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Multiple Conformational States of Repair Patches in Chromatin during DNA Excision Repair†

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ABSTRACT: In mammalian cells, newly synthesized DNA repair patches are highly sensitive to digestion by staphylococcal nuclease (SN), but with time, they acquire approximately the same nuclease resistance as the DNA in bulk chromatin. We refer to the process which restores native SN sensitivity to repaired DNA as chromatin rearrangement. We find that during repair of ultraviolet damage in human fibroblasts, repair patch synthesis and ligation occur at approximately the same rate, with ligation delayed by about 4 min, but that chromatin rearrangement is only 75% as rapid. Thus, repair-incorporated nucleotides can exist in at least three distinct states: unligated/unrearranged, ligated/unrearranged, and ligated/rearranged. Inhibition of repair patch synthesis by aphidicolin or hydroxyurea results in inhibition of both patch ligation and chromatin rearrangement, confirming that repair patch completion and/or ligation are prerequisites for rearrangement. We also analyze the kinetics of SN digestion of repair-incorporated nucleotides at various extents of rearrangment and find the data to be consistent with the existence of two or more forms of unrearranged repair patch which have different sensitivities to digestion by SN. These data indicate that the chromatin rearrangement which restores native SN sensitivity to repaired DNA is a multistep process. The multiple forms of unrearranged chromatin with different SN sensitivities may include the unligated/unrearranged and ligated/unrearranged states. If so, the differences in SN sensitivity must arise from differences in chromatin structure, because SN does not differentiate between ligated and unligated repair patches in naked DNA.

Excision repair of DNA in mammalian cells involves transient changes in chromatin structure (Smerdon & Lieberman, 1978, 1980; Smerdon et al., 1979; Williams & Friedberg, 1979; Bodell & Cleaver, 1981) which have the following characteristics: repair-incorporated nucleotides are initially highly sensitive to staphylococcal nuclease (SN),[1] but with time, they acquire approximately the same degree of nuclease resistance as nucleotides in bulk chromatin; repair-incorporated nucleotides are initially underrepresented in nucleosome core-length DNA fragments produced by SN digestion, but with time, they acquire the same distribution in these fragments as nucleotides of bulk chromatin. We call the process by which repaired DNA regains the native level of SN sensitivity chromatin rearrangement. It has been suggested that the extreme SN sensitivity of newly synthesized repair patches results from an unfolding or sliding of nucleosomes required for repair enzymes to gain access to the damaged DNA and that the rearrangement process reestablishes native chromatin

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[1] Abbreviations: SN, staphylococcal nuclease; UV, ultraviolet; dThd, thymidine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid.

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conformation following completion of a patch (Lieberman et al., 1979). Although this hypothesis implies that the rearrangement of repaired chromatin is the terminal event in the repair process, the temporal relationship of chromatin rearrangement to other steps in DNA excision repair has not been clearly determined. Further, it is not known whether repaired chromatin regains the native level of SN resistance as the result of a single structural change or of several stepwise changes. The data reported here indicate that rearrangement of repaired chromatin follows synthesis and ligation of the repair patch and that rearrangement is a multistep process.

Materials and Methods

Cell Culture. Normal diploid human fibroblasts (AG1518; Institute for Medical Research) were cultured in monolayers, labeled with [^{14}C]dThd (20 nCi/mL; 50–60 mCi/mmol) for 1 week, and grown to confluence for 3 weeks as described (Dresler et al., 1982).

Damaging with UV Radiation and Labeling of Repair Patches. All operations were carried out at 37 °C. The medium was removed from monolayer cultures of confluent fibroblasts, and the cultures were irradiated with 10 J/m^2 of UV radiation from a G15T8 germicidal lamp at a flux of 2 W/m^2. The medium, supplemented with [^{3}H]dThd (20 μCi/mL, 40–60 Ci/mmol) and with aphidicolin and/or hydroxyurea as indicated, was added back to the cultures which were then incubated at 37 °C for the indicated time.

Preparation of Nuclei and Digestion with Staphylococcal Nuclease. Following UV irradiation and labeling with [^{3}H]Thd, cells were harvested, nuclei were prepared, and some of the nuclei were digested with SN (Worthington Biochemical Corp.) as described previously (Smerdon et al., 1978) for evaluation of the extent of rearrangement of repaired chromatin. The remaining nuclei were used to prepare DNA for the ligation assay (see below).

Quantitation of Rearrangement of Repair-Incorporated Nucleotides in Chromatin. We have developed a method for calculating ϕ_u, the fraction of the repair-incorporated nucleotides in repair patches which are unrearranged (i.e., which have not acquired the native level of SN sensitivity). As shown in the Appendix (eq A12)

$$\phi_u = \frac{\sigma'(\eta_I - 1)}{(\sigma' - 1)(1/\xi_{app} - 1)}$$

where σ' = the ratio of total nucleotide incorporation in UV-irradiated cells to total nucleotide incorporation in unirradiated cells. η_I = the initial slope of the curve relating release by SN of repair-incorporated nucleotides to release of bulk nucleotides (see Figure 4A), and ξ_{app} = the fraction of bulk nucleotide release corresponding to the point of intersection of lines tangent to the initial and final portions of the curve relating release by SN of repair-incorporated nucleotides to release of bulk nucleotides (see Figure 4A). This equation arises from a general model of SN digestion of repaired chromatin developed in the Appendix; however, an identical expression for ϕ_u can be derived from eq A6 of Smerdon et al. (1978) and eq A3 of Smerdon et al. (1982). Thus, the validity of ϕ_u as a measure of rearrangement does not depend on the choice of model. We calculate R, the radioactivity of repair-incorporated nucleotides in rearranged repair patches (i.e., in repair patches which have regained the native level of SN sensitivity), as follows:

$$R = (1 - \phi_u)S$$

where S = the total radioactivity of repair-incorporated nucleotides.

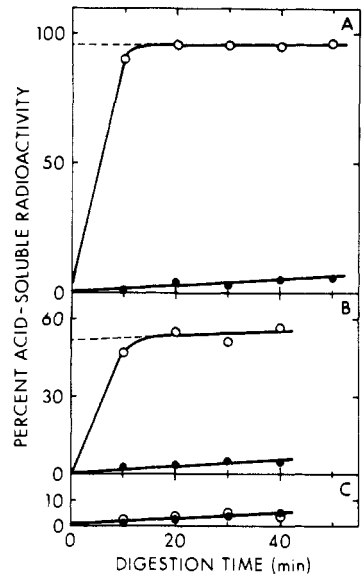

FIGURE 1: DNA repair patch ligation during UV-induced excision repair, determined by exonuclease III digestion of purified DNA. (A) Confluent fibroblasts, prelabeled with [^{14}C]dThd, were damaged with 40 J/m^2 UV radiation and incubated for 30 min with [^{3}H]dThd in the presence of 10 mM hydroxyurea and 7.7 μM aphidicolin. (B and C) Confluent human fibroblasts, prelabeled with [^{14}C]dThd, were damaged with 10 J/m^2 UV radiation and incubated with [^{3}H]dThd for 5 (B) or 120 min (C). In all cases, DNA was isolated and digested with exonuclease III for the times indicated, and acid-soluble release of ^{3}H (○) and ^{14}C (●) was determined.

Measurement of the Extent of Repair Patch Ligation Using Exonuclease III. The assay for unligated DNA repair patches is based on the fact that such patches have free 3′ ends and will be sensitive to digestion by exonuclease III, a 3′–5′-exonuclease. Under the digestion conditions used, exonuclease III activity is nonprocessive (Thomas & Olivera, 1978); thus, repair-incorporated nucleotides present in unligated patches will be digested much more rapidly than either nucleotides in bulk DNA or repair-incorporated nucleotides in ligated patches. To prepare DNA for the assay, nuclei were incubated with proteinase K (0.5 mg/mL) in the presence of 0.2 M Tris and 0.1 M EDTA, pH 8.0 at 47 °C, for 2 h. Samples were extracted 3 times with isoamyl alcohol/chloroform (1:24), and DNA was precipitated with ethanol and dissolved in digestion buffer (50 mM Tris, pH 8.0 at 37 °C, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.5 mg/mL nuclease-free bovine serum albumin) overnight at 4 °C on a rotating wheel. All manipulations were designed to minimize shearing of the DNA. Samples were digested at 37 °C with 0.2 unit of exonuclease III (Bethesda Research Laboratories) per microgram of DNA, and aliquots were removed at several times during the 40–50-min digestion period for determination of perchloric acid soluble ^{3}H (repair label) radioactivity and ^{14}C (bulk label) radioactivity, which were each expressed as a fraction of the total radioactivity in the DNA being digested. The release of ^{14}C from bulk DNA, which probably resulted from digestion at strand breaks produced by shearing during DNA isolation, was linear and was always less than 6% of the total ^{14}C at the end of the digestion (Figure 1). The release of ^{14}C from the DNA of unirradiated cells and that from irradiated cells were not significantly different (data not shown), indicating that continued digestion of bulk DNA 5′ to repair patches or at incised sites at which repair patches had not yet been synthesized did not contribute significantly to the ^{14}C release. The release of ^{3}H was biphasic with a rapid initial phase followed by a late slow linear phase (Figure 1A,B). It was assumed that the late slow phase of ^{3}H release, like ^{14}C release from

Table I: Effect of Hydroxyurea and Aphidicolin on Repair Synthesis, Repair Patch Ligation, and Chromatin Rearrangement[a]

treatment	relative repair synthesis	σ'	ξ_{app}	ϕ_u	fraction unligated
none	1.0	10.8	0.16	0.37	0.08
hydroxyurea	0.68	27.2	0.14	0.56	0.25
aphidicolin	0.51	10.2	0.12	0.55	0.35
aphidicolin plus hydroxyurea	0.37	19.0	0.11	0.75	0.64

[a] Confluent human fibroblasts, prelabeled with [^{14}C]dThd, were damaged with 10 J/m^2 UV radiation and incubated with [^{3}H]dThd for 30 min with no inhibitor, with 10 mM hydroxyurea, with 0.77 μM aphidicolin, or with both inhibitors. Relative repair synthesis was calculated as the difference in the specific activities of the DNA from irradiated and unirradiated cells (i.e., total incorporation − replicative incorporation) relative to control cells. σ' is the ratio of the specific activities of the DNA from irradiated and unirradiated cells. ξ_{app} is determined as shown in Figure 4A. ϕ_u is the fraction of repair-incorporated nucleotides present in unrearranged patches, calculated as described under Materials and Methods. Fraction unligated is the fraction of repair-incorporated nucleotides in unligated repair patches, determined as described under Materials and Methods.

bulk DNA, resulted from exonuclease III digestion at strand breaks not related to the repair process. The value for the fraction of ^{3}H present in unligated repair patches is therefore given by extrapolation of the final slope of the ^{3}H digestion curve back to zero digestion time. To validate this assay, we irradiated cells with 10 J/m^2 UV radiation and labeled them with [^{3}H]dThd in the presence of 10 mM hydroxyurea and 7.7 μM aphidicolin, inhibitors of UV-induced repair patch synthesis which also retard repair patch ligation [see below and Ben-Hur & Ben-Ishai (1971), Collins et al. (1977), Erixon & Ahnstrom (1979), Francis et al. (1979), and Snyder & Regan (1981)]. When DNA from these cells was digested with exonuclease III, essentially all of the ^{3}H radioactivity was rapidly released (Figure 1A), demonstrating that exonuclease III is capable of digesting all of the nucleotides in unligated repair patches. These data are in apparent conflict with the results of Cleaver (1981) but are consistent with results reported by Smith & Okumoto (1984). When DNA from cells labeled for 120 min without inhibitors was digested with exonuclease III, ^{3}H and ^{14}C were released at the same slow rate (Figure 1C), indicating that virtually all repair-incorporated nucleotides were contained in ligated patches, as one would expect. The data show that digestion of DNA with exonuclease III under the conditions used here is a valid means for measuring the fraction of repair-incorporated nucleotides in unligated repair patches.

In all of the experiments reported here, replicative synthesis represented less than 10% of the total ^{3}H incorporation, and therefore, no correction was made for the release of replicative ^{3}H by exonuclease III. We calculate L, the radioactivity of repair-incorporated nucleotides in ligated repair patches, as follows:

$$L = (1 - f_u)S$$

where f_u = the fraction of repair-incorporated nucleotides in unligated repair patches.

Results

Suppression of Replicative Synthesis. In these studies, we suppressed replicative synthesis by growing fibroblasts to a high degree of confluence (4 weeks in culture; Collins et al., 1982), and in all experiments, the ratio of the specific [^{3}H]dThd incorporation in UV-irradiated cells to the specific [^{3}H]dThd incorporation in unirradiated cells (which we call σ'; see above) was greater than 10 (see, for example, Table I). Since the residual replicative synthesis in cells irradiated with 10 J/m^2 UV radiation would be severalfold less than that in unirradiated cells, due to suppression of replication by the high dose of UV radiation (Smerdon et al., 1979; Dahle et al., 1980), the ratios of repair incorporation to replicative incorporation in irradiated cells were undoubtedly much greater than 10.

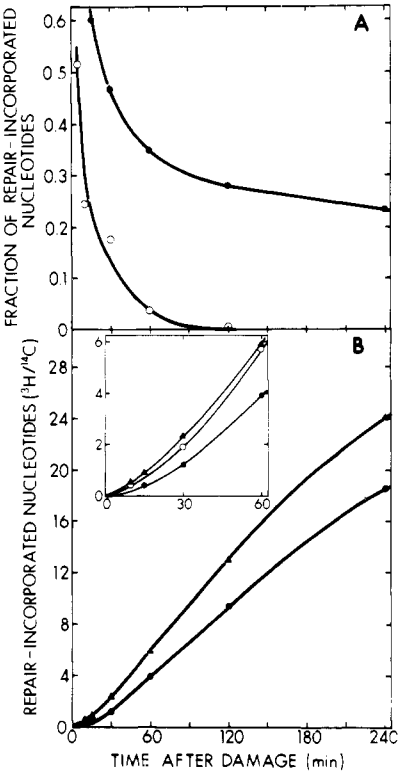

FIGURE 2: Time courses of DNA repair synthesis, repair patch ligation, and rearrangement of repaired chromatin during UV-induced excision repair. Confluent fibroblasts, prelabeled with [^{14}C]dThd, were damaged with 10 J/m^2 UV radiation and incubated with [^{3}H]dThd for the indicated times. (A) Fractions of repair-incorportated nucleotides present in unligated (O) and in unrearranged (●) repair patches were determined. (B) In the inset, the radioactivities of repair-incorporated nucleotides present in ligated (O) and rearranged (●) repair patches, calculated as described under Materials and Methods, are compared with total repair synthesis (▲) over the initial 60 min following damage. In panel B the radioactivity of repair-incorporated nucleotides present in rearranged repair patches is compared with total repair synthesis over a 240-min period. All data in Figure 2 are from the same experiment.

Relative Rates of Repair Patch Synthesis, Repair Patch Ligation, and Rearrangement of Repaired Chromatin. We have compared the rate of chromatin rearrangement with the rates of repair synthesis and of repair patch ligation, the final step in the restoration of the primary structure of the DNA to its predamaged state (Figure 2). Confluent cells, prelabeled with [^{14}C]dThd during exponential growth, were damaged with 10 J/m^2 of UV radiation and incubated with [^{3}H]dThd for the times shown. The cells were then harvested, and nuclei were prepared and either digested with SN to measure the extent of chromatin rearrangement or used to prepare DNA for the exonuclease III ligation assay. Completion and ligation of repair patches were very rapid: after a 5-min pulse, almost 50% of the repair-incorporated nucleotides were present in ligated patches (Figure 2A). Chromatin rearrangement was slower than patch ligation. At 15 min, 60% of repair-incorporated nucleotides were still in unrearranged repair patches, whereas only 25% of these nucleotides were in unligated patches (Figure 2A). In Figure 2B, the rates of repair patch ligation and chromatin rearrangement (measured in terms of the radioactivity of repair-incorporated nucleotides present in

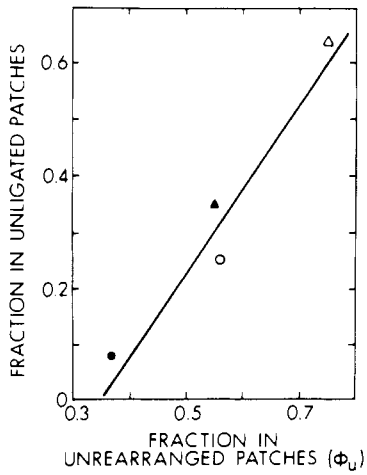

FIGURE 3: Relationship between repair patch ligation and rearrangement of repaired chromatin in cells treated with hydroxyurea and/or aphidicolin. Cells were damaged with 10 J/m^2 UV radiation and labeled for 30 min in the presence of no inhibitor (●), 10 mM hydroxyurea (○), 0.77 μM aphidicolin (▲), or 10 mM hydroxyurea plus 0.77 μM aphidicolin (△). Data are from Table I, and further details are given in the legend to that table.

ligated and rearranged repair patches, respectively) are compared with the rate of repair synthesis. Repair patch ligation occurred at a rate equal to that of repair synthesis but was delayed by about 4 min. This delay provides an estimate of the time required to synthesize and ligate a repair patch and is consistent with a previous estimate (3–10 min) made by Erixon & Ahnstrom (1979). Chromatin rearrangment, like repair synthesis, showed an initial lag and then was linear for 2–4 h; however, the rate of rearrangement was only about 75% of the rate of repair synthesis and repair patch ligation. The finding that repair patch ligation is more rapid than chromatin rearrangement indicates the existence of at least three states of the repair patch during the excision repair process: unligated/unrearranged, ligated/unrearranged, and ligated/rearranged.

Effect of Inhibition of Repair Synthesis on Repair Patch Ligation and Chromatin Rearrangement. To characterize further the relationship of repair patch synthesis and ligation to the rearrangement of repaired chromatin, the three parameters were measured in cells irradiated with 10 J/m^2 UV radiation and labeled with [^{3}H]dThd for 30 min in the presence of 10 mM hydroxyurea, 0.77 μM aphidicolin, or the two drugs together. Hydroxyurea, a direct inhibitor of ribonucleotide reductase, apparently retards repair patch synthesis by altering cellular deoxyribonucleoside triphosphate concentrations (Downes et al., 1983; Snyder, 1984) and has previously been shown to inhibit repair patch ligation (Ben-Hur & Ben-Ishai, 1971; Collins et al., 1977; Erixon & Ahnstrom, 1979; Francis et al., 1979). Aphidicolin directly inhibits DNA polymerase α (Huberman, 1981), the polymerase responsible for 80–90% of repair patch synthesis following 10 J/m^2 UV radiation (Dresler & Lieberman, 1983), and also has been shown to retard repair patch ligation (Snyder & Regan, 1982; Collins et al., 1982). Hydroxyurea, aphidicolin, and hydroxyurea plus aphidicolin all retarded repair synthesis, repair patch ligation, and chromatin rearrangement (Table I), and there was a strong positive correlation between the degree of inhibition of ligation and the degree of inhibition of rearrangement (Figure 3). There was also a positive correlation between the inhibition of repair synthesis and the inhibition of repair patch ligation, in apparent contradiction to the conclusions of Smith & Okumoto (1984). The finding that treatments which slow the rate of repair patch synthesis and ligation also retard

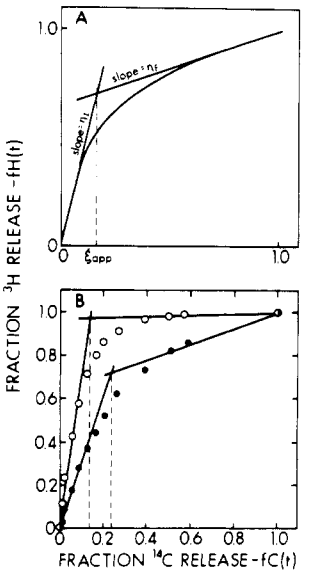

FIGURE 4: (A) Idealized curve relating fractional release of ^{3}H-labeled repair-incorporated nucleotides [$fH(t)$] to fractional release of ^{14}C-labeled bulk nucleotides [$fC(t)$] during staphylococcal nuclease digestion of repaired chromatin. DNA was uniformly prelabeled with [^{14}C]dThd, and excision repair took place in the presence of [^{3}H]dThd. The terms η_I and η_F represent the initial and final slopes of the digestion curve. The value of $fC(t)$ at the intersection of lines tangent to the initial and final segments is defined as ξ_{app}. This method of analysis is similar to that shown in Figure 9 of Smerdon et al. (1978). (B) Determination of ξ_{app} at two extents of rearrangement. Confluent human fibroblasts, prelabeled with [^{14}C]dThd, were damaged with 10 J/m^2 UV radiation and incubated with [^{3}H]dThd at 37 °C for 30 min in the presence (○) or absence (●) of 10 mM hydroxyurea and 7.7 μM aphidicolin. The cells were harvested, nuclei were prepared and digested with SN, and the fractions of total ^{3}H and ^{14}C released at various digestion times were measured and plotted (see Materials and Methods). For cells labeled without inhibitor, ξ_{app} was 0.24 and ϕ_u, calculated as described under Materials and Methods, was 0.76. For cells labeled in the presence of the inhibitors, ξ_{app} was 0.14, and ϕ_u was 1.0.

chromatin rearrangement supports the conclusion that completion and/or ligation of repair patches are necessary prerequisites of chromatin rearrangement.

Existence of Multiple Forms of Unrearranged Repair Patches Studied by Analysis of SN Digestion Kinetics. The data presented above indicate that during the excision repair process, unrearranged repair patches exist in at least two distinct states, unligated/unrearranged and ligated/unrearranged. As described in the Appendix (eq A10 and A11), it is possible by analyzing the kinetics of SN digestion of repair-incorporated nucleotides to detemine independently whether unrearranged repair patches all have the same degree of SN sensitivity or whether there are two or more forms of unrearranged repair patch with different SN sensitivities. The analysis depends on measurement, at various extents of rearrangement, of the parameter ξ_{app}, defined as the fraction of bulk chromatin released at the point of intersection of lines tangent to the initial and fnal segments of the curve relating SN digestion of repair-incorporated nucleotides [$fH(t)$] to digestion of bulk nucleotides [$fC(t)$]; this parameter is determined graphically (Figure 4A). If all unrearranged repair patches have the same degree of SN sensitivity, ξ_{app} will be constant throughout the course of chromatin rearrangement. If, however, there are several states of unrearranged chromatin with different nuclease sensitivities, and if the proportions of repair-incorporated nucleotides in each of these states change during the course of rearrangement, then ξ_{app} will vary. As seen in Figure 4B, ξ_{app} does not remain constant. Cells irradiated with 10 J/m^2 UV radiation and pulse labeled for 30

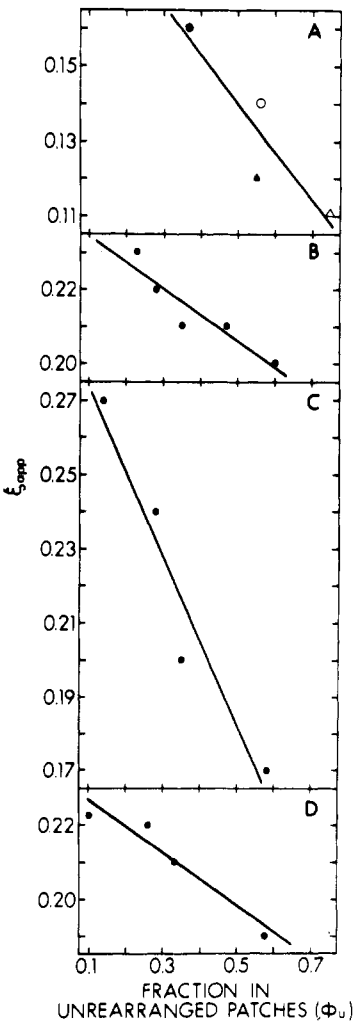

FIGURE 5: Variation of ξ_{app} with extent of chromatin rearrangement. (A) Cells were damaged with 10 J/m^2 UV radiation and labeled for 30 min with [^{3}H]dThd. Different extents of rearrangement were obtained by including no inhibitor (●), 10 mM hydroxyurea (○), 0.77 μM aphidicolin (▲), or 10 mM hydroxyurea plus 0.77 μM aphidicolin (△) during the labeling period (data from Table I; see legend to that table for further details). (B) Cells were irradiated with 10 J/m^2 UV radiation and incubated for various times with [^{3}H]dThd to attain different degrees of rearrangement (same experiment as Figure 2; see that legend for further details). (C and D) Analysis of data from Table I of Smerdon et al. (1979). Confluent human fibroblasts (IMR 90) were irradiated with 12 J/m^2 UV radiation, pulse labeled for 30 min with [^{3}H]dThd, and chased for various periods of time in medium without radioactive label, yielding various extents of rearrangement. Pulse and chase were done in the presence (panel C) or absence (panel D) of 10 mM hydroxyurea. Values of ξ_{app} are taken directly from the table. Values of ϕ_u were calculated as follows. Values of η_I were back-calculated for each time point by using values of f_s, ξ, and σ from the table and eq A6 of Smerdon et al. (1978). Values of ϕ_u were then calculated by using these values of η_I and the values of ξ and σ from the table.

min with [^{3}H]dThd had a fraction of unrearranged repair-incorporated nucleotides (ϕ_u) of 0.76 and an ξ_{app} of 0.24. Cells labeled in the presence of 10 mM hydroxyurea and 7.7 μM aphidicolin showed essentially complete inhibition of rearrangment (ϕ_u = 1.0) and had a much lower ξ_{app} of 0.14. We have also compared ξ_{app} and ϕ_u using data from two other experiments; in one of these, different extents of rearrangement were generated during a 30-min pulse labeling by the use of aphidicolin and/or hydroxyurea (Figure 5A), and in the other, repaired chromatin was examined at different extents of rearrangement during a time course experiment performed without inhibitors (Figure 5B). In both cases, there was a strong negative correlation between ξ_{app} and ϕ_u; as rearrangement proceeds, ξ_{app} increases. The finding that the same general relationship exists between ξ_{app} and ϕ_u in both experiments indicates that the changes in ξ_{app} do not result from direct effects of hydroxyurea and/or aphidicolin on chromatin structure. The data are consistent with the existence of two or more forms of unrearranged repair patch which have different SN sensitivities.

Data previously published by Smerdon et al. (1979) are also consistent with the existence of multiple forms of unrearranged repaired chromatin. The value of ξ_{app} also changed in their experiments during the course of chromatin rearrangement, in both the presence (Figure 5C) and absence (Figure 5D) of 10 mM hydroxyurea. As evidence for the reproducibility of the changes in ξ_{app} during rearrangement, we note the similarity between the data of Smedon et al. (1979) in Figure 5D and our data in Figure 5B, which were obtained from two experiments of similar design.

Discussion

We have compared the rates of repair synthesis, repair patch ligation, and rearrangement of repaired chromatin in diploid human fibroblasts damaged with UV radiation. The rate of repair patch ligation parallels the rate of repair synthesis with a lag of about 4 min. The rate of rearrangement is slower than the rate of ligation, suggesting that during the excision repair process repair patches pass serially through at least three states: unligated/unrearranged, ligated/unrearranged, and ligated/rearranged. Comparison of these results with those of Kastan et al. (1982), who measured methylation of repair-incorporated deoxycytidine using experimental conditions and cells identical with those used here, demonstrates that methylation is substantially slower than the rearrangement of repair patches in chromatin.

We have also studied the relationship between chromatin rearrangement and repair patch synthesis and ligation using aphidicolin and hydroxyurea. These agents, which primarily inhibit repair patch synthesis, also retard patch ligation and chromatin rearrangment, indicating that completion and/or ligation of the repair patch necesarily precede rearrangement. This result supports the multistep scheme of repair patch maturation proposed above and is consistent with previous findings that arabinosylcytosine, an inhibitor of DNA polymerase α, inhibits repair patch ligation (Erixon & Ahnstrom, 1979; Snyder et al., 1981; Bodell et al., 1982) and chromatin rearrangement (Bodell et al., 1982) in human fibroblasts damaged with UV radiation. Our data are also consistent with a previous report (Smerdon, 1983) that hydroxyurea inhibits rearrangement of repaired chromatin in UV-damaged human fibroblasts.

As an independent test of the existence of multiple forms of unrearranged repair patches, we analyzed the kinetics of SN digestion of repair patches at various extents of rearrangement, using a method developed in the Appendix. The test requires that one measure ξ_{app}, a parameter determined from the SN digestion curve (see Figure 4A), and determine whether ξ_{app} changes as rearrangement proceeds. Both our data and those of Smerdon et al. (1979) show changes in ξ_{app}, a result which is consistent with the existence of two or more forms of unrearranged repair patch with different sensitivities to digestion by SN. This finding suggests that restoration of native SN sensitivity to repaired DNA is a multistep process. Restoration of native chromatin structure to newly replicated DNA also apparently involves more than one step (Annunziato et al., 1981; Annunziato & Seale, 1982; Cusick et al., 1983).

Lending credence to our conclusion that rearrangement of repaired chromatin is a multistep process is the fact that, for

confluent human fibroblasts irradiated with UV radiation and allowed to repair without the use of inhibitors, our data and those of Smerdon et al. (1979) show very similar relationships between ξ_{app} and the extent of rearrangement (compare panels B and D of Figure 5). Data obtained from cells treated with inhibitors of repair patch synthesis (Figure 5A,C) show a very different relationship between ξ_{app} and the extent of rearrangement, however, suggesting that the changes in chromatin structure associated with inhibited repair patch synthesis may be quite different from the chromatin changes associated with normal repair synthesis. This possibility is consistent with recent suggestions (Cleaver, 1983; Collins, 1983; Smith & Okumoto, 1984) that the mechanism of repair patch synthesis utilized in the presence of DNA polymerase inhibitors may be different from that which predominates in cells not treated with inhibitors.

As discussed above, comparison of the rates of repair patch ligation and chromatin rearrangement establishes that repair patches pass through unligated/unrearranged and ligated/unrearranged states prior to rearrangement. This result raises the possibility that the multiple states of unrearranged chromatin with different SN sensitivities may include the unligated/unrearranged and ligated/unrearranged states. Currently, we have no way of evaluating this possibility. We do know, however, that if ligated and unligated patches in chromatin have different SN sensitivities, the difference must arise from differences in the chromatin structures associated with these two forms of repair patch, because ligated and unligated patches in naked DNA have the same sensitivity to digestion by SN (data not shown; Smerdon et al., 1979).

Our repair patch ligation data provide an alternate interpretation for previous studies (Smerdon & Lieberman, 1980) in which the structure of newly repaired chromatin was analyzed by DNase I digestion. In those experiments, cells were damaged with 12 J/m^2 UV radiation, labeled with [^{3}H]dThd in the presence of 10 mM hydroxyurea, and chased for various times, and nuclei were isolated and digested with the nuclease. Using denaturing gel electrophoresis, it was found that, following a 30-min pulse, the DNA fragments which contained repair-incorporated nucleotides did not show the characteristic 10 base pair DNase I repeat; however, after a 30-min chase, the 10 base pair repeat became apparent, and after a 4.75-h chase, the repeat pattern was essentially the same as the pattern observed with bulk chromatin. The authors suggested that the initial absence of a 10 base pair repeat indicated that repaired DNA was not initially packaged in nucleosomal cores. Our observation that a significant fraction of repair-incorporated nucleotides is present in unligated patches after a 30-min pulse in the presence of hydroxyurea (see Table I) suggests that the experimental approach used by Smerdon & Lieberman (1980) may not have a valid test for the presence of newly repaired DNA in nucleosomal cores. At early times of repair, many of the rapair-labeled fragments released by DNase I could have contained unligated repair patches, and the additional single-strand breaks present at the 3′ ends of these patches could have obscured the 10 base pair repeat. As ligation proceeded, these additional strand breaks would have disappeared, but as well, a substantial amount of chromatin rearrangement would have occurred, preventing a distinction between the role of ligation and chromatin rearrangement in the restoration of the 10 base pair repeat.

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APPENDIX

Here we describe a general model of the digestion by SN of chromatin from cells prelabeled with [^{14}C]dThd, grown to confluence, and pulse labeled with [^{3}H]dThd during DNA repair. We assume that DNA repair patches in chromatin exist in either of two general states: *rearranged*, in which the patches are uniformly distributed between the "nuclease-sensitive" and "nuclease-resistant" compartments of bulk chromatin, and *unrearranged*, in which all of the radioactivity in the repair patches has a high degree of nuclease sensitivity. This assumption is consistent with data from a number of laboratories (Smerdon et al., 1978, 1979; Williams & Friedberg, 1979; Smerdon & Lieberman, 1980; Bodell & Cleaver, 1981). We also assume that unrearranged repair patches are distributed among a number of subdomains which differ in their specific degrees of nuclease sensitivity. This assumption is consistent with the multistep nature of excision repair [reviewed by Hanawalt et al. (1979)] and with studies of gene expression which have established the existence of multiple states of chromatin structure with different degress of nuclease sensitivity [reviewed in Igo-Kemenes et al. (1982)].

The following definitions [similar to those of Smerdon et al. (1978, 1982)] are used. The term ξ is the fraction of bulk chromatin which is nuclease sensitive (and thus also the fraction of rearranged repaired chromatin in the nuclease-sensitive compartment of bulk chromatin), ϕ_u is the fraction of repaired chromatin which is unrearranged, and δ_i is the fraction of unrearranged repaired chromatin in the ith unrearranged subdomain. We let $\alpha(t)$ and $\beta(t)$ to be the fractions of DNA released at time t during SN digestion from the nuclease-sensitive and nuclease-resistant compartments of bulk chromatin, respectively. Similarly, $\gamma_i(t)$ is the fraction of DNA released at time t from the ith subdomain of unrearranged repaired chromatin. We let σ be the ratio of the total [^{3}H]-dThd incorporation in damaged cells (i.e., repair synthesis plus reisudal replication) to the amount of [^{3}H]dThd incorporation due to residual replication.

Using these definitions, we see that $fH(t)$ and $fC(t)$, the fractions of ^{3}H and ^{14}C released at time t during SN digestion, are given by

$$fH(t) = \xi\left[(1-\phi_u)\left(\frac{\sigma-1}{\sigma}\right)+1/\sigma\right]\alpha(t) + (1-\xi)\left[(1-\phi_u)\left(\frac{\sigma-1}{\sigma}\right)+1/\sigma\right]\beta(t) + \phi_u\left(\frac{\sigma-1}{\sigma}\right)\sum_{i=1}^{n}\delta_i\gamma_i(t) \quad \text{(A1)}$$

where n is the number of subdomains of unrearranged chromatin and

$$fC(t) = \xi\alpha(t) + (1-\xi)\beta(t) \quad \text{(A2)}$$

We assume that at early times of digestion, the rate of release of nucleotides from each chromatin compartment is constant. [This is consistent with available data; see Smerdon et al. (1978), Tlsty & Lieberman (1978), and Oleson et al. (1979).] Thus, at early times, $\alpha(t) = at$, $\beta(t) = bt$, and $\gamma_i(t) = c_i t$, where a, b, and c_i are constants. If we let $K = a/b$ and $N_i = c_i/a$, eq A1 becomes

$$fH(t) = [\xi(K-1)+1]\left[(1-\phi_u)\left(\frac{\sigma-1}{\sigma}\right)+1/\sigma\right]bt + \left(\frac{\sigma-1}{\sigma}\right)\phi_u Kbt\sum_{i=1}^{n}\delta_i N_i \quad \text{(A3)}$$

eq A2 becomes

$$fC(t) = [\xi(K-1)+1]bt \quad \text{(A4)}$$

and we see that when one plots $fH(t)$ against $fC(t)$ (Figure 4A), the curve originates at (0, 0) and its initial slope, η_I, is obtained by dividing $fH(t)$ by $fC(t)$, giving

$$\eta_I = \left[(1-\phi_u)\left(\frac{\sigma-1}{\sigma}\right) + 1/\sigma\right] + \left(\frac{\sigma-1}{\sigma}\right)\phi_u K \sum_{i=1}^{n} \delta_i N_i / [\xi(K-1)+1] \quad \text{(A5)}$$

At late digestion times, DNA in the nuclease-sensitive compartment of bulk chromatin and in the various subdomains of unrearranged repaired chromatin, all of which are assumed to be highly nuclease sensitive (see above), is virtually completely digested. In other words, at late times $\alpha(t) \simeq 1$ and, for all i, $\gamma_i(t) \simeq 1$. Then, from eq A1

$$fH(t) = \xi\left[(1-\phi_u)\left(\frac{\sigma-1}{\sigma}\right) + 1/\sigma\right] + \phi_u\left(\frac{\sigma-1}{\sigma}\right) + (1-\xi)\left[(1-\phi_u)\left(\frac{\sigma-1}{\sigma}\right) + 1/\sigma\right]\beta(t) \quad \text{(A6)}$$

and from eq A2

$$fC(t) = \xi + (1-\xi)\beta(t) \quad \text{(A7)}$$

We see that the curve relating $fH(t)$ to $fC(t)$ terminates at (1, 1) and that the final slope, η_F (see Figure 4A), can be obtained by simple differentiation, yielding

$$\eta_F = (1-\phi_u)\left(\frac{\sigma-1}{\sigma}\right) + 1/\sigma \quad \text{(A8)}$$

We define ξ_{app} as the value of $fC(t)$ at the intersection of lines tangent to the initial and final segments of the $fH(t)$ vs. $fC(t)$ curve (Figure 4A). This value is given by

$$\xi_{app} = \frac{1-\eta_F}{\eta_I - \eta_F}$$

or, from eq A5 and A8

$$\xi_{app} = \frac{\xi(K-1)+1}{K\sum_{i=1}^{n}\delta_i N_i} \quad \text{(A9)}$$

This relation allows one to determine whether SN digestion data are consistent with the existence of one or of more than one subdomain of unrearranged chromatin. If there is a single subdomain of unrearranged chromatin, then all unrearranged chromatin has the same SN sensitivity. In other words, for all i, $N_i = \mu$, here μ is a constant, and eq A9 can be rewritten as

$$\xi_{app} = \frac{\xi(K-1)+1}{\mu K} \quad \text{(A10)}$$

Because ξ and K are constant for any set of experimental conditions, eq A10 yields a ξ_{app} which is constant. If, however, there are two or more subdomains of unrearranged repaired chromatin with different nuclease sensitivities, and if the fractions of repair-incorporated nucleotides in each of these compartments change as rearrangement proceeds, then $\sum\delta_i N_i$ will change, and, as a result, ξ_{app} will change according to the equation

$$\xi_{app} = \chi\frac{1}{\sum_{i=1}^{n}\delta_i N_i} \quad \text{(A11)}$$

where $\chi = [\xi(K-1)+1]/K$, which is a constant. Determination of ξ_{app} at different extents of rearrangement provides a method for determining whether SN digestion kinetics are consistent with the existence of a single unrearranged state or of multiple unrearranged states with different SN sensitivities. In addition, the fraction of unrearranged repair-incorporated nucleotides, ϕ_u, can be calculated by using the values of η_I and ξ_{app} determined as shown in Figure 4A. From eq A5 and A9, it follows that

$$\phi_u = \frac{\sigma(\eta_I - 1)}{(\sigma-1)(1/\xi_{app} - 1)} \quad \text{(A12)}$$

Equations A6 of Smerdon et al. (1978) and A3 of Smerdon et al. (1982) yield an identical expression for ϕ_u, indicating that calculated values of ϕ_u do not depend on whether a two-state or greater than two-state model is postulated.

To calculate ϕ_u, one must know the value of σ. Measurement of σ is difficult, and we use σ', the ratio of [^{3}H]dThd incorporation in damaged cells to [^{3}H]dThd incorporation in undamaged cells, as an estimate of σ. Because UV irradiation suppresses residual replication (Smerdon et al., 1979), this procedure will underestimate σ. In the expts. reported here, however σ' is always greater than 10 (see, for example, Table I), and it can be shown that the error arising from using σ' rather than σ to calculate ϕ_u is less than 10% of the value of ϕ_u.

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Networks of DNA and RecA Protein Are Intermediates in Homologous Pairing†

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ABSTRACT: Partial coating of single-stranded DNA by recA protein causes its aggregation, but conditions that promote complete coating inhibit independent aggregation of single strands and, instead, cause the mutually dependent conjunction of single- and double-stranded DNA in complexes that sediment at more than 10000 S. This coaggregation is independent of homology but otherwise shares key properties of homologous pairing of single strands with duplex DNA: both processes require ATP, $MgCl_2$, and stoichiometric amounts of recA protein; both are very sensitive to inhibition by salt and ADP. Coaggregates are closed domains that are intermediates in homologous pairing: they form faster than joint molecules, they include virtually all of the DNA in the reaction mixture, and they yield joint molecules nearly an order of magnitude faster than they exchange DNA molecules with the surrounding solution. The independent aggregation of single-stranded DNA differs in all respects except the requirement for Mg^{2+}, and its properties correlate instead with those associated with the renaturation of complementary single strands by recA protein.

Escherichia coli recA protein promotes two kinds of homologous pairing of DNA molecules in vitro, each of which may play a direct role in genetic recombination, namely, the renaturation of complementary single strands (Weinstock et al., 1979) and the formation of joint molecules from single-stranded or partially single-stranded DNA plus duplex DNA (Shibata et al., 1979; Radding, 1982). The latter of these two kinds of pairing reaction is an ordered process in which at least three sequential phases can be distinguished: (1) A slow *presynaptic phase* consists of the polymerization of recA protein on single-stranded DNA (Cox & Lehman, 1982; Dunn et al., 1982; Kahn & Radding, 1984; Radding, 1982; Flory et al., 1984a,b), a reaction that is hindered by secondary structure in the single strands (Muniyappa et al., 1984). (2) A rapid *synaptic* phase can be subdivided into two sequential steps, which are *conjunction*, the coming together of single-stranded DNA and duplex DNA that is mediated by recA protein independent of homology, and *homologous alignment*, which occurs at least in part by facilitated diffusion within the complex of DNA and protein that results from conjunction (Gonda & Radding, 1983). (3) A slow *postsynaptic phase* of strand exchange produces heteroduplex DNA and correspondingly displaces an old strand (DasGupta et al., 1980; Cox & Lehman, 1981; Kahn & Radding, 1984).

A few of the intermediates in this sequence of reactions have been isolated or identified. The product of the presynaptic reaction is a highly ordered nucleoprotein filament which is at least 1.5 times the length of duplex DNA and consists of repeating subunits of about 6 molecules of recA protein and 20 nucleotide residues (Flory et al., 1984a,b; Tsang et al., 1985). Homologous alignment involves a nascent three-stranded intermediate in which the incoming single strand can pair with its complement in the duplex DNA without the true interwinding of strands that exists in duplex DNA (Bianchi et al., 1983).

The experiments described in this paper concern additional putative intermediates in the two distinct pairing reactions that are promoted by recA protein: an intermediate that is associated with *conjunction*, the step between the formation of a presynaptic complex and homologous alignment in the formation of D loops, and an aggregated form of single-stranded

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