Nucleosome Rearrangement in Vitro. 2. Formation of Nucleosomes in Newly Repaired Regions of DNA†

John F. Watkins and Michael J. Smerdon*
Biochemistry/Biophysics Program, Washington State University, Pullman, Washington 99164-4660
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ABSTRACT: We have reported previously that immediately following nucleotide excision repair in human cells the newly repaired DNA lacks a nucleosome conformation [Smerdon, M. J., & Lieberman, M. W. (1980) *Biochemistry 19*, 2992–3000]. In this study, we have examined the ability of these nascent DNA regions to acquire a nucleosome structure in vitro by incubating intact or H1-depleted nuclei in buffers containing different salt concentrations (0.025–0.625 M KCl) at 0 or 37 °C. Nucleosomes were detected in these regions by an increase in the level of repair-incorporated nucleotides associated with isolated nucleosome core particle DNA. Our results indicate that the nascent DNA is resistant to nucleosome formation during the low-salt transition where the limiting repeat length decreases from ~190 to 168 base pairs (bp) [Watkins, J. F., & Smerdon, M. J. (1985) *Biochemistry* (preceding paper in this issue)]. This result provides further evidence that the nascent DNA is indeed in a nonnucleosomal state. At higher salt concentrations (>0.4 M), where the nucleosome repeat length decreases to a limiting value of ~146 bp, there was an increase in nucleosome formation in nascent DNA that correlated with the decrease in limiting repeat length. However, we did not observe a *complete* randomization of the repair-incorporated nucleotides. Indeed, even at the highest salt concentration used (0.625 M), we never observed more than 50% of the nascent DNA associated with the isolated core particles. This was the case even though a major portion of the nucleosomes had a limiting value repeat length following the high-salt incubation. Furthermore, the removal of histone H1 prior to salt treatment did not allow complete randomization during the higher salt transition. Since it was shown in the preceding paper that nucleosome exchange can occur during this higher salt transition, it is possible that the formation of nucleosomes in the nascent DNA involves an exchange mechanism. The presence of hydroxyurea during the repair labeling step had little or no effect on the fraction of newly repaired DNA that was refractory to nucleosome formation. This result indicates that the incomplete randomization of repair-incorporated nucleotides following salt-induced rearrangement does not result from the presence of single-strand nicks, gaps, or displaced strands in many of the newly repaired regions. However, a complete randomization was observed when the core histones were dissociated from the DNA in 2 M salt and the chromatin reconstituted. Therefore, newly repaired DNA *alone* can be folded into nucleosomes in vitro, following the removal of most of the nuclear proteins, and factors other than simply the presence of incomplete 3′ ends in this regions must be responsible for maintaining the nonnucleosomal state in vivo.

The rearrangement of chromatin structure during nucleotide excision repair of adducts induced by ultraviolet (UV)[1] radiation and UV-mimetic chemicals is well documented [for review, see Lieberman et al. (1979), Lieberman (1982), Bohr & Hanawalt (1984), and Friedberg (1985)]. The evidence to date suggests that immediately following repair synthesis the nascent DNA is in an "unfolded" or nonnucleosomal state (Lieberman et al., 1979). For example, these regions do not yield a 10.4-base repeat pattern on denaturing gels following digestion of isolated nuclei with DNase I (Smerdon & Lieberman, 1980). This pattern appears, however, following the (presumed) refolding of the nascent DNA into nucleosome structures. Furthermore, removal of both UV-induced pyrimidine dimers (Williams & Friedberg, 1979) and the adducts formed by at least one UV-mimetic chemical (Oleson et al., 1979) occurs at approximately equal rates from linker and core DNA. An alternative explanation that we originally considered (Smerdon & Lieberman, 1978) is that repair synthesis occurs primarily in linker DNA and, with time, this nonuniform distribution is randomized by nucleosome sliding. In this case, however, additional assumptions must be imposed (Lieberman et al., 1979). Indeed, it is difficult to explain the DNase I results by this type of redistribution mechanism (Smerdon & Lieberman, 1980). However, this latter possibility cannot be completely ruled out by the evidence to date. Therefore, one of the goals of the present study was to compare the salt-induced formation of nucleosome-like structures in newly repaired DNA to the rearrangements that occur in bulk chromatin reportedin the preceding manuscript (Watkins & Smerdon, 1985). The rationale for these experiments was the following: If repair patches are initially located in linker regions of nucleosomes having a native structure, then there should be a tight "coupling" between the induced migration of nucleosomes and the randomization of repair patches between linker and core regions. On the other hand, if repair patches have a nonnucleosomal structure, this coupling may not occur. Thus, we have examined the ability of newly repaired regions of DNA to form nucleosome-like structures in vitro under conditions that promote nucleosome rearrangements in the mature chromatin. The results are compared to those of the preceding report (Watkins & Smerdon, 1985), demonstrating two phases of nucleosome rearrangement in

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[1] Abbreviations: UV, ultraviolet; dThd, thymidine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; bp, base pair(s); EDTA, ethylenediaminetetraacetic acid.

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nuclei. Furthermore, we have examined the effect of incomplete repair patches on the formation of nucleosomes in these regions.

Materials and Methods

A flow chart of the procedures used in this paper is shown in Figure 1 of the preceding paper (Watkins & Smerdon, 1985).

Cell Culture, Damage, and Labeling. Human diploid fibroblasts (strain AG1518) were prelabeled during the growth phase with 25–50 nCi/mL [^{14}C]dThd (50 mCi/mmol; New England Nuclear), grown to confluence, treated with 2 mM hydroxyurea for 45 min prior to irradiation, and irradiated with 12 J/m^2 UV light (predominantly 254 nm; 2 W/m^2) as previously described (Smerdon et al., 1978, 1982a). The hydroxyurea step was omitted in some experiments to determine the effects of hydroxyurea on the salt-induced rearrangements. Immediately after irradiation, the cells were pulse-labeled for 40 min with 2.5–5.0 μCi/mL [^{3}H]dThd (50–80 Ci/mmol; New England Nuclear) in the medium and then harvested. In one set of experiments, some of the cells were subjected to a 24-h chase period with conditioned medium containing 50 μM unlabeled dThd prior to harvest.

For experiments measuring nuclease digestion kinetics, the cells were split 1:2 and labeled with 25 nCi/mL [^{14}C]dThd or 10 nCi/mL [^{3}H]dThd for 4 days. The medium was then changed, and the cells were grown to confluence prior to harvest.

Preparation of Intact and H1-Depleted Nuclei. Nuclei were prepared, and histone H1 was selectively extracted, according to the method of Lawson & Cole (1979) [also see Smerdon & Lieberman (1981), Smerdon et al. (1982b), and Watkins & Smerdon (1985)]. All buffers contained 0.1 mM phenylmethanesulfonyl fluoride. The number of nuclei in each sample was determined by counting an aliquot of the nuclei on a "bright-line" hemacytometer (American Optical Corp.) viewed through a phase-contrast microscope.

Chromatin Preparations and Reconstitution. Chromatin was prepared from nuclei by three different methods: sonication, hypotonic lysis, and digestion of hypotonically lysed nuclei with the restriction enzyme *Bam*HI. Sonication was performed with a Heat Systems-Ultrasonics, Inc. sonicator (Model W-225R), equipped with a microtip, using a setting of 3.5 for 15 s. The hypotonically lysed preparation was made by dialysis of nuclei against 1 mM Tris (pH 7.4) as described in the preceding paper (Watkins& Smerdon, 1985). For restriction-cut chromatin, hypotonically lysed nuclei ($\sim 4 \times 10^7$) were dialyzed overnight against *Bam*HI digestion buffer (50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM dithiothreitol). The resulting chromatin suspension was incubated with 500 units of *Bam*HI (Bethesda Research Laboratories) at 37 °C for 21 h. All samples were resuspended in buffer A (50 mM Tris, pH 7.5, 25 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.25 M sucrose) by extensive dialysis before high-salt treatment.

Chromatin was reconstituted by using a method similar to those reported by Steinmetz et al. (1978) and Tatchell & Van Holde (1977). Hypotonically lysed nuclei were dialyzed at 4 °C for 16 h against reconstitution buffer (10 mM Tris, pH 7.4, and 2 mM EDTA) containing 2 M KCl. Subsequent dialysis steps, each at 4 °C for at least 16 h, consisted of successive dialyses against solutions containing reconstitution buffer and decreasing concentrations of KCl in the following order: 1.5, 1.0, 0.75, 0.5, 0.4, and 0.1 M. Finally, the reconstituted chromatin was dialyzed against buffer A for 24 h.

Salt Incubations. Suspended nuclei or chromatin preparations were exposed to elevated salt concentrations (see text), incubated at 37 °C for 1 h, and resuspended in buffer A as described in the preceding paper (Watkins & Smerdon, 1985). In one experiment, some of the nuclei were incubated for an additional 14 h at 37 °C before being resuspended in buffer A. For control samples, nuclei (or chromatin) were suspended in low-salt buffer (buffer A, 2 mM EDTA, pH 7.5) and either incubated at 37 °C or kept on ice during the high-salt incubation.

Nuclease Digestion, Monomer Nucleosome Isolation, and Electrophoresis. Staphylococcal nuclease digestions of nuclei (or chromatin) suspended in buffer A, isolation of monomer nucleosomes, agarose gel electrophoresis, and determination of the radioactive profile of mononucleosome core DNA were all carried out as described in the preceding paper (Watkins & Smerdon, 1985). The size of core DNA was determined by calibration of gels with *Hin*fI restriction fragments of ϕX174 RF DNA.

Analysis of Salt-Induced Rearrangement. A simplified representation of chromatin structure is one in which different structural regions can be approximated as either nuclease sensitive or nuclease resistant. Nucleotides incorporated during repair synthesis are initially staphylococcal nuclease sensitive and become increasing resistant during the subsequent nucleosome rearrangement. This loss of nuclease sensitivity is coupled to the appearance of these nucleotides in nucleosome core DNA (Smerdon & Lieberman, 1978, 1980). By analogy, repaired DNA in isolated chromatin can be represented as either "unfolded" or "folded" into a nucleosome structure. [We note that the terms unfolded and folded are used in this paper for convenience only and that the actual structure(s) of newly repaired DNA in chromatin is (are) unknown.] Using this simplified representation of repaired DNA in chromatin, we can quantitate nucleosome core particle formation in newly repaired DNA, as the result of salt-induced rearrangement, in the following manner: Let f_u and f_f be the fraction of repair-incorporated nucleotides in chromatin in the unfolded and folded states, respectively. Thus

$$f_u + f_f = 1 \tag{1}$$

If we let F_i be the fraction of newly repaired DNA, initially in the unfolded state, which undergoes salt-induced rearrangement (i.e., "fraction induced"), then

$$F_i = \frac{f_u(0) - f_u(S)}{f_u(0)} \tag{2}$$

where $f_u(0)$ and $f_u(S)$ represent the fractions of repaired DNA in the unfolded state following incubation in buffer A (at 0 °C) or in buffer A and salt concentration S (at 37 °C), respectively. Therefore, from eq 1

$$F_i = \frac{f_f(S) - f_f(0)}{1 - f_f(0)} \tag{3}$$

This equation can be rewritten in terms of the ^{3}H dpm (repair-incorporated nucleotides) associated with isolated core particle DNA following the different incubations [i.e., $^3H(0)$ and $^3H(S)$] and the ^{3}H dpm associated with isolated core particle DNA for the case where *all* of the newly repaired DNA is folded into nucleosomes (i.e., $^3H_{100\%}$). Now if the same number of core particles is isolated following the different incubations

$$F_i = \frac{^3H(S)/^3H_{100\%} - ^3H(0)/^3H_{100\%}}{1 - ^3H(0)/^3H_{100\%}} \tag{4}$$

However, the amount of DNA in each core particle preparation is directly proportional to the amount of ^{14}C dpm in that preparation, and eq 4 can be written such that the number of core particles in each preparation need not be the same. Therefore

$$F_i = \frac{R_S - R_0}{R_{100} - R_0} \quad (5)$$

where R_X = the ratio of ^3H and ^{14}C dpm for the isolated core DNA from nuclei (or chromatin) incubated in buffer A and salt concentration X and R_{100} = the ratio of ^3H and ^{14}C dpm for the total DNA (i.e., the 100% digestion value). The values of F_i were corrected for the contribution of replicative synthesis to the total ^3H dpm incorporated by using the method outlined in Smerdon (1983).

RESULTS

Effect of H1 Removal and Hydroxyurea. The ability of newly repaired regions of DNA to "accept" a nucleosome corelike structure was examined in a series of salt-induced rearrangement experiments. In each case, normal human fibroblasts were labeled in the growth phase with [^{14}C]dThd, grown to confluence, and irradiated with 12 J/m^2 UV light. Immediately following irradiation, the cells were allowed to repair for 40 min in the presence of [^3H]dThd and 2 mM hydroxyurea. Nuclei were isolated from these cells and incubated for 1 h in low-salt buffer (buffer A and 2 mM EDTA) at 0 or 37 °C or in high-salt buffer (low-salt buffer and 0.6 M KCl) at 37 °C. The nuclei were then resuspended in buffer A and digested with staphylococcal nuclease, and the core particle DNA was isolated. Two criteria were used as a measure of salt-induced nucleosome rearrangement: (1) a decrease in the average nucleosome repeat length of the bulk DNA [presented in detail in the preceding paper (Watkins & Smerdon, 1985)], and (2) an increase in the level of repair-incorporated nucleotides in nucleosome core DNA.

Originally, we determined the fraction of (presumably) unfolded, newly repaired DNA which underwent salt-induced rearrangement (F_i; see Materials and Methods) from the monomer peak of total nuclease digestion products electrophoresed on agarose gels. These gels showed a high background for nuclei that were incubated in high-salt buffer at 37 °C (Figure 1B), while a nominal background was observed for nuclei incubated in low-salt buffer at either 0 or 37 °C. Thus, to determine the value of F_i, a background level had to be estimated for the high-salt samples and subtracted from the integration value of the peak. The validity of the integrated peak value determined in this manner was in doubt, however, since random digestion of long stretches of DNA left devoid of nucleosome cores following high-salt treatment (Spadafora et al., 1979) could lead to nonnucleosomal DNA migrating with the monomer peak. Therefore, we modified our experiments to include a sucrose gradient purification step prior to running the mononucleosome fraction on the agarose gel (Figure 1A). In this manner, any nonnucleosomal DNA fragments which were migrating on the gel with bona fide core DNA would be separated in the gradient (i.e., sediment more slowly), and any DNA which sedimented with monomer nucleosomes in the gradient would migrate separately from the core DNA on the gels (i.e., at higher molecular weights). The result of isolating mononucleosomes on sucrose gradients and subsequent electrophoresis of the core DNA on agarose gels is shown in Figure 1C. By comparing panels B and C of Figure 1, the effect of the gradient step is clear: The high background, observed prior to centrifugation, is abolished. Furthermore, even by subtracting the high background in

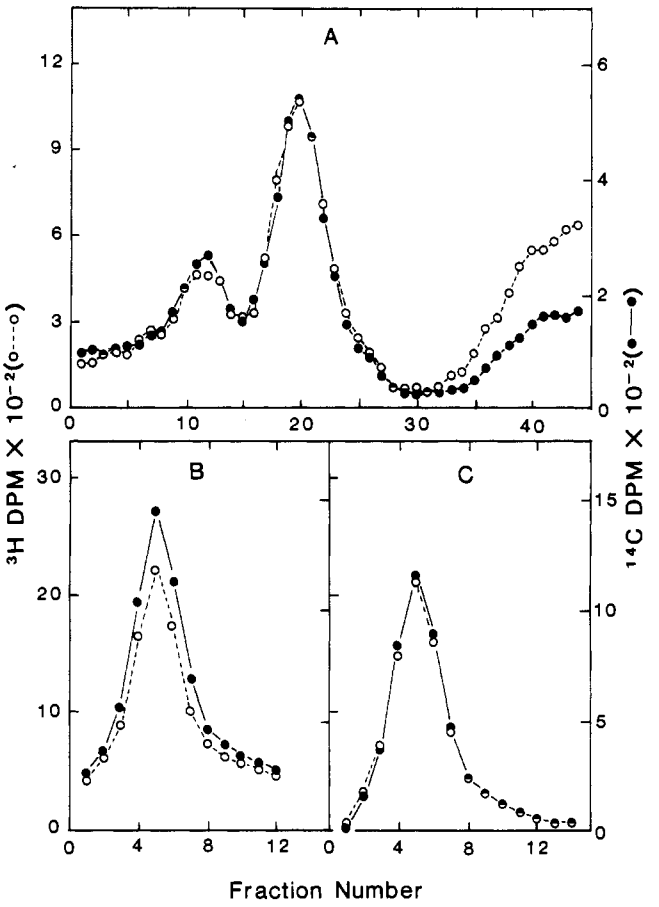

FIGURE 1: Sucrose gradient profile (A) and gel electrophoresis profiles of core DNA before (B) and after (C) isolation of mononucleosomes. Confluent human fibroblasts, prelabeled with [^{14}C]dThd, were irradiated with 12 J/m^2 UV light and labeled with [^3H]dTHd, in the presence of 2 mM hydroxyurea, for 40 min immediately after irradiation. Intact nuclei were prepared, suspended in high-salt buffer, and incubated for 1 h at 37 °C (Materials and Methods). The nuclei were resuspended in buffer A and incubated with staphylococcal nuclease until the fraction of ^{14}C rendered acid soluble was 0.34. An aliquot of the incubation mixture was then treated with proteinase K and ethanol-precipitated, and the DNA was electrophoresed on a 2.8% agarose gel (B). Alternatively, mononucleosomes in the incubation mixture were first isolated on a 5–20% sucrose gradient (A), and the DNA in the monomer peak (fractions 17–23) was prepared and electrophoresed as described previously (C). The region of the gel containing core particle DNA (~146 bp) was cut out and sliced (2 mm/fraction), and the fractions were assayed for radioactivity. The direction of sedimentation or migration is from right to left.

Figure 1B, the resulting $^3H/^{14}C$ ratio is different than the $^3H/^{14}C$ ratio obtained from the isolated monomer DNA. The difference between these two ratios varied from 10% to 33% for both intact and H1-depleted nuclei incubated in high-salt buffer at 37 °C (four different experiments), while the maximum difference for nuclei incubated in low-salt buffer at either 0 or 37 °C was only 2%. Also, in each of the high-salt experiments, the isolated core DNA had a greater $^3H/^{14}C$ ratio than the monomer peak prior to purification of mononucleosomes. Since the sucrose gradient cannot increase the amount of ^3H-labeled DNA in the monomer, the change in the ratio can only be attributed to a selective removal of ^{14}C-labeled (noncore) DNA during the centrifugation step. Thus, the centrifugation step is important in these experiments for the accurate determination of the $^3H/^{14}C$ ratio in core DNA.

When intact nuclei were incubated in low-salt buffer at 0 or 37 °C and the core DNA was isolated and analyzed for the levels of ^3H and ^{14}C, no differences were observed (Table I). This result is consistent with our findings that during the

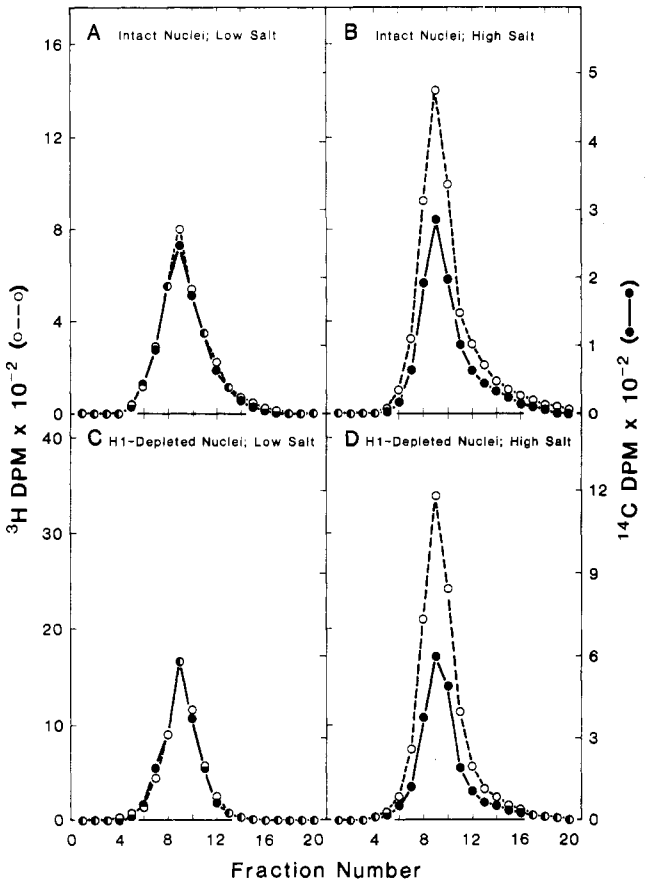

FIGURE 2: Gel electrophoresis profiles of nucleosome core DNA from intact (upper panels) and H1-depleted (lower panels) nuclei incubated in low-salt or high-salt buffers. Nuclei were isolated from confluent human fibroblasts labeled with [^{14}C]dThd and [^{3}H]dThd as described in Figure 1. The nuclei were suspended in low-salt buffer (A and C) or in high-salt buffer (B and D) and incubated at 37 °C for 1 h. The nuclei were then resuspended in buffer A and incubated with staphylococcal nuclease until 23–27% of the bulk DNA (^{14}C) was rendered acid soluble. Mononucleosomes were isolated on 5–20% sucrose gradients, and the core DNA was electrophoresed on 2.8% agarose slab gels. The direction of migration is from right to left.

incubation period, increased temperature *alone* was insufficient to induce changes in the nucleosome repeat length in intact nuclei (Watkins & Smerdon, 1985). However, when intact nuclei were exposed to high salt at 37 °C, there was a significant increase in the level of ^{3}H associated with nucleosome core DNA (Figure 2, panels A and B). (We note that following this high-salt incubation, most of the nuclei appear ruptured even though we refer to them as "intact nuclei".) This change is also consistent with our preceding report demonstrating a decrease in the average nucleosome repeat length from 190 to ~146 bp (for a 20% digestion; Watkins & Smerdon, 1985) following the high-salt incubation step. These data demonstrate that at least a portion of the newly repaired DNA, which lacks a nucleosome structure prior to the incubation step, can be folded into nucleosomes during this in vitro rearrangement process.

To quantitate the degree of salt-induced nucleosome core formation in newly repaired regions of DNA, we have determined the fraction of unfolded, nascent DNA that acquires a nucleosome structure during the high-salt incubation step (F_i; see Materials and Methods). As shown in Table II (control), this fraction is dependent on the extent of nuclease digestion and essentially doubles in going from low to high extents of digestion. This dependence arises from the following observations: (1) The ^{3}H/^{14}C ratio value of the core DNA decreases with increasing extents of digestion; (2) the decrease in the ^{3}H/^{14}C ratio value of core DNA occurs more rapidly in low-salt-treated (0 °C) nuclei than in high-salt-treated (37 °C) nuclei. Thus, the value of F_i, by definition (see Materials and Methods), decreases with larger extents of digestion. The difference in the rates at which the ^{3}H/^{14}C ratio decreased may be due to differences in chromatin structure between the two samples: The high-salt samples have undergone salt-in-

Table I: Measurement of the Fractional Change in the ^{3}H/^{14}C Ratio of Core DNA following Incubation in Low-Salt Buffer

nuclei[a]					
sample A		sample B			
H1	temp (°C)	H1	temp (°C)	f_c[b]	no. of determinations
+	0	+	37	−0.030 ± 0.034	5
+	0	−	0	−0.054 ± 0.031	5
−	0	−	37	−0.024 ± 0.003	2
+	0	+ (C)	0	−0.073 ± 0.004	3

[a] Intact (+) and H1-depleted (−) nuclei were prepared from cells prelabeled during the growth phase with [^{14}C]dThd and repair labeled with [^{3}H]dThd for 40 min following damage with UV light. The nuclei were then incubated in low-salt buffer for 1 h at the indicated temperatures. The samples were then resuspended in buffer A and incubated with staphylococcal nuclease, and mononucleosomes were isolated on sucrose gradients. In each case, the difference in the extent of digestion between samples A and B was <3%. DNA from the isolated mononucleosomes was then electrophoresed on 2.8% agarose gels, the gels were sliced, and the radioactive profiles were determined (e.g., Figure 1C). The last set of data are for the use of chromatin (C), prepared by hypotonic lysis of intact nuclei, as sample B. [b] The fractional change in the ^{3}H/^{14}C ratio of core DNA between the A and B samples is expressed as $f_c = (R_B - R_A)/R_B$ where R is the ratio of ^{3}H dpm to ^{14}C dpm in isolated core DNA and A and B denote the A and B samples, respectively. The values shown are the mean ± 1 standard deviation.

Table II: Extent of Salt-Induced Nucleosome Core Formation in Newly Repaired Regions of DNA

sample[a]	$f(^{14}C)$[b]	F_i[c]	expt[d]
control	0.12	0.26	1
	0.22	0.33	1
	0.25	0.40	2
	0.25	0.45	3
	0.29	0.41	4
	0.42	0.58	5
H1 depleted	0.23	0.44	1
	0.31	0.66	2
no hydroxyurea	0.10	0.38	6
	0.20	0.39	6
chromatin	0.14	0.26	3
	0.21	0.25	3
	0.33	0.29	4
reconstituted chromatin	0.21	0.98	3
	0.24	0.90	3
	0.29	1.00	4
	0.32	0.94	4

[a] Intact (control) and H1-depleted nuclei were isolated from cells labeled during repair synthesis in the presence of 2 mM hydroxyurea. The "no hydroxyurea" samples denote intact nuclei prepared from cells not treated with hydroxyurea. Chromatin samples were prepared by hypotonic lysis of intact nuclei. Reconstituted chromatin was prepared from hypotonically lysed nuclei as described under Materials and Methods. All samples were incubated with staphylococcal nuclease in buffer A. [b] Each value denotes the average of the low-salt and high-salt values for the fraction of bulk DNA rendered acid soluble. In each case, these values were within 0.02 of the value reported. [c] Fractional increase of repair-incorporated nucleotides in core DNA due to salt-incuded nucleosome rearrangement (see Materials and Methods for details). Low-salt samples, incubated at 0 °C, were compared to high-salt samples (0.625 M KCl) incubated at 37 °C. A value of F_i = 1 is expected for complete randomization. Values were corrected for the contribution of replicative synthesis to the total ^{3}H dpm incorporated. [d] Different numbers indicate experiments performed on different batches of nuclei.

duced nucleosome rearrangement and have formed "compact" dimers and oligomers (Watkins & Smerdon, 1985) which yield "trimmed" nucleosome core particles upon nuclease digestion, whereas the low-salt samples yield core particles containing varying amounts of linker DNA which is then trimmed by the exonucleolytic action of staphylococcal nuclease (Steinmetz et al., 1978). Thus, the values of F_i for low extents of digestion may be underestimated since the "core particle DNA" obtained from the low-salt samples contains some added linker DNA which may be more enhanced in ^3H label (Lan & Smerdon, 1985). Alternatively, these results may indicate that the extent of salt-induced nucleosome core formation in newly repaired regions of DNA varies between different structural regions of chromatin and is, therefore, dependent upon the location of the newly repaired DNA in chromatin.

Since the value of F_i changes with the extent of digestion, it is difficult to determine what fraction of the newly repaired DNA, lacking a nucleosome conformation in the intact cell, acquired such a structure during the incubation in high salt. However, given the first possibility discussed above, the best approximation of F_i should be for higher extents of digestion. In any event, one can specify the extent of digestion used to determine F_i. Thus, for intact nuclei, ~50% of the unfolded repair "patches" acquired a nucleosome-like conformation following incubation in high-salt buffer at 37 °C when the nuclei were digested to ~40%.

When histone H1 was selectively removed from nuclei, very little change in the $^3H/^{14}C$ ratio of core DNA was observed when compared to intact nuclei (Figure 2; Table I). This result is consistent with previous reports demonstrating that removal of histone H1 by low pH does not cause nucleosome rearrangement (Lawson & Cole, 1979; Weischet et al., 1979; Smerdon & Lieberman, 1981; Watkins & Smerdon, 1985). As with intact nuclei, the incubation of H1-depleted nuclei in low-salt buffer at 37 °C (instead of 0 °C) had little effect on the $^3H/^{14}C$ ratio in the isolated core DNA (Table I). This is the case even though the limiting repeat length for H1-depleted nuclei was markedly reduced (by ~25 bp) following incubation in low-salt buffer at 37 °C (Watkins & Smerdon, 1985). Furthermore, gel electrophoresis profiles of the total nuclease digestion products from these nuclei showed no preferential shift in repeat length of the bulk (^{14}C-labeled) DNA over that of the repair-labeled (^3H) DNA; that is, the shift in size of oligomer bands was coincident for both DNA labels (data not shown). These results indicate that although a large fraction of the H1-depleted chromatin underwent structural rearrangement during the low-salt incubation at 37 °C, only those newly repaired regions which had undergone refolding *in the intact cell* participated in the in vitro rearrangement. Moreover, when nuclei were pulse-labeled for 40 min and chased for 24 h [to allow all of the labeled repair patches to undergo rearrangement back to a nucleosome structure; see Smerdon & Lieberman (1980)], no change was observed in the $^3H/^{14}C$ ratio of core DNA between nuclie incubated at high salt (37 °C) and nuclei incubated in low salt (0 °C) (data not shown). This is a further indication that once the newly repaired regions of DNA have undergone the refolding process in the intact cell, their participation in the salt-induced rearrangement is the same as that of the bulk of the DNA in chromatin.

Incubation of H1-depleted nuclei in high-salt buffer at 37 °C was shown to decrease the repeat length to a "limiting" value of ~146 bp (Watkins & Smerdon, 1985). As shown in Figure 2, this treatment also increased the amount of repair label (^3H), relative to bulk label (^{14}C), in isolated core particles

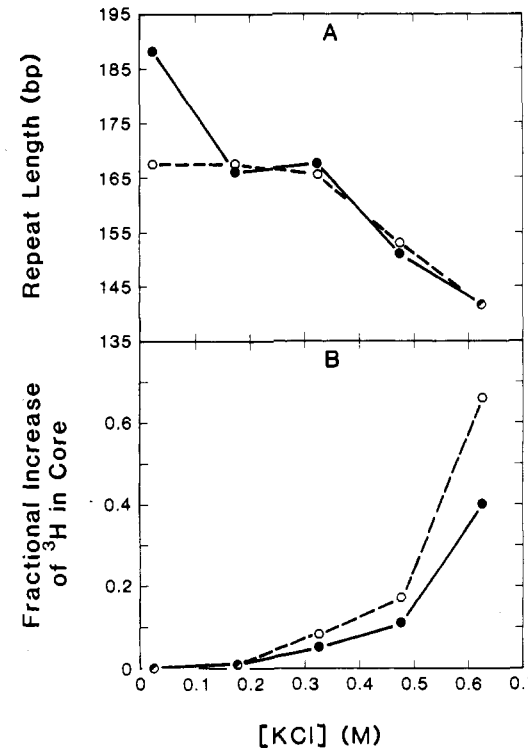

FIGURE 3: Nucleosome repeat length (A) and fractional increase of repair-incorporated nucleotides in nucleosome core DNA (B) as a function of the salt concentration used during the 1-h incubation of intact (●) and H1-depleted (○) nuclei at 37 °C. Confluent human fibroblasts were labeled with [^{14}C]dThd and [^3H]dThd as described in Figure 1. The fractional increase of ^3H in the nucleosome core DNA (F_i) is equivalent to the fraction of newly repaired DNA, lacking a nucleosome core structure in the intact cell, that became associated with nucleosome corelike structures following treatment with elevated salt concentrations. A value of $F_i = 1$ is expected for complete randomization during the salt treatment. Values were corrected for the contribution of replicative synthesis to the total ^3H dpm incorporated (see Materials and Methods for details).

(Figure 2C,D). This increase in the $^3H/^{14}C$ ratio of core DNA was somewhat larger than the increase obtained for intact nuclei prepared from the same cells (Figure 2A,B); however, this increase was much less than required for a complete randomization (i.e., for $F_i = 1$; Table II). Thus, even when the conditions for salt-induced rearrangment were optimized (i.e., by prior removal of histone H1), a significant fraction of the newly repaired DNA did not acquire a nucleosome corelike structure.

The effect of intermediate salt concentrations on the in vitro rearrangement in intact and H1-depleted nuclei was also examined. As shown in Figure 3, the initial drop in repeat length to 168 bp for *intact nuclei* (Figure 3A, closed circles) was not accompanied by an increase in the value of F_i (Figure 3B). Moreover, this shift in repeat length was coincident for both the ^3H and ^{14}C labels, as demonstrated by gel electrophoresis profiles of total nuclease digestion products, but did not involve a significant increase in the background levels of these labels (data not shown). (This is yet another indication that, under our conditions, the intermediate drop in repeat length to 168 bp involved nucleosome rearrangement of bulk DNA and only those regions of repaired DNA which had undergone nucleosome refolding in the intact cell.) However, for salt concentrations >0.325 M, increases in the background level, as well as further decreases in the repeat length, were observed. These changes were accompanied by an increase in the value of F_i (Figure 3). We also note that the value of F_i was always slightly greater for H1-depleted nuclei than for intact nuclei,

even though the extents of digestion were comparable. Nevertheless, even at the highest salt concentration used (0.625 M KCl), the maximum value of F_i was less than 70%. Thus, at least 30% of the newly repaired regions of DNA in H1-depleted nuclei were refractory to the formation of corelike structures as a result of the salt treatment.

The possibility existed that incomplete randomization of repair-incorporated nucleotides was due to insufficient time allowed for salt-induced rearrangement to take place, even though exchange of nucleosome cores between chromatin and naked DNA reached equilibrium during the 1-h incubation, and the average repeat length dropped to ~146 bp (Watkins & Smerdon, 1985). To test for this, nuclei were incubated in high-salt buffer at 37 °C for 1 or 15 h. The isolated core DNA from the two samples gave identical $^3H/^{14}C$ ratios (data not shown), indicating no further rearrangement occurred during the addition 14 h of incubation. Thus, it appears that 1 h is sufficient for the maximum amount of salt-induced rearrangement to take place under these conditions.

We also tested the possibility that repair labeling in the presence of hydroxyurea may have affected the salt-induced rearrangement of nucleosome cores. This idea was particularly intriguing since we have recently found that following a 30-min repair labeling period in the presence of hydroxyurea, ~50% of the repair patches in these cells are unligated (Smerdon, 1985). This is in contrast to the results obtained when hydroxyurea is omitted, where ~93% of the newly repaired DNA is ligated following the same 30-min labeling time. Therefore, we examined nuclei from cells not treated with hydroxyurea. As shown in Table II, the omission of this drug had little effect on the fraction of newly repaired DNA that was refractory to the salt-induced formation of nucleosome corelike structures. Thus, the incomplete randomization of repair-incorporated nucleotides following salt-induced rearrangement does not appear to result from the presence of single-strand nicks or gaps in many of the newly repaired regions of DNA.

Effect of Chromatin Preparations and Reconstitution. Since structural constraints on chromatin in nuclei may limit the ability of nucleosomes to rearrange in vitro, the salt-induced rearrangement of nucleosome cores was also examined in chromatin. The first priority of such a study is to prepare chromatin by a process which does not induce rearrangements in nucleosome structure. Therefore, the relative nuclease sensitivity of repair-incorporated nucleotides was determined for several different chromatin preparations and compared to the results for intact nuclei (Smerdon & Lieberman, 1978, 1980; Smerdon et al., 1978, 1982b). The chromatin preparations tested were sonication, hypotonic lysis, and hypotonic lysis plus digestion with the restriction enzyme *Bam*HI. (We note that the common method of preparing chromatin fragments by a very light nuclease digestion of nuclei cannot be used in these studies since the unfolded repair patches are attacked preferentially by nuclease.) As shown in Figure 4A, the enhanced nuclease sensitivity of newly repaired DNA was observed in the chromatin prepared by both sonication and hypotonic lysis. (The repair-labeled DNA in restriction enzyme digested chromatin preparation was preferentially digested by endogenous nucleases during the long incubation step of this method.) However, the enhanced nuclease sensitivity of newly repaired DNA (relative to bulk DNA) is slightly reduced following preparation of chromatin by either method. This reduced relative sensitivity could arise from either an actual decrease in the overall sensitivity of repaired DNA in chromatin or an increase in the overall sensitivity of the bulk DNA in chromatin. To test this latter possibility, we prepared

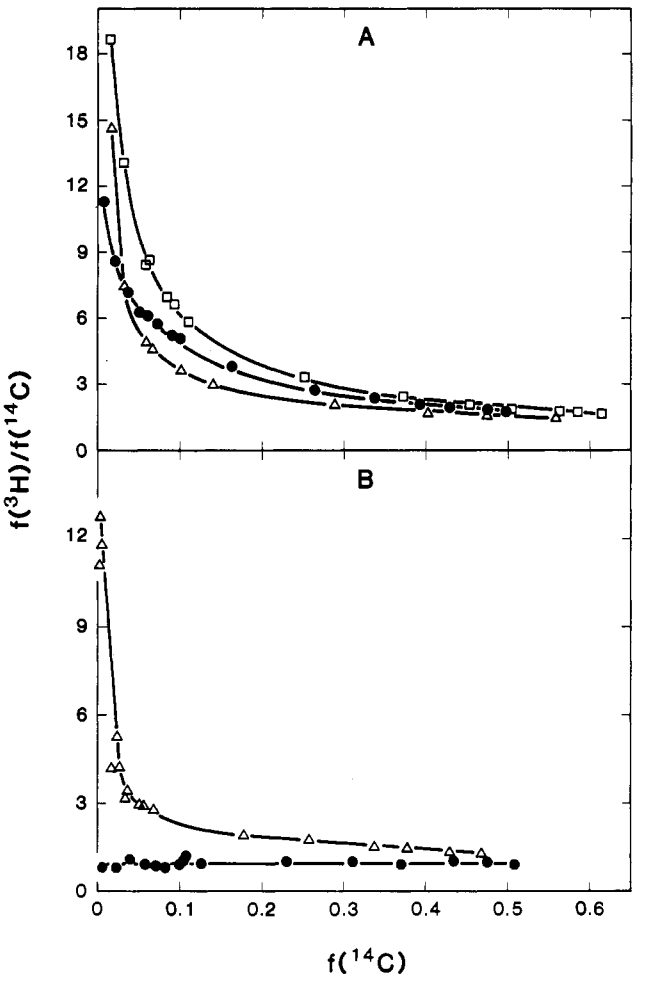

FIGURE 4: Ratio of the fraction of ^3H dpm [$f(^3H)$] to the fraction of ^{14}C dpm [$f(^{14}C)$] rendered acid soluble by staphylococcal nuclease as a function of the fraction of ^{14}C dpm rendered acid soluble. (A) Human cells were prelabeled with [^{14}C]dThd, grown to confluence, irradiated with 12 J/m^2 UV light, and labeled during repair with [^3H]dThd for 40 min. Nuclei (□) and chromatin prepared by sonication (△) or by hypotonic lysis (●) of nuclei were digested at 37 °C by adding staphylococcal nuclease to a final concentration of 0.15 unit/10^6 nuclei (or its chromatin equivalent) at time zero and taking aliquots at 2, 4, 6, 8, 10, 12, 14, and 16 min for the determination of acid-soluble dpm (Smerdon et al., 1978; Watkins & Smerdon, 1985). Additional nuclease was added at 18 min to a final concentration of 1.5 units/10^6 nuclei, and aliquots were taken at 5-min intervals, starting at 20 min, until the sample was exhausted. (B) Human cells were prelabeled with either [^3H]dThd or [^{14}C]dThd, grown to confluence, and harvested, and the nuclei were isolated. The ^3H-labeled nuclei were divided into two aliquots and used to prepare chromatin. One aliquot was sonicated (△) while the other was treated with hypotonic buffer (●) (Materials and Methods). Each chromatin preparation was then mixed with half of the ^{14}C-labeled nuclei and digested with staphylococcal nuclease as in (A).

intact nuclei from cells prelabeled with [^{14}C]dThd and the two different chromatins from cells *prelabeled* with [^3H]dThd. The nuclei and chromatin were then combined, and the mixture was digested with staphylococcal nuclease. As shown in Figure 4B, the bulk DNA in chromatin prepared from sonicated nuclei (triangles) was much more sensitive to staphylococcal nuclease than the DNA in chromatin of intact nuclei. On the other hand, identical rates of digestion of bulk DNA were observed for nuclei and chromatin prepared by hypotonic lysis (Figure 4B, circles). Therefore, evaluation of the extent of nucleosome rearrangement in newly repaired regions of hypotonically lysed nuclei is not complicated by an increase in sensitivity of bulk DNA, as seen for sonicated nuclei. Furthermore, as a final test for nucleosome rearrangement in

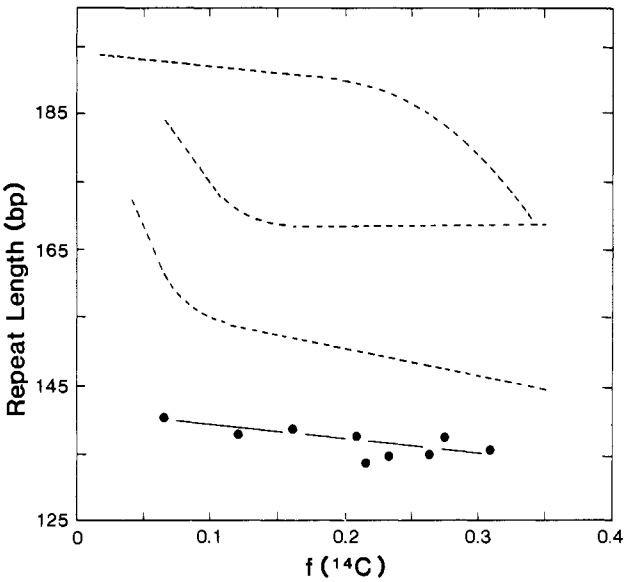

FIGURE 5: Repeat length of reconstituted chromatin as a function of the fraction of bulk DNA [$f(^{14}C)$] rendered acid soluble. Chromatin was reconstituted by suspending hypotonically lysed nuclei in buffer containing 2 M KCl and then removing the excess salt in a series of dialysis steps (Materials and Methods). The reconstituted chromatin was incubated with staphylococcal nuclease in buffer A, and the nuclease digestion products were coelectrophoresed on 2.8% agarose gels with *Hin*fI restriction fragments of ϕX174 RF DNA for the determination of the repeat length. The dashed lines represent the fits to the repeat length data presented in Figure 3 of the preceding paper (Watkins & Smerdon, 1985).

newly repaired DNA of hypotonically lysed nuclei, the $^3H/^{14}C$ ratio was determined for isolated core DNA from this chromatin preparation and compared to the $^3H/^{14}C$ ratio of core DNA from intact nuclei (Table I, bottom row). Although we observed a small difference between the two ratios, the change was not in the direction expected for a redistribution of nucleosomes onto unfolded, newly repaired regions of DNA during the chromatin preparation. Thus, by all the criteria examined, the preparation of chromatin by hypotonic lysis of nuclei does not cause any major rearrangements in the structure of newly repaired regions of chromatin.

Having prepared chromatin without inducing significant nucleosome rearrangement, we examined the amount of newly repaired DNA in hypotonically lysed nuclei which underwent the rearrangement induced by high salt. As shown in Table II, removal of the nuclear membrane constraints on chromatin did not allow complete randomization of the repair-incorporated nucleotides. In fact, if anything, the level of randomization was less for the chromatin than for control nuclei.

Another possibility to explain why a significant fraction of the newly repaired DNA is refractory to nucleosome formation is that the newly repaired DNA *itself* has a unique feature which is inhibitory to the formation of nucleosome cores. To test this possibility, we prepared chromatin by hypotonic lysis of nuclei and suspended the chromatin in buffer containing 2 M salt to dissociate histones. The chromatin was then reconstituted during a series of dialysis steps (Materials and Methods). As shown in Figure 5, a limiting nucleosome repeat of ~137 bp was obtained following digestion of the reconstituted chromatin with staphylococcal nuclease, which agrees well with previous reports on the repeat length of chromatin reconstituted in this manner (Tatchell & Van Holde, 1977; Steinmetz et al., 1978). When the value of F_i was determined for the isolated mononucleosomes from the reconstituted chromatin, complete (or near-complete) randomization was obtained (i.e., $F_i \simeq 1.0$; Table II). This result shows that the newly repaired DNA alone is not responsible for the limited salt-induced nucleosome rearrangement in the previous experiments. Furthermore, since the reconstituted chromatin was prepared from cells treated with hydroxyurea, these results represent another indication that single-strand nicks or gaps in a significant fraction of the newly repaired DNA do not affect the ability of nucleosomes to form on these regions.

Discussion

We have shown that newly repaired DNA, not associated with a nucleosome core structure in vivo, can be folded into a nucleosome corelike structure under the proper conditions in vitro. This nucleosome formation, however, does not occur during the first phase of nucleosome rearrangement reported in the preceding paper (Watkins & Smerdon, 1985) (i.e., when the limiting repeat length shifts from ~190 to 168 bp). As discussed in the introduction, this result provides further evidence that newly repaired regions in chromatin lack a native nucleosome structure. This altered structure must provide a "barrier" to nucleosome migration during the formation of the more closely packed chromatosome repeat at low (for H1-depleted nuclei) or intermediate (for intact nuclei) salt concentrations. This barrier may be due to the loss of cooperative interactions between nucleosomes across the more "open" repair patches. In this case, the nucleosomes would be expected to migrate away from these regions rather than toward them. Alternatively, the unfolded repair patches may be associated with proteins that block nucleosome formation in these regions. Indeed, Bodell et al. (1982) have suggested that proteins are associated with incomplete repair patches in nuclei based on the reduced sensitivity of these regions to exonuclease III.

The formation of nucleosomes in newly repaired regions correlates well with the shift in limiting repeat length to ~146 bp [i.e., the second phase of rearrangement reported in the preceding paper (Watkins & Smerdon, 1985); Figure 3]. It is important to note that there is significant exchange of (presumably) core histones at these higher salt concentrations (Watkins & Smerdon, 1985). Thus, since in nuclei or chromatin the newly repaired DNA is presumably in a very high (local) concentration of histones, it is clearly possible that nucleosomes are formed in these regions through an exchange mechanism. Furthermore, if proteins are present in repaired regions that block nucleosome formation during the low salt transition, these proteins may dissociate from the DNA at the elevated salt concentrations required for the higher salt transition and allow nucleosome formation to take place. Indeed, most non-histone proteins will dissociate at these salt concentrations (Comings, 1978).

Although a significant fraction of the newly repaired DNA became associated with a nucleosome corelike structure during the high-salt transition, not all of these regions were folded into nucleosomes. Even when histone H1 was removed from nuclei prior to the high-salt treatment, ~30% of the newly repaired DNA did not acquire a nucleosome structure (Figure 3). This was the case for both nuclei and chromatin (hypotonically lysed nuclei; Table II), indicating that this resistance to nucleosome formation is not due to constraints placed upon these regions by the nuclear envelope. Furthermore, repair patches labeled in the absence of hydroxyurea gave the same result as those labeled in the presence of this drug. This result indicates that the presence of nicks, gaps, or displaced parental strands is probably not responsible for the lack of complete randomization. This conclusion is supported by the fact that when chromatin was reconstituted by dissociation/reassociation of histones, a random distribution of nucleosomes between the

newly repaired DNA and bulk DNA was obtained. Therefore, when the histones are removed and then reassociated, the repaired DNA and bulk DNA acquire the same number of nucleosomes per DNA length, and no selective formation occurs. This is the case, even though (under the conditions used for the repair labeling) many of the repair patches were incomplete at the 3′ end (Smerdon, 1985). These results suggest that repair patches that are unfolded in the intact cell are capable of forming a nucleosome structure. Thus, the state of the repaired DNA alone (i.e., nicked, gapped, displaced, or intact) is probably not responsible for maintaining a non-nucleosomal structure in the intact cell, and other factors may be present (e.g., DNA binding proteins) which stabilize the unfolded form.

Finally, we observed that the repaired DNA that was folded into a nucleosome structure in the intact cell underwent the same salt-induced rearrangements as the bulk (or mature) chromatin. There was no evidence for a selective shift in the repeat length of these regions in intact nuclei incubated in low salt at 37 °C even though these conditions are sufficient to decrease the limiting repeat length in H1-depleted nuclei (Watkins & Smerdon, 1985). This finding supports our previous results indicating that following the refolding of repair patches into nucleosomes, these regions are rapidly associated with histone H1 (Smerdon et al., 1982b).

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