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Preferential DNA repair of an active gene in human cells

(chromatin/dihydrofolate reductase/pyrimidine dimer/bacteriophage T4 endonuclease V)

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ABSTRACT **Removal of pyrimidine dimers was measured in defined sequences in human cells amplified for the dihydrofolate reductase (*DHFR*) gene. We quantitated repair in specific restriction fragments by using the dimer-specific bacteriophage T4 endonuclease V and analysis by Southern blotting. Within 4 hr after 5- or 10-J/m^2 UV irradiation, more than 60% of the dimers had been removed from a 20-kilobase fragment that lies entirely within the transcription unit of the *DHFR* gene and from a 25-kilobase fragment located in the 5′ flanking region of the gene. Repair in the overall genome was measured by analyzing cellular DNA treated with T4 endonuclease V in alkaline sucrose gradients. Sixty-nine percent of the dimers were removed from the genome overall within 24 hr after irradiation, but only 25% were removed within 4 hr and 38% were removed within 8 hr. These results demonstrate a strong preferential *rate* of removal of dimers from the 50-kilobase region that includes the transcriptionally active *DHFR* gene compared to that in total cellular DNA. We confirmed that *DHFR*-containing DNA is repaired more rapidly than bulk DNA by using an approach that provides a direct comparison between repair in specific sequences and repair in total cellular DNA. We also show that the *DHFR*-containing sequences are repaired more rapidly than the nontranscribed repetitive α DNA sequences. Our finding of preferential early repair in a transcriptionally active region in overall repair-proficient cells suggests that selective dimer removal from active sequences may be a general characteristic of mammalian DNA repair.**

The biological consequences of unrepaired DNA damage, such as cell death, mutation, and neoplasia, in human cells have been inferred from hereditary diseases (1, 2). Cells from cancer-prone patients with the disease xeroderma pigmentosum are hypersensitive to the killing and mutagenic effects of UV light (3) and are defective in excision repair of their DNA (4).

Although the details of enzymatic repair are not well understood in mammalian cells, there is evidence that the organization of DNA into the complex and dynamic structure of chromatin has significant consequences for the efficiency of damage recognition and removal. Approximately 50% of the pyrimidine dimers in the DNA of permeabilized human cells are refractory to the activity of an exogenously supplied pyrimidine-dimer-specific endonuclease (5). However, pretreatment of the permeabilized cells with 2 M NaCl, which dissociates core histones, renders most of the dimers accessible to the endonuclease, indicating that chromosomal proteins are capable of shielding damaged DNA from a repair enzyme. Although xeroderma pigmentosum cells are generally defective in the incision event of the excision-repair pathway, it has been reported that cellular extracts from some complementation groups of xeroderma pigmentosum can promote the removal of pyrimidine dimers from pure DNA (6, 7) or from chromatin depleted of some proteins by treatment with 0.35 M NaCl (7). This suggests that the deficiency can arise from a defect in rendering chromatin accessible to the endonucleolytic activity. A correlation between chromatin structure and the repairability of genomic domains was demonstrated *in vivo* by the finding of deficient removal of chemical adducts from the heterochromatic nontranscribed α sequences of monkey cells (8). More recently, the demonstration of highly efficient cellular removal of pyrimidine dimers from the dihydrofolate reductase (*DHFR*) gene of Chinese hamster ovary (CHO) cells (9, 10) and the c-*abl* gene of mouse 3T3 cells (11) suggests that, at least in these rodent cells in culture, pyrimidine dimers are preferentially removed from transcriptionally active gene sequences. In the CHO cells, 60% of the dimers were removed from the *DHFR* transcription unit in 8 hr, while only 10% were removed from a sequence at least 30 kilobases (kb) upstream from the gene and only 15% were removed from total cellular DNA in 24 hr. The organization of transcriptionally active chromatin clearly affects its accessibility to nucleases (12, 13). Most active genes are preferentially sensitive to digestion by DNase I, indicating a more extended conformation of this chromatin. Preferential repair of active sequences could be a consequence of increased accessibility to repair enzymes of this more "open" chromatin state.

In general, rodent cells in culture are deficient in their overall ability to remove pyrimidine dimers (14). In contrast, normal human cells remove most of the dimers from their genomes (5). Both cell types exhibit similar survival after UV irradiation even though they differ so dramatically in their repair levels (14, 15). Transcription is blocked by dimers in template DNA (16), and it seems likely that removal of these lesions from essential sequences is necessary for cell survival. The high level of rodent cell survival could be due to selective repair in transcriptionally active sequences, and preferential repair of active genes could be an atypical process unique to repair-deficient rodent cells.

To determine if selective dimer removal from active sequences is a general characteristic of the mammalian repair process or a process peculiar to the repair-deficient rodent cells, we examined the rate of removal of pyrimidine dimers from functionally different sequences by repair-proficient human cells. Since these cells remove 60–80% of the dimers from their total cellular DNA within 24 hr after UV irradiation (5), significant differences in the long-term levels of repair in transcriptionally active and inactive sequences would not be expected. However, preferential repair might instead be reflected in the *rate* of damage removal. In the present study, we show that dimers are indeed removed much more rapidly from the transcriptionally active human *DHFR* domain than from the nontranscribed α DNA sequences and the total cellular DNA. Thus preferential early repair of a transcriptionally active sequence in repair-proficient cells has now been demonstrated. In the course of this study, we developed

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Abbreviations: CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; kb, kilobase(s); T4 endo V, bacteriophage T4 endonuclease V.

a method that provides a relatively rapid and easy way to quantitate dimer removal from a variety of defined DNA sequences in the same biological experiment.

MATERIALS AND METHODS

Cell Culture. The methotrexate-resistant human cell line 6A3 (obtained from G. Attardi, California Institute of Technology, Pasadena, CA) was grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum, penicillin, streptomycin, and 180 μM methotrexate. Actively growing cells were used in all experiments.

Repair Analysis by Southern Blotting. Standard procedures for blotting and hybridization (17–19), modified as described by Bohr *et al.* (10), were followed. C_0t_{100} (9) human DNA was used with the probe designated 2.6.

Repair of Total Cellular DNA. The procedure of van Zeeland *et al.* (5) was used. After treatment with bacteriophage T4 endonuclease V (T4 endo V), samples from 0-, 4-, and 8-hr repair incubations were centrifuged for 96 min at 45,000 rpm, all others for 110 min at 30,000 rpm in a Beckman SW 50.1 rotor. Number-average molecular weight was calculated as described by Lehmann (20); the values were not significantly altered by choice of method.

Repair Analysis by Slot Blotting. The procedure for total cellular DNA was carried out with duplicate gradients as described above. Fractions of the duplicate gradients were neutralized with an equal volume of 2 M ammonium acetate and applied to a nitrocellulose filter by using a slot blot apparatus (Schleicher & Schuell). The wells were washed with 1 M ammonium acetate before and after samples were loaded. Filters were processed in the same manner as the Southern blots and the relative amount of ^{32}P-labeled probe hybridized to each fraction was quantitated by scanning densitometry of the autoradiograms. The number-average molecular weight of the DNA containing sequences homologous to the probe was then calculated from the ^{32}P profile relative to the position of the ^{14}C-labeled bacteriophage λ DNA present in the parallel gradient.

RESULTS

Repair in the Amplified *DHFR* Domain by Southern Analysis. To determine how rapidly dimers are removed from specific sequences in human cells we measured repair at very early times after UV irradiation. Repair was measured by treating purified restriction enzyme-digested DNA with the pyrimidine-dimer-specific T4 endo V and then quantitating the reappearance of specific restriction fragments after the cells were allowed increasing periods of time to remove dimers from their DNA. With increasing repair, fewer endonuclease-sensitive sites remain in the DNA, resulting in fewer strand breaks and more full-length restriction fragments. Cells were grown in 5-bromodeoxyuridine after irradiation and only purified parental density (unreplicated) DNA was analyzed. Replication would generate dimer-free DNA, which if included in the analysis would cause an overestimate of repair. Samples from each time point were treated or not treated with the enzyme and electrophoresed in parallel. The average number of endonuclease-sensitive sites per fragment was calculated in each case from the ratio of the amount of full-length restriction fragments in the enzyme-treated and untreated samples, using the Poisson expression. For clarity we will continue to use the term "dimer" or "pyrimidine dimer," although the measurements actually reflect endonuclease-sensitive sites.

To provide the optimal sensitivity for precise repair measurements, we used the human cell line 6A3, in which the *DHFR* domain has undergone an intrachromosomal amplification. Repair was examined in both a 20-kb *Kpn* I fragment that lies entirely within the *DHFR* transcription unit and in a 25-kb *Kpn* I fragment that directly flanks the gene on the 5′ side (Fig. 1) at 0, 1, 2, and 4 hr after 10-J/m^2 UV irradiation. A visual comparison of the enzyme-treated samples with increasing time indicates substantial reappearance of dimer-free fragments in both the gene and flanking sequence within 4 hr after irradiation. The initial level of damage (determined from the 0 time in Fig. 1) was not significantly different in the gene and the flanking region: 0.55 dimer and 0.53 dimer per 10 kb of single-strand DNA, respectively. These values are similar to the value determined for total cellular DNA (see below), indicating that this region of the *DHFR* domain is not detectably more exposed to or shielded from UV damage than are other sequences in the genome. There was no significant difference in dimer removal from the gene and flanking region at any time examined: more than 50% of the dimers were removed from both regions within only 4 hr (Table 1). The rate of repair in the *DHFR* domain appears to be considerably more rapid than that expected for the bulk of the DNA in these cells (5, 22, 23).

Repair of Total Cellular DNA. We examined the kinetics of dimer removal in the total cellular DNA to determine if the rate of dimer removal from the *DHFR* domain is indeed more rapid than the average rate of repair in the genome overall in these cells. Cells were prelabeled with [^{3}H]thymidine and permeabilized by freezing and thawing. To render all dimers accessible to the enzyme, the permeabilized cells were incubated with 2 M NaCl prior to treatment with T4 endo V, after which they were lysed directly on top of alkaline sucrose gradients and centrifuged. The single strand number-average

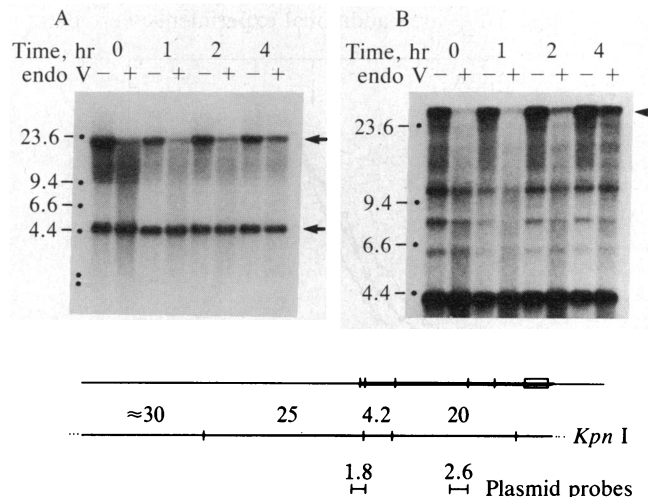

FIG. 1. Southern analysis of repair in the *DHFR* domain. DNA was isolated from cells irradiated with UV light at 10 J/m^2, harvested at the times indicated above the lanes, and digested with *Kpn* I. Samples (5 μg) of DNA were not treated (−) or treated (+) with T4 endo V prior to electrophoresis. The sizes (kb) and positions of *Hin*dIII-digested λ DNA markers are shown. In maps at the bottom (from ref. 21), the *DHFR* gene is indicated by a thick horizontal line and its exons are distinguished by the vertical lines and box. The *Kpn* I fragments and their sizes (kb) and the locations and our designations of the plasmid probes (provided by G. Attardi) are shown. The complete designation of plasmid 1.8 is pBH31R1.8; plasmid 2.6 (pUH6R2.6a) contains a 2.6-kb *Eco*RI fragment from Cha4aDM6 (21). (*A*) The filter was hybridized with plasmid 2.6 to measure repair in the 20-kb *Kpn* I fragment (upper arrow). pBR322 DNA was added to the samples of 6A3 DNA before samples were taken for enzyme treatment. Hybridization of pBR322 sequences in the probe to the pBR322 DNA (lower arrow) served as an internal standard to control for errors in pipetting or loading. (*B*) The filter was hybridized with plasmid 1.8 to measure repair in the ≈25-kb *Kpn* I fragment (arrow) upstream from the gene.

Table 1. Removal of endonuclease-sensitive sites as measured by Southern analysis

Dose, J/m^2	Time, hr	% sites removed	
		Gene	Flanking region
5	1	12	5
	2	23	20
	4	71	65
10	1	6	14
	2	24	21
	4	79	67

Data were obtained by liquid scintillation counting of bands excised from nitrocellulose filters.

molecular weight of the DNA was determined from the profile of the ^{3}H mass label relative to the position of ^{14}C-labeled λ marker DNA present in the gradient. This assay differs from the analysis described above in that the DNA is not digested with a restriction enzyme and random DNA fragments are resolved in sucrose gradients instead of gels. Fig. 2 shows the radioactivity profiles of cellular DNA from one repair experiment. Analysis of the average size of the DNA from the respective time points required different conditions of centrifugation. Hence, the 0-, 4-, and 8-hr time points (Fig. 2*A*) are presented separately from the 24- and 48-hr time points and the control (Fig. 2*B*). When the position of each peak is compared to the position of the λ DNA marker, dimer removal is evident from the progressive relative shift in the molecular weight of the cellular DNA with increasing repair time. The number-average molecular weights of the DNA samples from the experiment presented in Fig. 2 and from three additional experiments were determined. The average value of the initial dimer frequency was 0.5 dimer per 10 kb. As expected for these repair-proficient human cells, a large fraction (69%) of the dimers were removed from the bulk of the DNA by 24 hr (Table 2). However, at 4 hr only 25% were removed and at 8 hr 39% were removed. These results confirm a significant difference in the rate of removal of dimers from the *DHFR* domain and total cellular DNA in these cells.

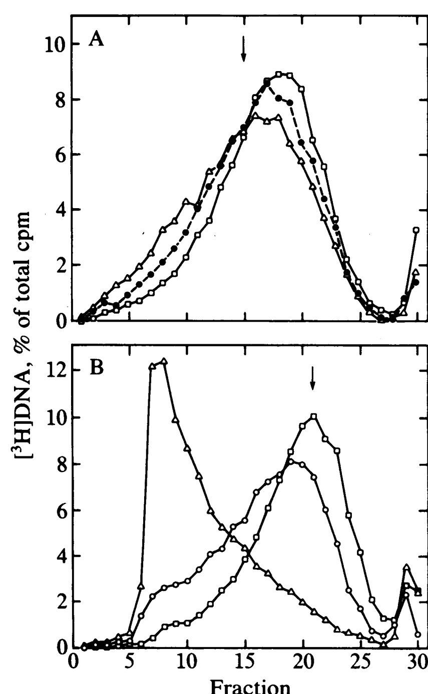

FIG. 2. Radioactivity profiles of alkaline sucrose gradients, showing repair in total DNA from cells UV-irradiated with 10 J/m^2. In both panels the position of ^{14}C-labeled λ DNA (48.5 kb) is indicated by an arrow. The direction of sedimentation is from right to left. (*A*) Cells harvested at 0 (□), 4 (●), and 8 (△) hr after irradiation and treated with T4 endo V. (*B*) Cells harvested at 24 (□) and 48 (○) hr after irradiation and treated with T4 endo V. Control cells harvested immediately after irradiation were not treated with T4 endo V (△). Profiles from unirradiated cells treated with T4 endo V were identical to those of the other controls.

Table 2. Removal of endonuclease-sensitive sites from total cellular DNA

Time, hr	% sites removed				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean
4	20	26	23	29	25
8	36	46	40	34	39
24	65	74	69	68	69
48	—	91	—	—	91

Cells were UV-irradiated with 10 J/m^2.

Repair of Defined Sequences by Slot-Blot Analysis. The difference in repair of the *DHFR* domain and total cellular DNA detected 4 hr after irradiation is striking, but it should be noted that two different techniques were used. Consequently, we developed a single method to compare measurements of bulk repair and sequence-specific repair. The method described above for assessing repair in bulk DNA was extended to include a duplicate gradient for each time point, in which the marker DNA was not included. The DNA in the fractions of these duplicate gradients was applied to nitrocellulose by slot blotting and probed for specific sequences. The relative amounts of hybridization in each fraction were determined from scanning densitometry. Profiles for bulk DNA (^{3}H label) are plotted together with those of *DHFR* and α DNA (^{32}P-labeled probes).

To examine repair in *DHFR*-containing sequences we used the plasmid probe 1.8 (see Fig. 1), which is located in the center of the 50-kb region of the *DHFR* domain examined by Southern analysis. The profiles of bulk and *DHFR*-containing DNA at 0 time were coincident (Fig. 3*A*), indicating that the initial dimer frequency is the same. However, at 4 hr (Fig. 3*B*), the average size of *DHFR*-containing DNA was larger than the average size of the total DNA, and at 8 hr (Fig. 3*C*) this difference was even more pronounced, indicating faster repair in the *DHFR* domain.

We also examined repair in human α DNA, a nontranscribed repetitive DNA sequence (Fig. 3 *D–F*). In this case the profiles were coincident at each time point, indicating that the initial dimer frequency was the same in α and bulk DNA (Fig. 3*D*) and the rates of repair in α and bulk DNA were similar. Number-average molecular weights were determined from these profiles and used to calculate repair (Table 3). These results confirm the observation by Southern analysis of preferential early repair of the *DHFR* domain compared with bulk DNA. In addition, the rate of dimer removal from *DHFR*-containing sequences is greater than that found in α DNA-containing sequences.

This slot-blot analysis (unlike the measurements of bulk repair derived from prelabeled DNA) does not distinguish between dimer-free DNA that is generated by repair and that

Table 3. Removal of endonuclease-sensitive sites as measured by slot-blot analysis

Time, hr	% sites removed			
	Bulk	*DHFR*	Bulk	α
4	23	48	29	29
8	40	73	34	35

Cells were UV-irradiated with 10 J/m^2.

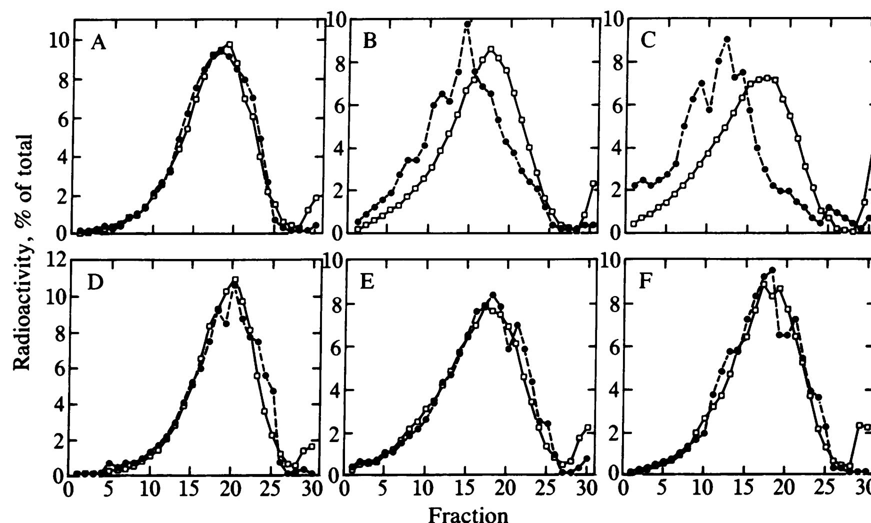

FIG. 3. Comparison of repair in defined sequences and in total cellular DNA. Cells were UV-irradiated with 10 J/m^2 and harvested after 0 hr (*A* and *D*), 4 hr (*B* and *E*), and 8 hr (*C* and *F*). All profiles shown are for cells treated with T4 endo V. In all panels, open squares represent the ^{3}H in total cellular DNA. The filled circles represent DNA containing specific sequences obtained from the relative amounts of ^{32}P-labeled probe bound to each fraction. In *A*, *B*, and *C* the profiles were obtained by hybridization with probe made from plasmid 1.8 (see Fig. 1). In *D*, *E*, and *F* they were obtained with probe made from plasmid pCA1004a, which contains a 344-base-pair *Hin*dIII dimer of monkey α DNA, provided by M. Singer (24). Repair in α and *DHFR*-containing DNA was examined in separate experiments.

generated by replication after irradiation. We therefore assessed the amount of DNA replication in the 6A3 cells under these conditions by incubating irradiated cells in the presence of density label and determining the amount of ^{3}H-prelabeled DNA appearing at hybrid density in CsCl density gradients. Only 3% and 6% of the DNA was replicated at 4 and 8 hr after 10 J/m^2. Therefore, it is unlikely that replication had a significant influence on the measurements of repair obtained by the slot-blot method. However, about 40% of the DNA was replicated in 24 hr after 10-J/m^2 irradiation, so this technique is not suitable for examining long-term levels of repair unless replication is inhibited.

DISCUSSION

We show here that pyrimidine dimers are preferentially removed from an approximately 50-kb region of DNA that includes the transcriptionally active *DHFR* gene in human cells. Activity of the *DHFR* gene in these experiments is ensured by using cells growing in methotrexate. The rate of repair in this region is more rapid than that found in the nontranscribed α DNA sequences or in the total cellular DNA. The different rates are not related to differences in substrate concentration, since the initial level of damage is not significantly different in any of the DNA species we examined. It is also unlikely that this is related to the amplification of the gene in the 6A3 cells, since a similar rapid repair has been found in a 25-kb region of the *DHFR* gene in a nonamplified cell line (I.M., unpublished data). Repair characteristics of the *DHFR* gene region in CHO cells are not altered by amplification of the region (9, 10), and preferential repair has also been shown for the nonamplified c-*abl* gene in mouse cells (11).

Proficient repair of pyrimidine dimers has been correlated with the transcriptional activity of sequences in generally repair-deficient rodent cells (9, 11). If removal of dimers in active regions is necessary for viability, one could speculate that rodent cells in culture have maintained proficient repair of essential transcribing sequences but have lost their ability to repair other sequences. Therefore, differential dimer removal from functionally distinct sequences might merely reflect a specific deficiency in repair of nontranscribing DNA. Our results presented here for human cells indicate that this is not the case. Even in cells that will have removed most dimers from all sequences within 24 hr, preferential removal of dimers from an active sequence is demonstrated at early times after irradiation. These results suggest that selective dimer removal from transcriptionally active regions is a process characteristic of mammalian DNA repair in general. Pyrimidine dimers in template DNA cause premature termination of transcription, apparently without inhibiting initiation (16); their selective removal from regions active in transcription would presumably be an advantage to the cell. Mayne and Lehmann (25) reported that overall transcription is initially inhibited in UV-irradiated human cells but returns to near normal levels before extensive overall repair has taken place. They suggested that selective rapid repair in transcribing regions could explain their results. Studies with inhibitors of DNA repair (26) and the results presented here support this explanation. It will be important to examine other active genes to determine whether rapid repair is common to active sequences.

We found similar preferential early repair in both the gene and a 5′ flanking sequence in human cells. This does not contradict the results of Bohr *et al.* (9), since the upstream fragment examined in that study was at least 30 kb from the active gene. More recent examination of CHO cells has shown proficient repair of a fragment in the 5′ flanking region of the gene (31). Preferential repair could be specifically related to the transcriptional activity of both sequences, since a divergent transcript that initiates in the 5′ region of the *DHFR* gene but extends in the opposite direction for several kb has been reported in mouse cells (27) and CHO cells (28). Although no similar transcript has been reported in human cells, it is possible that the rapid repair found in the human *DHFR* gene and 5′ flanking sequences is related to transcription in both regions. Rapid repair could also be related to chromatin structure. Active genes in general are preferentially sensitive to digestion by DNase I, indicating greater accessibility of this DNA in chromatin. In some cases this preferential sensitivity has been found to extend for several kb in both the 5′- and 3′-flanking regions of genes (13). If chromatin structure strongly influences damage removal, flanking sequences could be repaired as well or with the same kinetics as gene sequences because of similarities in their chromatin structure. Furthermore, since potentially active

genes also exhibit increased DNase sensitivity (13), they might also be repaired rapidly.

We have developed a method to quantitate dimer removal in specific sequences by probing DNA resolved in alkaline sucrose gradients. Using this method, we found that the rate of repair in α DNA is similar to that found in total DNA within 8 hr after irradiation. Zolan *et al.* (8) had previously examined repair of α DNA in green monkey cells *in vivo* by purifying α DNA from other genomic sequences. This was technically feasible because α makes up 15–20% of the total DNA in these cells. They found that although removal of chemical adducts was deficient in α, repair synthesis in response to UV was similar to that found in bulk DNA. Our examination of dimer removal by using the slot-blot method is consistent with this previous observation. Furthermore, we found similar rates of repair in the *DHFR* domain when we assayed dimer removal by using the Southern method or the slot-blot method. In the 6A3 cells, at 4 hr after irradiation the slot-blot analysis showed 48% repair in sequences that contain the 1.8-kb *Eco*RI fragment. Our Southern analysis after 4 hr showed greater than 60% repair in the 20-kb gene sequence and the 25-kb flanking sequence, indicating that approximately 50 kb of contiguous DNA was repaired similarly. When the Southern assay is used, measurements are not influenced by the repair characteristics of sequences located 5′ or 3′ to the restriction fragment being examined. However since the DNA is not digested with restriction endonuclease during the slot-blot analysis, repair measurements in *DHFR*-containing sequences will be influenced by the amount of repair in contiguous DNA. Consequently, if dimers are not removed as rapidly from DNA 5′ and/or 3′ to the 50-kb *DHFR* domain, the amount of repair measured by this assay will be less than that determined from the Southern analysis. This could account for the slight difference in the repair measured for the *DHFR* domain with the two different techniques.

Our results have potentially significant implications concerning the elucidation of factors required for the recognition and removal of damage from DNA as it exists in chromatin. Several models could explain the differences in kinetics we observe: (*i*) DNA in active chromatin could be readily accessible to repair enzymes but damaged sequences in more condensed regions may be shielded from them by chromosomal proteins and require an additional processing step to render the DNA accessible. This preincision processing step may be deficient in rodent cells, resulting in low levels of repair outside of active domains. (*ii*) The actual conformation of the DNA as it exists in functionally different states of chromatin rather than its accessibility may determine whether damage is recognized and/or removed by repair enzymes. Proper superhelical conformation of the DNA may be necessary for binding of repair enzymes. Evidence is accumulating that a specific DNA tertiary structure is required for transcription in eukaryotic cells (29, 30). A similar specific superhelical density could be functionally significant to mechanisms of repair. (*iii*) The rapid rate of repair of an active sequence of human cells and the proficient repair of active sequences in rodent cells could simply reflect the existence of separate, independent pathways for processing damage in active and inactive DNA. (*iv*) The arrest of transcription at lesions and release of RNA polymerase from the template could serve as a specific signal to accelerate repair in active domains. Examination of repair in genomic sequences that are in different states of transcriptional activity such as those that are inducible or developmentally regulated should clarify the relationship between chromatin structure and mechanisms of DNA repair and help to support or reject these models.

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