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Enzyme Digestion of Intermediates of Excision Repair in Human Cells Irradiated with Ultraviolet Light†

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ABSTRACT: During excision repair of DNA in ultraviolet-irradiated human fibroblasts there was an alteration of protein–nucleic acid interactions that resulted in a nearly 2-fold increase in sensitivity of the repair patch to digestion by micrococcal nuclease. This was followed by a decrease in nuclease sensitivity of the repair patch during the reassembly of chromatin structure, which had a half-time of about 30 min. This decrease in nuclease sensitivity was not influenced by the presence of cycloheximide during the labeling period. The mechanism of reassembly of the DNA repair patch into a nucleosomal structure is therefore different from reassembly of newly replicated DNA strands and does not require de novo protein synthesis. Treatment of UV-irradiated human fibroblasts with 1-β-D-arabinofuranosylcytosine (ara-C) resulted in a dose-dependent inhibition of DNA repair synthesis and an accumulation of incomplete patches with 3′-OH termini. Maximum (65%) inhibition of DNA repair synthesis occurred at ≥25 μM ara-C. Continuous incubation of cells for up to 4 h in the presence of ara-C also resulted in a dose-dependent increase in micrococcal nuclease sensitivity of the DNA repair patch. Reassembly of the nucleosomal structure therefore occurs after synthesis of the DNA repair patch and its ligation into the strand. Treatment with ara-C also resulted in a 2.5–10-fold increase in the susceptibility of the repair patch in DNA and chromatin to enzymatic digestion by exonuclease III. However, in ara-C-treated cells the amount of the repair patch susceptible to exonuclease III digestion was 3-fold greater in purified DNA than in isolated nuclei. These results, in conjunction with the results from digestion by micrococcal nuclease, suggest that although there is an alteration in the protein–nucleic acid interactions in the newly synthesized repair patch, they are not completely eliminated. Incubation of ultraviolet-irradiated cells with [^{3}H]ara-C resulted in the incorporation of [^{3}H]ara-C into purified DNA. Exonuclease III removed only 48% of the [^{3}H]ara-C, indicating that ara-C did not block completion of ligation at every repair site. Estimates of the number of repair events from the number of single-strand breaks that accumulate in the presence of ara-C will therefore be in error by as much as a factor of 2, and use of this inhibitor for quantitative comparisons of repair between different cell types will be unreliable.

Excision repair of ultraviolet (UV)[1]-induced damage to DNA involves the synthesis of short patches throughout the genome. When irradiated cells have been incubated with [^{3}H]thymidine, which labels these repair patches, it has been shown that repaired DNA undergoes a transition from being initially highly susceptible to enzymatic digestion to eventually having the same susceptibility to digestion as bulk chromatin (Bodell & Cleaver, 1981; Smerdon & Lieberman, 1978, 1980). This structural transition represents one of the final steps of

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[1] Abbreviations: UV, ultraviolet; ara-C, 1-β-D-arabinofuranosylcytosine; HU, hydroxyurea; dThd, thymidine; dCyd, 2′-deoxycytidine; Tris, tris(hydroxymethyl)aminomethane.

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excision repair, the reassembly of chromatin structure. Similar processes have been shown to occur in replicating DNA in both cellular (Hewish, 1977; Hildebrand & Walters, 1976; Seale, 1975) and viral systems (Klempnauer et al., 1980; Cusick et al., 1981). However, the processes involved in reassembly of DNA repair patches and DNA replication strands are very likely to be different.

The major difficulty in studying the reassembly of the DNA repair patch is that repair is rapid and occurs on very few sites at any one time. There are consequently few intermediate structures available for biochemical analysis. The frequency of actively repairing sites is less than one per 2×10^8 daltons (Cleaver et al., 1972; Cleaver, 1974), and reassembly of the UV-induced repair patch occurs with a half-time of 15–30 min (Bodell & Cleaver, 1981). Therefore, to identify the intermediates in repair, it is necessary to use very short labeling times, which result in low amounts of incorporated radioactivity, or to block their completion with polymerase inhibitors such as 1-β-D-arabinofuranosylcytosine (Hiss & Preston, 1977; Hashem et al., 1980; Cleaver, 1981; Johnson & Collins, 1978; Dunn & Regan, 1979).

We therefore used ara-C to accumulate intermediates in UV repair and investigated the reassembly of DNA repair patches with several enzymes. Micrococcal nuclease was used to analyze changes in protein–nucleic acid interactions in the repair patch (Bodell & Cleaver, 1981; Smerdon & Lieberman, 1978, 1980). Exonuclease III was used to analyze the repair intermediates in cells treated with ara-C. If ara-C prevents completion of synthesis of the DNA repair patch, it should result in the formation of 3′-OH termini, in duplex DNA which are preferred substrates for this enzyme (Rogers & Weiss, 1980). Since exonuclease III digestion is blocked by proteins bound to DNA (Cusick et al., 1981; Herman et al., 1981; Shalloway et al., 1980), by comparing exonuclease III digestion of the repair patch in nuclei and in purified DNA, an analysis of protein binding at the site of repair was made. S-1 nuclease was used to analyze for the accumulation of single-stranded intermediates during excision repair, because previous studies revealed possible single strandedness in repair sites blocked by ara-C (Cleaver, 1981).

Materials and Methods

Cell Culture. Human fibroblasts (HS-1) were grown in Eagle's minimum essential medium supplemented with 15% fetal calf serum. One day after subculturing the cells were grown in medium containing 0.01 μCi/mL [^{14}C]dThd (56 mCi/mM) for 2–3 days. The medium was changed, and the cells were allowed to grow to confluence in unlabeled medium.

Irradiation and Labeling Conditions. Confluent cultures were incubated with 2 mM HU for 2 h before UV irradiation. The medium was then removed, and the cells were irradiated with 254-nm UV light at an incident fluence rate of 1.3 J m^{-2} s^{-1}. Immediately after irradiation the cultures were incubated for 30 min or longer in medium containing 10 μCi/mL [^{3}H]dThd (60 mCi/mM) and 2 mM HU. Under these conditions essentially all the [^{3}H]dThd incorporated into DNA after irradiation represents repair synthesis (Cleaver, 1982). For pulse–chase experiments the radioactive medium was replaced with medium containing 2 mM HU, 10^{-4} M dThd, and 10^{-5} M dCyd. In studies with cycloheximide or ara-C, the confluent cells were treated as described above, but with cycloheximide (10 μg/mL) or ara-C (various concentrations) present during both the HU pretreatment and the [^{3}H]-dThd-labeling periods.

The incorporation of [^{3}H]ara-C into DNA was determined by first treating cells for 2 h with 2 mM HU plus 1 μM ara-C. The cells were irradiated and incubated in medium containing 2 mM HU, 25 μL of [^{3}H]ara-C (Amersham; 1 mCi/mL, 15.5 Ci/mmol), and unlabeled ara-C to give a final concentration of 1 μM ara-C. The cells were collected by scraping, washed with saline, and pelleted by centrifugation. The cellular pellets were frozen in liquid N_2 and stored at −80 °C.

Isolation of Nuclei. The frozen pellet of cells from three to six 100-mm^2 dishes was suspended in 10 mM Tris-HCl, 10 mM NaCl, and 1 mM $MgCl_2$, pH 7.4 (STM buffer), for 10 min. The cells were Dounce homogenized, and the nuclei were collected by centrifugation at 1500 rpm for 10 min in an IEC model PR-J centrifuge. The nuclei were washed with STM plus 1% Triton X-100 and centrifuged. The nuclear pellet was again suspended in STM buffer and centrifuged. The nuclear pellet was suspended in 1.0 mL of STM buffer, and the concentration of nuclei was determined by hemocytometer counting.

Isolation of DNA. To 1 mL of isolated nuclei was added 100 μL of 10% sodium dodecyl sulfate. Crystalline NaCl was added to the nuclear lysate to give a final concentration of 1 M. The nuclear lysate was incubated at 37 °C with heat-treated RNase (100 μg/mL) for 30 min followed by proteinase K (1 mg/mL) for 30 min. Samples were then extracted with 2 mL of chloroform–isoamyl alcohol (24:1) and centrifuged at 8000 rpm for 10 min. The aqueous phase was collected and then reextracted as described above. For the exonuclease III and S-1 digestions of ^{3}H-repair-labeled DNA, the DNA was used at this step. For the [^{3}H]ara-C studies the DNA was precipitated by the addition of 100 μL of 4 M sodium acetate and 2 volumes of 100% ethanol. The DNA was collected by centrifugation at 10 000 rpm for 10 min and dissolved in 1.0 mL of STM buffer, pH 7.8.

Nuclease Digestions. Isolated nuclei (100 μL) containing approximately 4×10^5 nuclei were added to STM buffer containing 0.2 mM $CaCl_2$. Micrococcal nuclease (2.7 units) (Worthington Corp.) in STM buffer was added to give a final volume of 1 mL. For exonuclease III digestion, 12 units of exonuclease III (Bethesda Research Laboratory) was added to 100 μL of isolated nuclei in STM buffer, pH 7.8, at 37 °C.

For the digestion of purified DNA, 100 μL of labeled DNA plus 50 μg of unlabeled salmon DNA as carrier was added to either STM buffer, pH 7.8, for exonuclease III digestion or to 1 mM $ZnCl_2$, 0.1 M NaCl, and 0.25 M sodium acetate, pH 4.5, for S-1 nuclease digestion. Exonuclease III (48 units) or S-1 nuclease (20 units) (P-L Biochemicals) was incubated with the samples at 37 °C for various lengths of time. The enzymatic digestions were stopped by the addition of an equal volume of 1 N perchloric acid and 1.4 M NaCl. The samples were centrifuged at 10 000 rpm for 10 min, and the acid-soluble supernatant was collected. The acid-insoluble pellet was dissolved in 2.0 mL of 1 N NaOH. For liquid scintillation counting 0.5 mL of the acid-soluble supernatant was added to 1 mL of H_2O plus 10 mL of Aquasol; 0.5 mL of the alkali-solubilized pellet was added to a mixture of 0.5 mL of H_2O, 0.5 mL of 1 N glacial acetic acid, and 10 mL of Aquasol. Counting efficiencies and ^{14}C spillover into the ^{3}H channel were determined by the use of internal standards and were corrected for.

Results

[^{3}H]DNA of nuclei isolated from UV-irradiated HS-1 cells that had been labeled with [^{3}H]dThd for 30 min after UV irradiation was 1.7-fold more sensitive to digestion by micrococcal nuclease than was the bulk DNA (Figure 1). The ratio of the percent acid solubility of the ^{3}H-repair-labeled DNA to the percent acid solubility of the ^{14}C-bulk-labeled

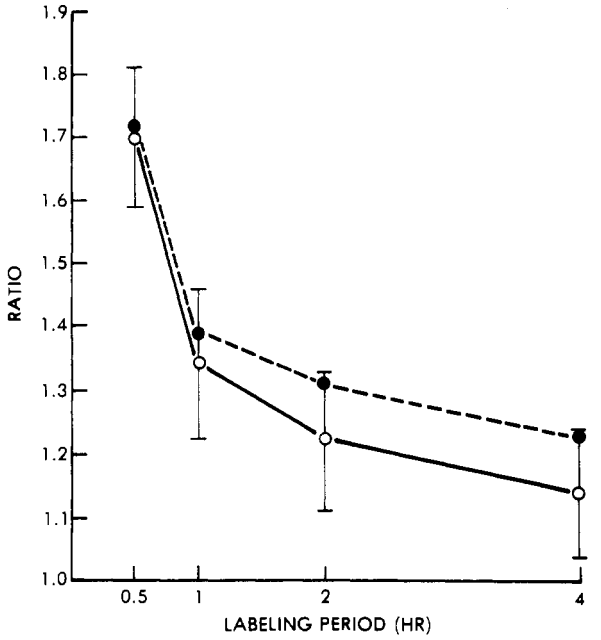

FIGURE 1: Micrococcal nuclease digestion of repair-labeled nuclei isolated from HS-1 cells that were labeled for various lengths of time with [^{3}H]dThd in medium containing 2 mM HU (○) or 2 mM HU plus 10 μg/mL cycloheximide (●). The ratio is the percentage of ^{3}H that was acid soluble divided by the percentage of ^{14}C that was acid soluble. When 20–25% of the ^{14}C has been converted to an acid-soluble form, this point in the digestion was used for calculation of the ratios. Bars show standard deviation of the mean.

DNA is highly dependent on the extent of digestion of the ^{14}C-bulk-labeled DNA. In this work the percentage of the ^{3}H-repair-labeled DNA that was acid soluble when 20–25% of the ^{14}C-bulk-labeled DNA was acid soluble was used to calculate the ratios presented. As the length of the labeling period was extended from 30 min to 4 h, there was a marked decrease in the nuclease sensitivity of the ^{3}H repair label. After 4 h of continuous [^{3}H]dThd labeling, the ^{3}H-repair-labeled DNA had about the same nuclease sensitivity as parental DNA (Figure 1).

Similarly, when UV-irradiated HS-1 cells were labeled for 30 min with [^{3}H]dThd and then chased for various lengths of time in medium containing unlabeled dThd and dCyd, the nuclease sensitivity of the repair patch also decreased (Figure 2). During this chase, the change in nuclease sensitivity occurred with a half-time of about 30 min.[2] Measurement of the ^{3}H/^{14}C ratio showed that there was insufficient incorporation of the [^{3}H]dThd during the chase period to result in significant changes in nuclease sensitivity (results not presented).

Cycloheximide is a strong inhibitor of protein and replicative DNA synthesis, but it does not influence DNA repair synthesis (Gautschi et al., 1973; Cleaver, 1969). Treatment of cells with cycloheximide did not significantly influence the change in micrococcal nuclease sensitivity of the repair patch (Figure 1), indicating that protein synthesis is not required during nucleosomal reassembly of repaired regions.

[2] The formula used to calculate the change in nuclease sensitivity of the ^{3}H repair-labeled DNA was

$$\frac{\text{ratio of time } (x) - 1}{(\text{ratio of 30-min label}) - 1}$$

This formula was incorrectly presented previously (Bodell & Cleaver, 1981).

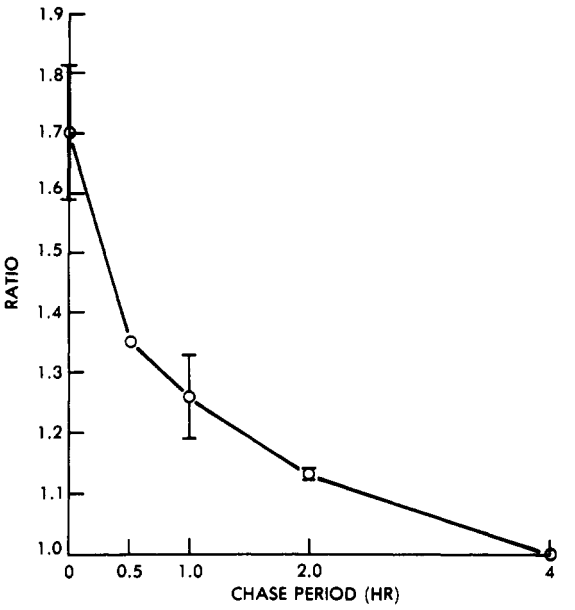

FIGURE 2: Micrococcal nuclease digestion of repair-labeled nuclei isolated from UV-irradiated HS-1 cells that were labeled with [^{3}H]dThd for 30 min and then chased for various lengths of time in unlabeled medium. For an explanation of the calculated ratio of micrococcal nuclease digestion of ^{3}H-repair-labeled DNA, and the ^{14}C-labeled DNA, see the legend to Figure 1. Bars show standard deviation of the mean.

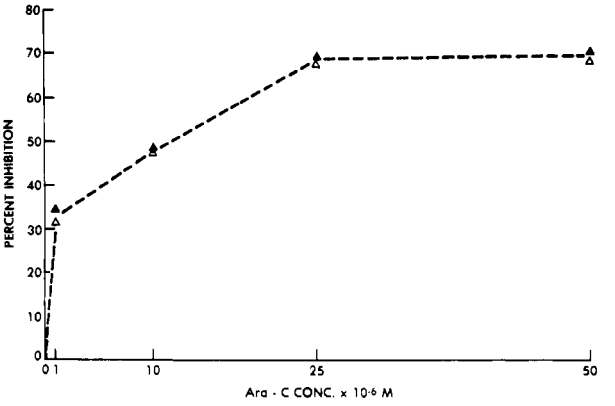

FIGURE 3: Inhibition of DNA repair synthesis in UV-irradiated HS-1 cells treated with varying concentrations of ara-C. Cells were irradiated with 13 J/m^2 UV light and then labeled with [^{3}H]dThd in medium containing 2 mM HU plus various concentrations of ara-C. 2 h (△) or 4 h (▲).

Ara-C caused a dose-dependent inhibition of DNA repair synthesis, with maximum inhibition occurring at 25 μM ara-C (Figure 3). These results were independent of the length of the labeling periods from 30 min to 4 h (data not shown). This inhibition is accompanied by an accumulation of four to five single-strand breaks per 10^8 daltons of DNA at blocked repair sites as previously shown (Cleaver, 1981).

Micrococcal nuclease digestion of ^{3}H-repair-labeled DNA of nuclei isolated from cells treated with various concentrations of ara-C showed a dose-dependent increase in the nuclease sensitivity of the repair patch (Figure 4). In cells treated with 10 μM ara-C, the sensitivity of the repair patch decreased slowly with continuous labeling. After 4 h the repair patch was still 1.7-fold more sensitive to nuclease digestion than the [^{14}C]DNA. At 10 μM ara-C DNA repair synthesis was inhibited by 49% (Figure 3). Therefore, the amount of incorporation of [^{3}H]dThd by ara-C-treated cells after 4 h was equivalent to that in HU-treated cells after 2 h. In cells treated for 2 h with HU alone, the micrococcal nuclease digestion ratio

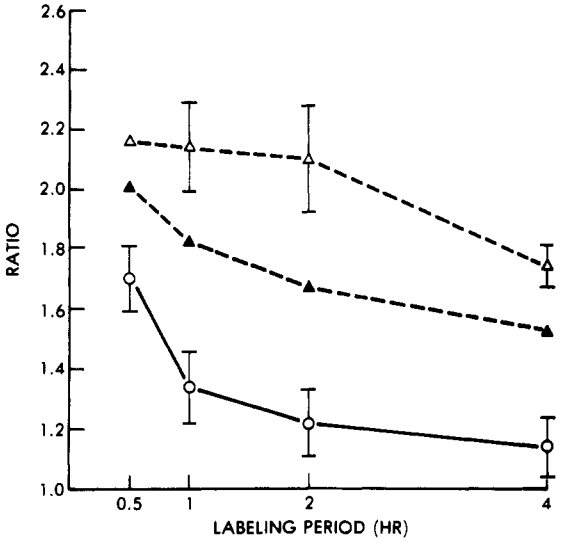

FIGURE 4: Micrococcal nuclease digestion of ^{3}H-repair-labeled nuclei isolated from UV-irradiated HS-1 cells that were labeled with [^{3}H]dThd for varying lengths of time in medium containing 2 mM HU (○), 2 mM HU plus 1 μM ara-C (▲), or 2 mM HU plus 10 μM ara-C (△). For an explanation of the calculated ratio of micrococcal nuclease digestion of the ^{3}H repair label and the ^{14}C-bulk-labeled DNA, see the legend to Figure 1. The length of the [^{3}H]Thd-labeling period is presented.

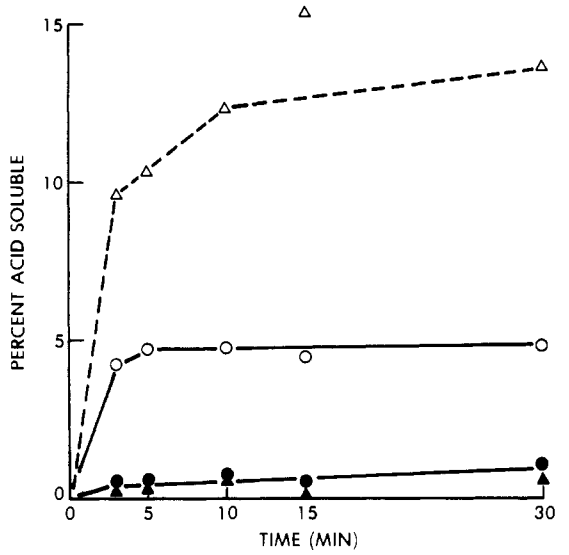

FIGURE 5: Exonuclease III digestion with time of incubation of the ^{3}H repair label and [^{14}C]DNA in nuclei isolated from UV-irradiated HS-1 cells that were labeled for 30 min with [^{3}H]dThd in medium containing 2 mM HU [^{3}H (○); ^{14}C (●)] or 2 mM HU plus 10 μM ara-C [^{3}H (△); ^{14}C (▲)].

was 1.2. Therefore, at equivalent amounts of repair synthesis the repair patch in cells treated with ara-C was more sensitive to micrococcal nuclease.

Further analysis of protein–nucleic acid interactions at the site of DNA repair was made with exonuclease III digestion. When nuclei were isolated from irradiated cells treated with HU and labeled with [^{3}H]dThd for 30 min, approximately 5% of the ^{3}H-repair-labeled DNA was susceptible to digestion by exonuclease III (Figure 5), compared to about 13% in irradiated cells treated with ara-C plus HU. In both cases digestion of the ^{3}H-repair-labeled DNA in nuclei occurred in the time that less than 1% of the bulk DNA was digested. In HU-treated cells as the length of the labeling period increased, the percentage of the ^{3}H-repair-labeled DNA susceptible to exonuclease III digestion decreased (Table I). In contrast,

Table I: Percentage of Repair Label Digested from Nuclei or Purified DNA Preparations by Exonuclease III[a]

		labeling times			
treatment	prepn	30 min	60 min	2 h	4 h
HU, 2 mM	nuclei	5.3 ± 3.1	3.2 ± 0.9	1.5 ± 0.4	0.8 ± 0.3
ara-C, 10 μM	nuclei	13.5 ± 7.4	13.0 ± 3.6	11.0 ± 3.2	8.2 ± 2.7
HU, 2 mM	DNA	15.5 ± 3.1	10.7 ± 3.9	9.4 ± 3.2	4.9 ± 1.5
ara-C, 1 μM	DNA	–[b]	–	32.0	29.1
ara-C, 10 μM	DNA	35.3 ± 4.9	39.6 ± 7.5	40.0 ± 9.7	44.0 ± 7.3
ara-C, 25 μM	DNA	–	–	52.2	52.1
ara-C, 50 μM	DNA	–	–	53.0	50.4

[a] Exonuclease III digestion of repair-labeled nuclei or purified DNA which were isolated from UV-irradiated HS-1 cells that had been labeled with [^{3}H]dThd in medium containing 2 mM HU or 2 mM HU plus the concentration of ara-C indicated. The extent of digestion of the [^{14}C]DNA was less than 1% after 30 min of exonuclease III digestion and was the same for both HU and HU plus ara-C-treated cells. The percentage of the ^{3}H-repair-labeled DNA digested by exonuclease III was determined from the plateau value of the enzyme digestion (see Figure 5). The extent of the digestion of the ^{14}C bulk label is not given, but for both nuclei and purified DNA, digestion of [^{14}C]DNA was less than 1% after 30 min of exonuclease III digestion and was the same for both HU and HU plus ara-C-treated cells. [b] Not determined.

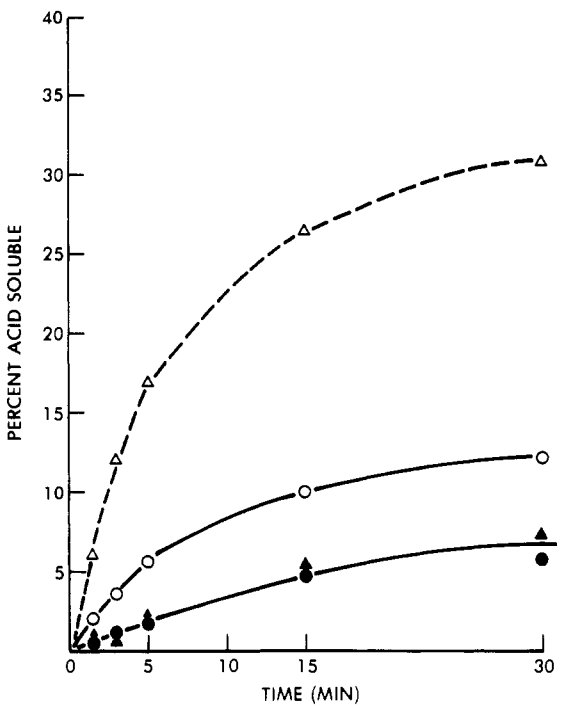

FIGURE 6: S-1 nuclease digestion with time of incubation of ^{3}H-repair-labeled DNA and the ^{14}C label isolated from UV-irradiated HS-1 cells that were labeled for 2 h with [^{3}H]dThd in medium containing 2 mM HU [^{3}H (○); ^{14}C (●)] or 2 mM HU plus 10 μM ara-C [^{3}H (△); ^{14}C (▲)].

the ^{3}H-repair-labeled DNA of nuclei isolated from cells treated with HU plus ara-C remained exonuclease III sensitive during a 4-h labeling period (Table I).

We wanted to determine if there was a difference in the susceptibility of the repair patch to exonuclease III digestion in purified DNA, as compared to the above results for isolated nuclei. For these experiments ^{3}H-repair-labeled DNA isolated from cells treated with HU or HU plus ara-C was digested with exonuclease III. In both cases removal of the chromosomal proteins increased the enzymatic digestion of the repair label by 3–6-fold (Table I).

S-1 nuclease digestions of repair-labeled DNA indicated that a significant amount of ^{3}H-repair-labeled DNA was sensitive

to digestion by S-1 nuclease (Figure 6). In HU-treated cells, when S-1 nuclease had digested 1% of the ^{14}C-labeled DNA, approximately 4% of the ^{3}H-repair-labeled DNA had been digested. In cells treated with HU plus ara-C, 12% of the ^{3}H-repair-labeled DNA had been digested by S-1 nuclease when 1% of the ^{14}C-labeled DNA was digested. Longer incubation with S-1 nuclease (30 min) resulted in digestion of 12% of the ^{3}H-repair-labeled DNA from HU-treated cells and 31% of the ^{3}H-repair-labeled DNA from cells treated with HU plus ara-C when 5–7% of the [^{14}C]DNA had been digested.

Irradiated HS-1 cells were incubated for 4 h with 1 μM [^{3}H]ara-C, and the DNA was isolated, purified, and digested with exonuclease III. An average of two experiments indicates that only 48% of the [^{3}H]ara-C incorporated during repair synthesis was susceptible to digestion by exonuclease III. [^{3}H]ara-C-labeled DNA not used for exonuclease III digestion was converted to nucleosides as previously described (Singer et al., 1978). Chromatographic analysis indicated that 86%, 4%, and 9% of the radioactivity comigrated with ara-C, deoxyadenosine, and dThd, respectively.

Discussion

Our results on nuclease sensitivity of UV-induced DNA repair synthesis in nuclei isolated from UV-irradiated HS-1 cells show that at short labeling periods the ^{3}H-repair-labeled DNA is more susceptible to nuclease digestion than the bulk DNA, as reported previously (Smerdon & Lieberman, 1978; Cleaver, 1977; Bodell & Cleaver, 1981). With longer labeling periods the nuclease sensitivity of the repair-labeled DNA approaches that of the bulk DNA. Pulse–chase experiments indicate that the repair patch returns to normal nuclease sensitivity with a half-time of about 30 min. Similar results in UV-irradiated human fibroblasts have been reported by Smerdon & Lieberman (1980).

These results suggest that during and immediately after synthesis the DNA repair patch is in a conformation that renders it more susceptible to digestion than the bulk DNA. However, with time the repair patch is reassembled into a nucleosomal structure with normal histone–DNA interactions. This interpretation is supported by our previous work showing that the ^{3}H-repair-labeled DNA resistant to micrococcal nuclease has the same size of core particle and subcore particle DNA fragments as that of the bulk DNA (Bodell & Cleaver, 1981).

Treatment of UV-irradiated cells with cycloheximide has been previously shown not to influence DNA repair synthesis (Cleaver, 1969; Gautschi et al., 1973). Our results show that cycloheximide also does not influence the reassembly of the repair patch, indicating that protein synthesis is not required for reassembly of the repair patch into a nucleosomal structure. This is in contrast to studies on DNA replication in which cycloheximide has been shown to prevent reassembly of the replicating DNA strands into a nucleosomal structure (Weintraub, 1976) due to the requirement of histone synthesis for replicating DNA reassembly. Therefore, although both replicating DNA strands and DNA repair patches undergo similar structural transitions, the processes for the reassembly of replicated and repaired DNA are different.

Treatment of UV-irradiated cells with ara-C caused a dose-dependent inhibition of DNA repair synthesis and an accumulation of single-strand breaks (Dunn & Regan, 1971; Cleaver, 1981). This inhibition of DNA repair synthesis may be explained by the incorporation of ara-C into the repair patch, with ara-C acting as a chain terminator. This is consistent with the observation that [^{3}H]ara-C is incorporated into repaired DNA as observed in this work and by Dunn & Regan (1979). However, the observed effects of ara-C may also be due to inhibition of DNA polymerase α (Graham & Whitmore, 1970; Furth & Cohen, 1968). Similar inhibition of repair has been found with inhibition of DNA polymerase α by aphidicolin (Snyder & Regan, 1981; Waters, 1981). Pulse–chase experiments indicate that the removal of UV-induced strand breaks accumulated in cells treated with aphidicolin occurs approximately 10 times faster than in cells treated with ara-C (J. E. Cleaver, unpublished observation). The above results indicate that ara-C may inhibit DNA repair by a combination of chain termination and polymerase inhibition.

Ara-C prevented the reassembly of the repair patch, indicating that reassembly of the repair patch is a sequential event and occurs only after completion of its synthesis. Thus ara-C can be used to accumulate intermediates of nucleosome reassembly even after labeling periods of 4 h.

The results from nuclease digestion of the repair patch indicate that during excision repair there is an alteration in protein–nucleic acid interactions. However, from micrococcal nuclease digestion alone we were not able to determine if during excision repair the repair patch was completely free of these interactions, representing a complete disassembly of the nucleosome during repair, or whether protein–nucleic acid interactions remained but were altered, in comparison to the bulk nucleosomes. Since exonuclease III has previously been used to probe for DNA–protein interactions on Okazaki fragments (Cusick et al., 1981; Herman et al., 1981; Shalloway et al., 1980), it was used in these experiments to study DNA–protein interactions in DNA repair patches.

Exonuclease III digestion of ^{3}H-repair-labeled nuclei isolated from cells treated with HU released a small amount of the ^{3}H repair label, but this amount decreased as the length of the [^{3}H]dThd-labeling period increased. Treatment of cells with ara-C increased the amount of the [^{3}H]dThd repair label that was susceptible to exonuclease III digestion by 2.5–10-fold. However, in absolute terms only about 10% of the ^{3}H repair label was susceptible to exonuclease III digestion. These results indicate that although the repair patch is sensitive to micrococcal nuclease, there are proteins at the repair sites blocking exonuclease III digestion (Cusick et al., 1981; Herman et al., 1981; Shalloway et al., 1980).

This was confirmed by exonuclease III digestion of ^{3}H-repair-labeled DNA purified from nuclei of ara-C-treated cells. Under these conditions 45% of the repair label was exonuclease III sensitive. Thus, removal of chromosomal proteins resulted in a 4-fold increase in digestion of the repair patch by exonuclease III. These results taken together suggest that there are protein–nucleic acid interactions in repairing sites that are qualitatively different than in the reassembled nucleosome. These protein–nucleic acid interactions at the repairing site have as yet to be identified. They may represent histone–DNA interactions, or the interaction of repair enzymes such as DNA polymerase with the repairing site.

The extent of exonuclease III digestion of the ^{3}H repair label in purified DNA isolated from ara-C-treated cells was only 45%. This value was found over a wide range of ara-C concentrations, and over a wide range of exonuclease III concentrations (unpublished observations). Under these conditions 100% of ^{32}P-terminally labeled, blunt-ended DNA was enzymatically removed by exonuclease III (J. E. Cleaver and W. Charles, unpublished results). Similar results with ara-C, aphidicolin, and dideoxythymidine individually or in pairs have also been observed (Cleaver, 1981; J. E. Cleaver and W. Charles, unpublished results). If ara-C blocked at each repair

site, then it should be possible for exonuclease III to enzymatically degrade 100% of the repair patches. There are two possible explanations for the observed results: (1) A fraction of the repair patch is in a single-stranded structure and hence is not exonuclease III sensitive, or (2) in a fraction of the DNA repair sites ara-C does not prevent completion of synthesis, and DNA ligation occurs at the repair patch, and hence these sites are not exonuclease III sensitive.

To determine if a significant fraction of the ^{3}H-repair-labeled DNA purified from ara-C-treated cells was in a single-stranded structure, we digested these samples with S-1 nuclease. At low levels of digestion of the [^{14}C]DNA (1%), 12% of the ^{3}H-repair-labeled DNA was digested. Further incubation resulted in 32% of the ^{3}H-repair-labeled DNA, and 6% of the [^{14}C]DNA becoming acid soluble. Under the conditions of digestion used, single-stranded DNA should be digested approximately 1000-fold faster than double-stranded DNA (Wiegand et al., 1975; Kroeker et al., 1976). The maximum ratio of ^{3}H-repair-labeled DNA to [^{14}C]DNA digested by S-1 nuclease was 10. This ratio suggests that the ^{3}H-repair-labeled DNA digested by S-1 nuclease was not single stranded but that digestion of the repair label occurred by nibbling from the ends (Kroeker et al., 1976). These results suggest that a large fraction of the ^{3}H-repair-labeled DNA in purified DNA preparations does not exist in a single-stranded form.

This interpretation of S-1 nuclease digestion of repair-labeled DNA isolated from ara-C-treated cells is in contrast to that previously presented by Cleaver (1981). The enzyme digestion conditions used in this study were chosen to maximize the distinction between S-1 nuclease digestions of single- and double-stranded DNA. Since the rate of digestion of the ^{3}H-repair-labeled DNA was at least 100-fold lower than expected for single-stranded DNA, we now attribute the observed S-1 nuclease digestion of the repair-labeled DNA to be due to nibbling from the ends. This result does not rule out the model proposed by Cleaver (1981) of displacement of the pyrimidine dimer containing strand during excision repair. However, it does mean that during isolation and enzyme digestion of the repair-labeled DNA, a significant fraction of the displaced dimer containing strand has not exchanged with the ^{3}H-repair-labeled strand as previously proposed by Cleaver (1981).

Isolation and purification of DNA from UV-irradiated cells incubated with [^{3}H]ara-C showed that [^{3}H]ara-C had been incorporated into the DNA as previously reported (Dunn & Regan, 1979). Exonuclease III digestion of this DNA resulted in release of only 48% of the [^{3}H]ara-C, suggesting that approximately half of the [^{3}H]ara-C had been incorporated into DNA repair patches which were ligated, thus not sensitive to exonuclease III. These results are similar to those obtained with exonuclease III digestion of the ^{3}H-labeled repair patch.

The above results indicate that ara-C does not prevent completion of ligation at all DNA repair sites. Numerous studies have used the incorporation of ara-C during UV-induced DNA repair to quantify the number of repair events by measuring the accumulation of strand breaks (Cleaver, 1981; Dunn & Regan, 1979; Hiss & Preston, 1977; Hashem et al., 1980; Snyder et al., 1981). Our results indicate that this procedure could underestimate the actual number of repair events by as much as a factor of 2 if the completed and inhibited patches have equal amounts of ^{3}H incorporated. Since it is probable that the inhibited patches are shorter, then the actual number of repair events will be greater than the number of single-strand breaks by a factor between 1 and 2.

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