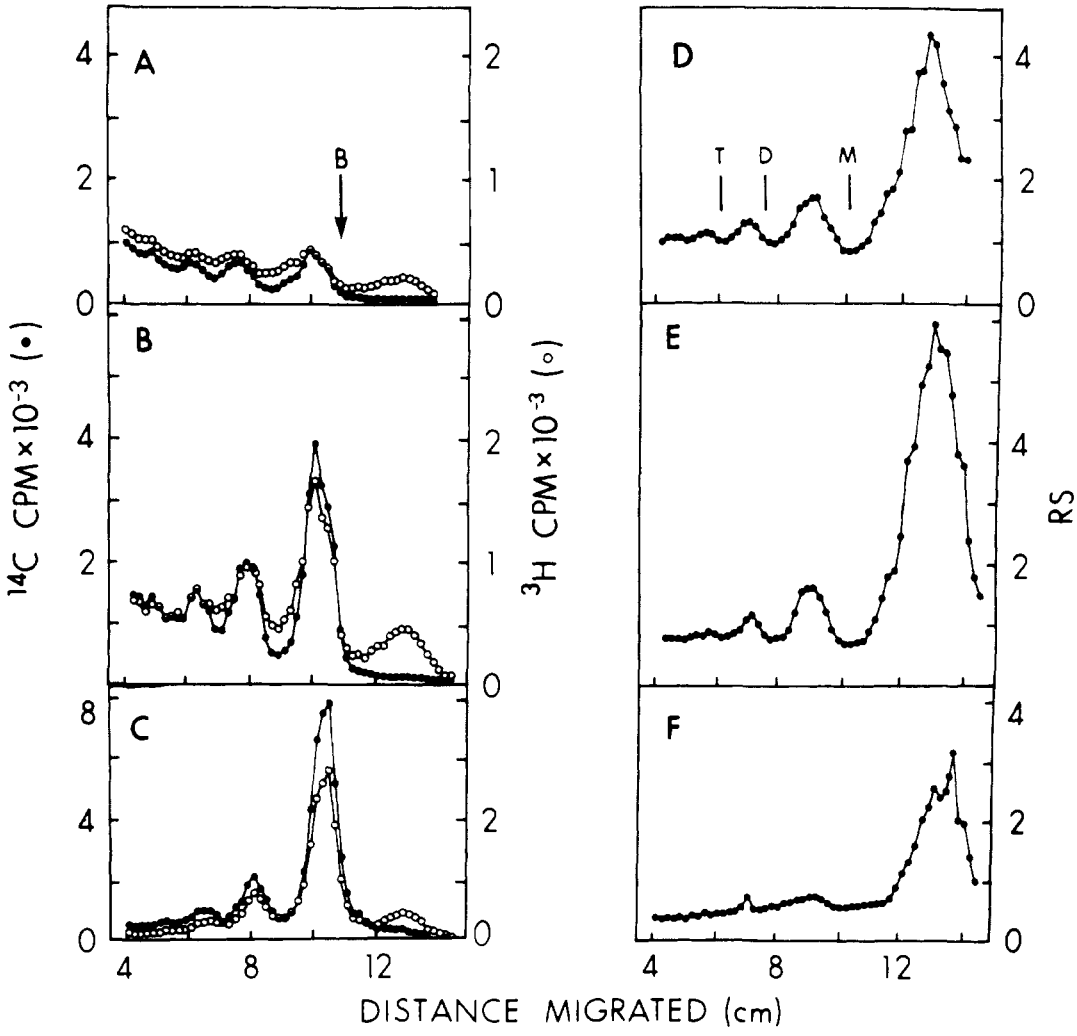

FIGURE 3: Agarose gel profiles of staphylococcal nuclease digestion products from cells pulse labeled for 1 h immediately after irradiation and harvested without subsequent chase. Digestion was terminated when the fraction of ^{14}C cpm rendered acid soluble was 0.022 (A), 0.063 (B), and 0.184 (C). Panels A–C show profiles for both the bulk DNA (^{14}C) and the DNA containing repair-incorporated nucleotides (^{3}H). To the right of these panels (panels D–F) is shown the corresponding relative specific activity (RS = ^{3}H cpm/^{14}C cpm of digested DNA divided by the ^{3}H cpm/^{14}C cpm of undigested DNA) as a function of migration. The arrow in panel A (designated B) indicates the position of the marker dye (~135 bp; Materials and Methods). Panel D includes the positions of the corresponding monomer (M), dimer (D), and trimer (T) peaks.

sensitive ($f_S/\xi \simeq 2.9$; see Figure 2A). As reported previously (Smerdon & Lieberman, 1978a), for low amounts of digestion the RS profile shows a periodic dependence on migration (parts D and E of Figure 3), with minima occurring at monomer, dimer, etc., demonstrating the underrepresentation of repair-incorporated nucleotides in native core structures. However, more extensive digestion yields an almost constant RS for the monomer and remaining multimer peaks (Figure 3F) and the value of RS is greater than 0 in these regions. Thus, some of the repair-incorporated nucleotides are in native core structures and are probably in regions that have undergone rearrangement during the pulse period. The latter conclusion is supported by data from pulse–chase experiments in which the extrapolation of RS for core DNA (145–165 bp) to one-half the pulse time was close to zero [data not shown; however, see Smerdon & Lieberman (1978a) and Tlsty & Lieberman (1978)].

Further inspection of Figure 3 indicates that the ^{3}H multimer peaks (i.e., dimer, trimer, etc.) are coincident with the ^{14}C multimer peaks at each digestion time. Pulse–chase experiments demonstrated that with increasing chase times the positions of the ^{3}H multimer peaks remain the same as those of the bulk DNA at differing extents of digestion even though the amount of ^{3}H label in these peaks increased (data not shown). Thus, following rearrangement the DNA labeled during repair synthesis has the same average nucleosome repeat length as bulk DNA.

For early times in the redistribution process, we routinely obtain a broad peak in the ^{3}H profile in the low molecular weight region of the gel (parts A–C of Figure 3; 12–14 cm). Electrophoresis of these samples on denaturing polyacrylamide gels, which yield a better resolution of the DNA fragments in this region (e.g., Figure 6), indicates that the front running "peak" observed on agarose gels (e.g., Figure 3) is composed of heterogeneous sizes of DNA (data not shown). This peak probably arises from the extreme nonlinearity of agarose gels in this size range.

The nuclease digestion products from cells pulse labeled during the 23–24-h interval were also examined on agarose gels. Parts A–D of Figure 4 show profiles for two different extents of digestion. In both cases, the profiles of ^{3}H, ^{14}C, and RS are similar to the corresponding profiles in Figure 3. These data agree with the digestion kinetic results (Figures 1 and 2) and suggest that nucleotides incorporated during repair synthesis occurring at late times after damage are also initially underrepresented in native core structures. With increasing

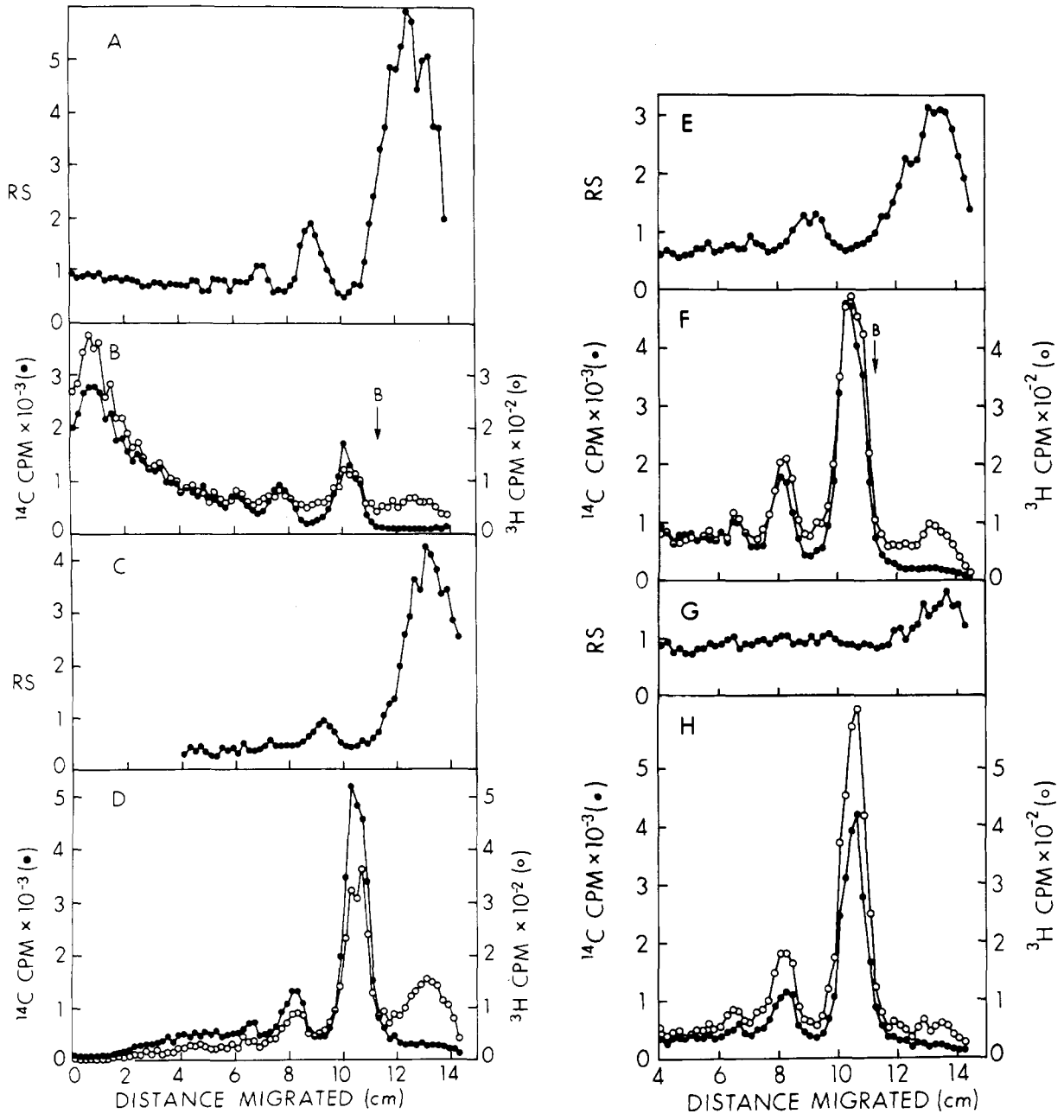

FIGURE 4: Agarose gel profiles of staphylococcal nuclease digestion products from cells pulse labeled for 1 h starting at 23 h after irradiation and subjected to chase periods of 0 (A–D), 0.5 (E and F), or 3 h (G and H). Panels B, D, F, and H show profiles for bulk DNA (^{14}C) and DNA containing repair-incorporated nucleotides (^{3}H). Above each of these panels (i.e., panels A, C, E, and G) are the corresponding relative specific activity profiles (RS; see Figure 3). Digestion was terminated when the fraction of ^{14}C cpm rendered acid soluble was 0.035 (B), 0.141 (D), 0.101 (F), and 0.124 (G). The arrow (B) indicates the position of the marker dye (~135 bp; Materials and Methods).

chase times more of these nucleotides appear in monomer and multimer DNA fragments (parts E–H of Figure 4).

Analysis of DNase I Digestion Kinetics. Nuclei from cells pulse labeled (or pulsed and then chased) during early and late times after damage were also digested with DNase I. Analysis of the kinetics of release of repair-incorporated nucleotides by DNase I gave results similar to those obtained by using staphylococcal nuclease as a probe (Figure 5). Thus, regardless of when after damage nucleotides are incorporated, they are initially DNase I sensitive and, with time, become DNase I resistant (see Discussion). (We note that in Figure 5 the pulse time immediately after damage was half the pulse time used in Figure 2A, and therefore the data cannot be directly compared.)

Electrophoretic Analysis of DNase I Digestion Products. The DNA fragments produced by digestion of nuclei with DNase I were analyzed on denaturing gels. Figure 6 shows the results for two different extents of digestion of nuclei from cells pulse labeled (0.5 h) immediately after irradiation and chased for 0, 0.5, and 4.75 h. The bulk DNA profiles (^{14}C) exhibit the familiar pattern of fragments in which each peak differs from the next by ~10 bases in length and has a characteristic relative area (Noll, 1974). The profiles of DNA labeled during repair synthesis (^{3}H) are different from those of bulk DNA. Immediately after the pulse period the repair-labeled DNA profiles show little of the ~10-base repeat pattern (parts A and D of Figure 6). Furthermore, even when the fraction of total label rendered acid soluble is similar for repair-labeled DNA and bulk DNA, there is a pronounced difference between their corresponding profiles [compare Figure 6A (open circles) and Figure 6D (closed circles)]. With increasing chase time, however, the repair-labeled DNA

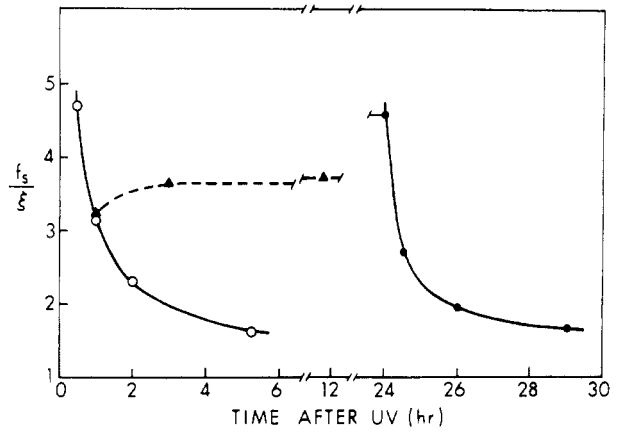

FIGURE 5: Kinetics of redistribution of nucleotides incorporated by repair synthesis at early and late times after damage using DNase I as a probe (definition of f_S/ξ is given in the legend to Figure 2). Cells were pulse labeled for 1 h starting at 0, 2, 11, or 23 h after damage and harvested without subsequent chase (▲). Alternatively, cells were either pulse labeled for 0.5 h immediately after damage (○) or pulse labeled for 1 h at 23 h after damage (●) and subjected to the chase periods shown.

profiles approach the patterns observed for bulk DNA (parts B, C, E, and F of Figure 6).

Similar profiles were obtained for cells pulse labeled during the 23–24-h interval (data not shown). Therefore, at both early and late times after damage the redistribution phenomenon involves the appearance of repair-incorporated nucleotides in the ~10-base repeat fragments characteristic of native nucleosome structures. We note, however, that although the repair-labeled DNA profile acquires the ~10-base repeat pattern after long chase periods, the relative areas of the peaks are not identical with those of the bulk DNA profiles (parts C and F of Figure 6; see Discussion).

Discussion

In the present study we have demonstrated that most or all of the nucleotides incorporated by DNA repair synthesis occurring at times as long as 24 h after damage are initially staphylococcal nuclease and DNase I sensitive. Subsequently, many of these nucleotides are involved in nucleosome rearrangement and become more uniformly distributed in chromatin. These results explain our previous findings that following an ~20-h pulse during the "slow phase" of repair (i.e., times greater than 6 h after damage) the distribution of label is more uniform than following a 3-h pulse immediately after UV (Smerdon et al., 1978). It is clear from our present study that the difference observed between these two pulse periods resulted primarily from more extensive redistribution of label during the longer pulse period. However, the present study suggests that there may be subtle differences in the regions within chromatin that are repaired during early and late times after damage. The difference in shape of the normalized difference curves (Figure 1) indicates that some of the nucleotides incorporated during the 1-h pulse at early times after

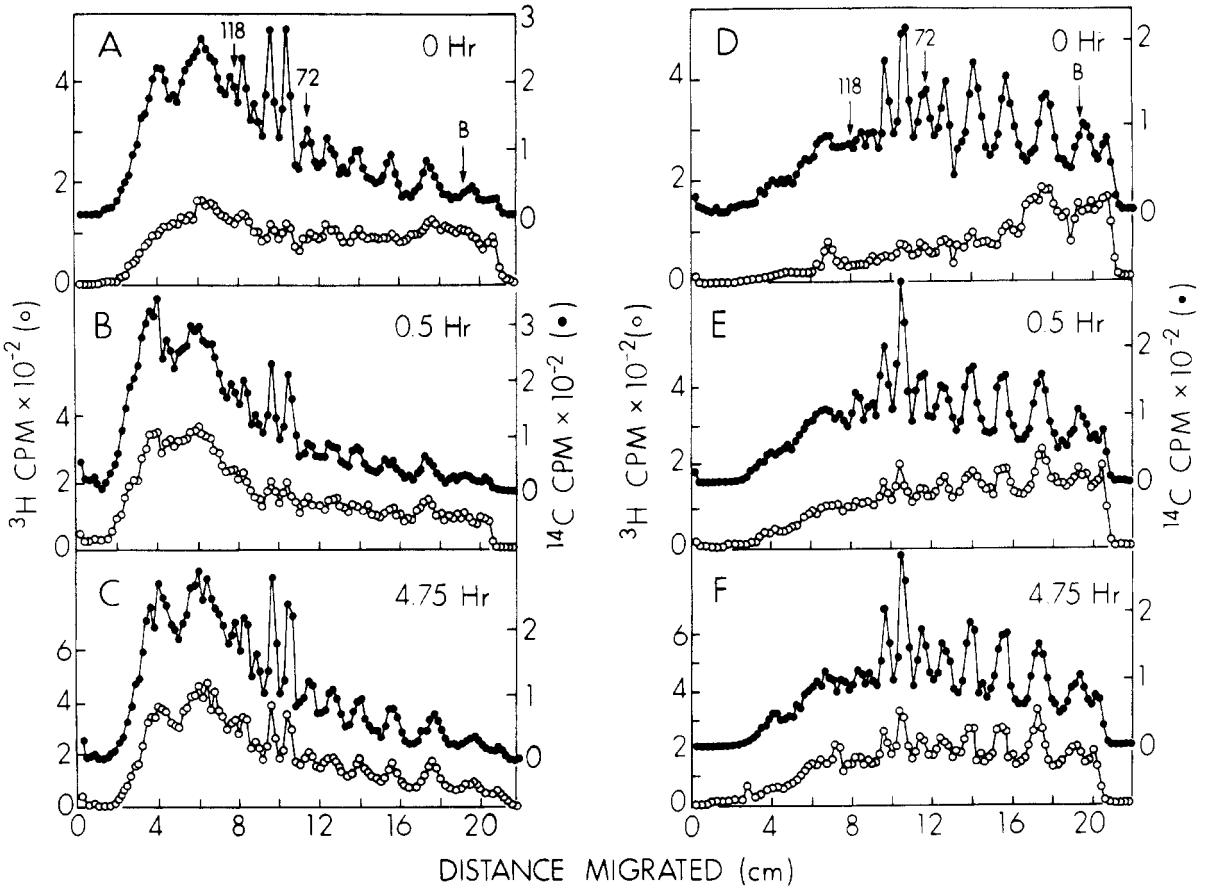

FIGURE 6: Denaturing gel profiles of DNase I digestion products from cells pulse labeled during early times after damage. Profiles shown are for the cells used for Figure 5 (0–0.5-h pulse) which were subjected to 0-, 0.5-, and 4.75-h chase periods. The fraction of total ^{14}C cpm rendered acid soluble was 0.15 (A), 0.09 (B), 0.12 (C), 0.45 (D), 0.34 (E), and 0.40 (F). The fraction of total ^{3}H cpm rendered acid soluble was 0.46 (A), 0.23 (B), 0.19 (C), 0.71 (D), 0.51 (E), and 0.49 (F). Arrows indicate the positions of two of the *Hae*III φX 174 RF marker fragments and the marker dye (B) which migrates at ~21 bases on these gels (Lutter, 1979). The ordinate values for ^{14}C cpm have been offset for clarity. Photographs of these gels, stained with ethidium bromide, revealed discrete fragments out to at least 200 bases for the early digestion times; however, slicing of these gels for scintillation counting (2-mm width) allows resolution out to only ~120 bases in length.

damage may be digested by staphylococcal nuclease at rates intermediate between those of sensitive and resistant DNA, whereas nucleotides incorporated at late times are essentially all digested at either the sensitive or the resistant rate. Also, the kinetics of redistribution of repair-incorporated nucleotides appear to be more rapid at late times after damage (Figure 2A). Clearly, further experimentation is required to elucidate the significance of these differences; however, an intriguing possibility is that they may reflect differences in the rates of repair of various *domains* within chromatin (e.g., euchromatin, heterochromatin, regions containing actively transcribed genes, etc.).

In assessing the possibility that staphylococcal nuclease sensitive regions of chromatin are repaired most rapidly (see introduction), it is important to consider the possibility that the repair process may *induce sensitivity* in a previously resistant region. A recent study by Williams & Friedberg (1979) indicates that UV-induced pyrimidine dimers are uniformly distributed in chromatin and are removed at similar rates from staphylococcal nuclease sensitive and resistant (core) DNA. We have obtained similar results by using [^{3}H]-7-(bromomethyl)benz[*a*]anthracene to damage cells (Oleson et al., 1979). These studies appear to rule out the possibility that only linker regions in chromatin are repaired. The apparent contradiction between the observation that damage is removed from both nuclease-sensitive and -resistant (core) DNA and the observation that initially most of the repair-incorporated nucleotides are nuclease sensitive can be explained by a model in which the repair process induces an "unfolding" of the nucleosomal DNA (Lieberman et al., 1979; Oleson et al., 1979). Thus, it is possible that the repair process "induces nuclease sensitivity" in a previously resistant region.[2]

Electrophoretic studies of the staphylococcal nuclease digestion products indicate that, for both early and late times after damage, redistribution results in the appearance of repair-incorporated nucleotides in DNA that coelectrophoreses with DNA from nucleosome cores. Following the rapid phase of redistribution (i.e., 2–3-h chase at early times and 1–1.5-h chase at late times; Figure 2A) the average repeat length of the DNA containing these nucleotides is the same as that of bulk DNA. Thus, following the rapid phase of redistribution the nucleosome structure of repair-labeled DNA is not measurably distinguished from that of bulk DNA by staphylococcal nuclease digestion. If rearrangement represents the restoration of the original repeat size of each repaired nucleosome (i.e., the length of DNA in linker and core present prior to damage), then these results suggest that repair synthesis occurs in nucleosomes of all repeat sizes and does not occur more rapidly in nucleosomes with longer linker regions as suggested by Cleaver (1977).

The kinetics of release of repair-incorporated nucleotides from nuclei by DNase I yield essentially the same results as obtained with staphylococcal nuclease (Figure 5). This correlation is in keeping with an "unfolding–refolding" model. If the repaired DNA is initially less tightly bound to core histones than bulk DNA, it is reasonable that this DNA will be more accessible to degradation by *any* nuclease. This consideration, along with the fact that the enhanced sensitivity to DNase I is observed at late times as well (Figure 5), disallows any inferences from these results about repair synthesis occurring preferentially in "active gene" regions (Weintraub & Groudine, 1976; Garel & Axel, 1976; Levy & Dixon, 1977).

The gel profiles of the DNase I digestion products provide a more detailed description of the distribution of repair-incorporated nucleotides within the nucleosome core. Many studies have shown that the digestion of isolated cores by DNase I results in an ~10-base repeat pattern similar to that observed for nuclei (Shaw et al., 1976; Simpson & Whitlock, 1976; Noll, 1977; Lutter, 1979). Furthermore, Lutter (1979) has shown that the frequencies of cutting by DNase I and the location of these sites within the nucleosome core can sufficiently describe the distribution of fragment lengths obtained from an extensive DNase I digestion of nuclei. These results suggest that the ~10-base repeat pattern seen in extensive DNase I digests of nuclei results primarily from digestion of nucleosome cores within nuclei. Therefore, since very little of the ~10-base repeat pattern is observed immediately after repair synthesis (i.e., before much rearrangement has occurred; parts A and D of Figure 6), the repaired DNA is not initially packaged in native nucleosome core structures.

From the results of Liu & Wang (1978), which predict the observation of an ~10-base repeat pattern for DNase I digestion of DNA lying on *any* surface, one can also argue that initially "repair patches" are not in close contact with core proteins. These observations may also indicate that "half-nucleosome" structures are not formed during repair synthesis (Weintraub et al., 1976; Oudet et al., 1977; Woodcock & Frado, 1977). In support of the latter view is the observation that urea-induced half-nucleosome structures retain their resistance to staphylococcal nuclease digestion (Woodcock & Frado, 1977), whereas DNA containing newly repair-incorporated nucleotides is rapidly degraded to small, nondiscrete fragments (see Results).

Following the rapid phase of redistribution, the DNA containing repair-incorporated nucleotides acquires the ~10-base repeat pattern generated by DNase I (parts C and F of Figure 6); however, the relative areas of the peaks are different from those of the bulk DNA in chromatin. This difference in relative areas is observed even though, following the rapid phase of redistribution, staphylococcal nuclease makes little or no distinction between repair-labeled nucleosomes and those of bulk DNA as judged by the similarity in repeat sizes (see above). At present we do not know if this result arises from a technical artifact or if subtle differences (i.e., undetectable by staphylococcal nuclease digestion) between repair-labeled cores and those of bulk chromatin persist for long periods of time and result in the differences seen in the DNase I gel patterns.

Finally, we note that, at present, we cannot explain why Cleaver failed to detect redistribution of repair-incorporated label in pulse–chase experiments (Cleaver, 1977). We have investigated the redistribution phenomena under many different conditions: (a) in the presence and absence of hydroxyurea (Smerdon et al., 1979); (b) over a large UV dose range (Smerdon et al., 1979); (c) following damage by the chemical agents 2-(acetylacetoxyamino)fluorene (Tlsty & Lieberman, 1978) and 7-(bromomethyl)benz[*a*]anthracene (Oleson et al., 1979); (d) in two normal cell lines and in two cell lines with reduced excision repair (xeroderma pigmentosum cells; Smerdon et al., 1979); (e) using four different nuclei preparation methods (Smerdon et al., 1978, 1979, and unpublished results), including the method used by Cleaver (1977

[2] One might think that nuclease digestion of unfolded core DNA would result in a value for ξ (i.e., the fraction of total DNA that is staphylococcal nuclease sensitive) in our analyses that is different from the fraction of the genome in linker regions, yet we observe these values to be similar. However, since both the repair-labeled DNA (prior to rearrangement) and linker DNA are digested much more rapidly than DNA in native core structures and the amount of repair-labeled DNA in chromatin is small [e.g., Williams & Cleaver (1978)], our determination of ξ may be insensitive to such an unfolding process.

and personal communication). Redistribution of repair-incorporated label during the chase period was observed in *all* of these studies. Furthermore, Williams & Friedberg (1979) have verified our findings following UV-induced repair synthesis. [It should be pointed out that these latter authors report that they find a more limited redistribution of repair-incorporated nucleotides than we observe; however, their calculations are for the redistribution occurring *after* a 1-h pulse time whereas our calculations are for an extrapolation to one-half the pulse time (see Results). Indeed, their calculations agree quite well with ours for the fraction of repair-incorporated nucleotides involved in redistribution *following* a 1-h pulse.] Although the differences between our results and those of Cleaver (1977) remain unresolved, an intriguing possibility is that the nucleosome conformation following rearrangement is less stable than bulk chromatin to certain perturbations during isolation and that these regions can be selectively altered.

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