Lack of Transcription-Coupled Repair in Mammalian Ribosomal RNA Genes†

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ABSTRACT: We studied the induction and removal of UV-induced cyclobutane pyrimidine dimers (CPDs) in the ribosomal RNA genes (rDNA) in cultured hamster and human cells. In these genes, which are transcribed by RNA polymerase I, we found no evidence for transcription-coupled repair. The induction of CPDs was heterogeneous in rDNA due to nucleotide sequence: it was lower on the transcribed strand than on the nontranscribed strand and slightly lower in the coding region than in the nontranscribed spacer. Nevertheless, no dramatic difference in CPD induction was observed between rDNA and the dihydrofolate reductase (DHFR) gene. In Chinese hamster ovary cells, we observed no removal of CPDs from either rDNA strand within 24 h after UV irradiation. In these experiments, we did observe efficient repair of the transcribed, but not the nontranscribed, strand of the DHFR gene, in agreement with published results. In human cells, repair of rDNA was observed, but it showed no strand preference and was slower than that reported for the genome overall. No significant differences in repair were observed between restriction fragments from transcribed and nontranscribed regions or between growth-arrested and proliferating human cells, with presumably different levels of transcription of rDNA. We conclude that the modest level of rDNA repair is accomplished by a transcription-independent repair system and that repair is impeded by the nucleolar compartmentalization of rDNA. We discuss the possibility that recombination, rather than repair, maintains the normal sequence of rDNA in mammalian cells.

Organisms encounter numerous sources of damage to their DNA. Oxidizing agents and other reactive chemicals, ionizing radiation, and UV are just some of the threats to the integrity of cellular DNA. To respond to these threats, a complex battery of repair mechanisms has evolved [see Friedberg (1985)]. Perhaps the most extensively studied is the excision repair system, especially with respect to its response to UV. It is well established that mammalian cells in culture display an intragenomic heterogeneity of repair of cyclobutane pyrimidine dimers (CPDs),[1] the predominant lesion introduced by 254-nm UV [for reviews, see Smith and Mellon (1990), Terleth et al. (1991), and Hanawalt (1993)]. Apparently at least two cellular repair systems exist. The genome overall is repaired at a slow rate. This genomic repair system is either absent or inefficient in hamster cells, which remove only 15–30% of the CPDs in 24 h (Bohr et al., 1985; Regan et al., 1990). A faster, transcription-coupled, repair system removes CPDs from active genes. The preferential repair of CPDs has been observed in a number of active genes transcribed by RNA polymerase II (pol II): dihydrofolate reductase (DHFR) in hamster (Bohr et al., 1985) and human (Mellon et al., 1986) cells; c-*abl* in mouse cells (Madhani et al., 1986); hypoxanthine phosphoribosyl-transferase in hamster cells (Vrieling et al., 1991); and adenosine deaminase (Venema et al., 1990), β-actin (Kantor et al., 1990), and active metallothionein genes (Leadon & Snowden, 1988) in human cells. In all cases examined, the preferential repair of active genes appears to result from an increase in the rate of repair of the transcribed (template) DNA strand (Mellon et al., 1987; Vrieling et al., 1991; Venema et al., 1991). We have previously demonstrated that the preferential repair of CPDs on the transcribed strand is dependent upon transcription (Christians & Hanawalt, 1992).

Mammalian ribosomal RNA genes (rDNA), transcribed by RNA polymerase I (pol I), differ in many ways from the genes transcribed by pol II. rDNA is naturally iterated. The haploid human genome, for example, contains 150–200 copies of a repeating unit distributed near the ends of 5 chromosomes [for reviews, see Lewin (1980) and Long and Dawid (1980)]. Each repeat is G+C-rich and encodes three highly evolutionarily conserved ribosomal RNA (rRNA) species, the 18S, 5.8S, and 28S ribosomal RNA molecules, which are spliced from a single 45S precursor transcript. Individual transcription units are separated by nontranscribed spacers, which contain a variable region just 3′ to the transcribed sequence (Arnheim & Southern, 1977; Erickson et al., 1981). The spliced rRNA species are used directly in the structure of the ribosomes. The level of transcription of rDNA can be phenomenonally high. Also, rRNA accounts for well over half of a growing cell's RNA while rDNA comprises less than 1% of the DNA (Sollner-Webb & Mougey, 1991). The rDNA transcription rate is reduced considerably in growth-arrested or cycloheximide-treated cells (Tower & Sollner-Webb, 1987; Conconi et al., 1989). rDNA is transcribed in the nucleolus, a dense aggregation of DNA, pol I, rRNA, and assembling ribosomes.

Very little is known about the repair of rDNA. Since the repair of the transcribed DNA strand is important to maintain transcript fidelity, and proper function of ribosomes is obviously important, one might expect the preferential repair system to extend to rDNA. Each of the unique features of rDNA may influence the introduction and repair of DNA damage. Two early reports suggested that while CPDs are introduced at a lower frequency in rDNA than in the genome overall, repair events can be detected in rDNA in human cells (Rajagopalan

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[1] Abbreviations: CHO, Chinese hamster ovary; CPD, cyclobutane pyrimidine dimer; DHFR, dihydrofolate reductase; pol I, RNA polymerase I; pol II, RNA polymerase II; rDNA, ribosomal RNA genes; rRNA, ribosomal RNA; TEV, T4 endonuclease V.

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et al., 1984; Cohn & Lieberman, 1984). Two more recent studies examined the repair of chemical-induced damage in rDNA. Matsumoto et al. (1989) measured 80% removal in 24 h of mitomycin C interstrand cross-links from rDNA in normal lymphoblastoid cells. Vos and Wauthier (1991) showed that psoralen monoadducts and interstrand cross-links were introduced and removed less efficiently in rDNA than in the DHFR gene in hamster and human cells. We examined the introduction and removal of UV-induced CPDs in human and hamster cells. We observed a slightly lower level of damage in rDNA than in the DHFR gene, a difference we attribute to nucleotide sequence and composition differences. rDNA was repaired less efficiently than total cellular DNA and exhibited no strand bias in repair.

MATERIALS AND METHODS

Probes. The plasmids pZH4, pZH8, and pZH9 were used to generate strand-specific RNA probes with Promega riboprobe kits. pZH4 contains both intron and exon sequences of the 5′ region of the hamster DHFR gene (Mellon et al., 1987). pZH8, constructed in this laboratory by I. Mellon and G. Spivak from a vector originally supplied by N. Arnheim, contains most of the murine 28S rRNA gene. The 4.8-kb *Sal*I–*Eco*RI genomic fragment (Arnheim, 1979) was cloned into pGEM-3Z (Promega). Because of the orientation of the fragment within the vector, SP6 RNA polymerase generated RNA probes specific for the transcribed strand of the 28S gene, and T7 RNA polymerase generated probes specific for the nontranscribed strand. pZH9, also constructed by I. Mellon and G. Spivak, contains most of the murine 18S rRNA gene. The 1.9-kb *Sal*I–*Eco*RI genomic DNA fragment was cloned into pGEM-3Z in the same orientation as pZH8. Although the rDNA in pZH8 and pZH9 is of murine origin, sequence conservation provided adequate hybridization to human and hamster DNA. The plasmids pC_{PE} and pD_{ES} (kindly provided by J. Sylvester) contain portions of the nontranscribed spacer region from human rDNA (see Figure 1) and were used to make double-stranded probes with nick-translation kits (Bethesda Research Laboratories), but not RNA probes since they lack RNA promoters. Double-stranded probes were also made from pZH8 and pZH9.

Cell Culture. Chinese hamster ovary (CHO) B11 cells (originally from R. T. Schimke, this department) have a 50-fold amplification of the DHFR gene, which makes them resistant to methotrexate (Johnston et al., 1983). This cell line was grown in minimal essential medium supplemented with 10% dialyzed fetal bovine serum, 2 mM glutamine, nonessential amino acids, and 500 nM methotrexate (Calbiochem). The human cell line HT1080 (American Type Culture Collection CCL-121) was derived from a fibrosarcoma and is near-diploid (Rasheed et al., 1974). HT1080 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and nonessential amino acids. Human GM38 primary fibroblasts (Human Genetic Mutant Cell Repository) were derived from a phenotypically normal individual. They were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and 2 mM glutamine. The GM38 medium was routinely changed every 2–3 days, and the cells were split 2:1 weekly.

Restriction Analysis. Five-microgram samples of B11, HT1080, or GM38 DNA were digested with the restriction endonucleases *Hin*dIII, *Sal*I, *Kpn*I, *Eco*RI, and *Pst*I (Bethesda Research Laboratories and New England Biolabs) singly and in some pairwise combinations. Restricted DNA was electrophoresed in neutral 0.5% agarose gels, transferred to nylon membranes (Amersham Hybond-N+), and hybridized with nick-translated ^{32}P-labeled probes made from pZH8 and pZH9.

CPD Induction. GM38 cells were washed twice with ice-cold phosphate-buffered saline and irradiated with 254-nm UV at an incident dose rate of 0.33 W/m^2 with a Westinghouse IL782-30 germicidal lamp. Cells irradiated with 0, 10, 20, 40, or 80 J/m^2 were immediately lysed with 0.5% sodium dodecyl sulfate in TE buffer [10 mM Tris/1 mM EDTA (pH 8.0)] and 0.1 mg/mL proteinase K. DNA was extracted with phenol and chloroform, precipitated with ammonium acetate and ethanol, resuspended in TE, and restricted with *Hin*dIII and *Eco*RI. Three-microgram samples (120 μL) of restricted DNA from unirradiated cells were placed in plastic 96-well microtiter plates and irradiated with 0, 10, 20, 40, or 80 J/m^2. For each dose, one sample was treated, and an equivalent sample was mock-treated with T4 endonuclease V (TEV), which makes single-stranded nicks at CPD sites in DNA. Both equivalent samples were then electrophoresed in parallel in an alkaline 0.5% agarose gel. The DNA was transferred to a nylon membrane (Amersham Hybond-N+) and hybridized with nick-translated ^{32}P-labeled probes made from pC_{PE}, pZH9, pZH8, and pD_{ES}. Autoradiography (Kodak X-AR5 film with no intensifying screens) and densitometry (Helena QuickScan) were performed to determine the relative intensity of each full-length (unnicked) fragment. CPD frequencies were calculated using the Poisson expression: CPDs/fragment = −ln[(band intensity of the TEV-treated sample)/(band intensity of the TEV-untreated sample)]

Repair Analysis. Detailed methodology can be found elsewhere (Bohr et al., 1985; Mellon et al., 1987). Exponentially growing cells were prelabeled for 3 days with 0.1 μCi/mL [^{3}H]thymidine and subcultured at a low density into fresh nonradioactive medium 24 h before irradiation. Growth-arrested GM38 cells were obtained by culturing the cells for 10 days with no medium changes. Cell samples were irradiated with either 10 or 20 J/m^2 UV as described above and incubated in medium containing 20 μM 5-bromodeoxyuridine and 1 μM 5-fluorodeoxyuridine. Cells were lysed 0, 4, 8, and 24 h post-UV. After extraction with phenol and chloroform or with the Amersham Elu-quick kit, DNA was restricted (*Pst*I for CHO cells and *Hin*dIII for human cells), and the unreplicated DNA, which did not contain 5-bromodeoxyuridine, was isolated by CsCl gradient centrifugation. TEV reactions, electrophoresis, and transfer to nylon membranes were performed on 5-μg unreplicated DNA samples as described in the preceding section. DNA was then hybridized with ^{32}P-labeled, strand-specific RNA probes made from pZH8 or pZH9. Membranes containing B11 DNA were also probed with strand-specific probes made from pZH4. Autoradiography and densitometry were performed, and the Poisson expression was used to determine the extent of repair.

RESULTS

The main aim of this work was to investigate the repair of rDNA, but it was first necessary to improve the characterization of the rDNA repeat. We performed restriction mapping of the cell lines used for repair analyses in order to select appropriate restriction fragments. We were concerned that the heterogeneity of the nucleotide sequence within an rDNA repeat, as well as regions of very high G+C content, might drastically influence the induction of CPDs, so we also analyzed this possibility carefully. Finally, the repair experiments were performed on UV-irradiated cells using an assay which involved restriction of genomic DNA, treatment

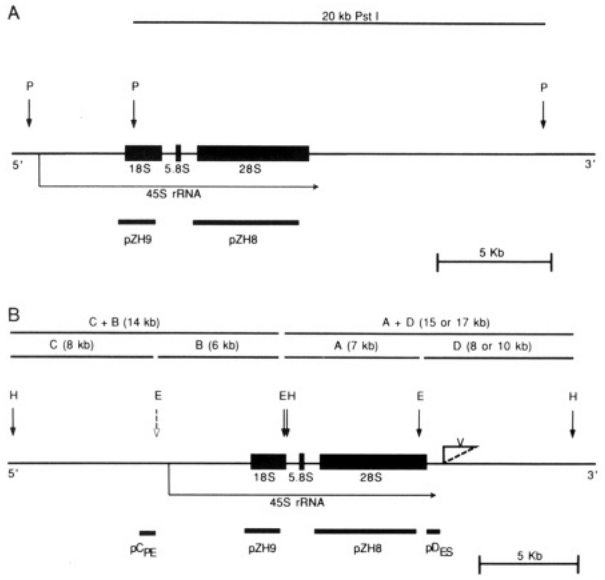

FIGURE 1: rDNA restriction maps. Genomic DNA of (A) hamster and (B) human cells was digested with various restriction endonucleases and analyzed with rDNA probes. Symbols: shaded boxes, rRNA genes; horizontal arrow, the 45S primary transcript; shaded boxes below the transcript, probes; horizontal lines above the map, restriction fragments used for subsequent analysis. Vertical arrows indicate restriction sites (P, *Pst*I; H, *Hin*dIII; E, *Eco*RI); the dashed tail and open arrowhead for the 5′ *Eco*RI site in (B) indicates that some rDNA repeats lack this site. The triangle marked "V" in (B) indicate a region that varies in length among repeats. Locations of pC_{PE} and pD_{ES} are courtesy of J. Sylvester. The designation of restriction fragments (C, B, A, and D) in (B) follows Sylvester et al. (1989).

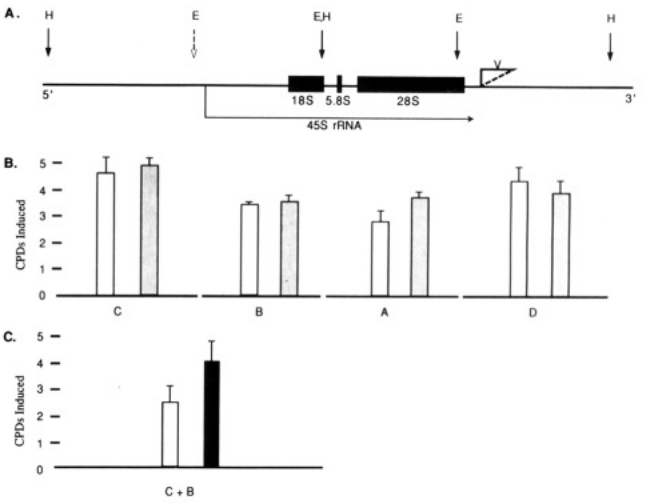

FIGURE 2: CPD induction in human rDNA. (A) Map of human rDNA (see Figure 1 legend for details). (B) GM38 cells were UV-irradiated (0, 10, 20, 40, and 80 J/m^2), and the DNA was immediately isolated, purified, and treated with *Hin*dIII and *Eco*RI. DNA from unirradiated cells was then irradiated *in vitro* at the same doses. TEV treatment, alkaline agarose gels, Southern transfer, hybridization with ^{32}P-labeled nick-translated DNA probes, and autoradiography were performed to determine the number of CPDs induced in each fragment (C, B, A, and D; see Figure 1B) for each sample. White bars, cells irradiated; stippled bars, purified DNA irradiated. The scale at the left represents the number of CPDs induced per 10^6 bp per J/m^2; average values and standard deviations for the four UV doses from one experiment are presented. (C) Initial CPD frequencies for each strand of the *Hin*dIII fragment C+B (see Figure 1B). White bar, transcribed strand; black bar, nontranscribed strand. The averages and standard deviations of three repair experiments with HT 1080 cells and two experiments each with GM38 proliferating and growth-arrested cells are presented; all experiments used 20 J/m^2 UV.

with TEV, denaturation of the DNA, and electrophoretic size fractionation. Any DNA strand with one or more CPDs was cleaved by the TEV and was thus resolved into smaller than full-length fragments. Parallel mock-treated samples were included for comparison with the amount of full-length (CPD-free) fragments remaining in the TEV-treated samples.

Restriction Mapping of rDNA. Two factors prompted us to characterize the restriction patterns in the rDNA of the cell lines used for repair analysis. First, despite the high sequence conservation in parts of the 18S and 28S rRNA genes, other portions of the rDNA repeat are highly divergent [see Gonzalez et al. (1990) and references cited therein]; the nontranscribed spacers vary even within an individual (Arnheim & Southern, 1977). Second, we needed to identify restriction fragments in which approximately one CPD, the optimum for our repair analysis using the Poisson expression, would be induced by biologically relevant UV doses. We considered the *Eco*RI fragments used in earlier studies (Cohn & Lieberman, 1984) to be smaller than optimal (6–7 kb in the coding region). We restricted genomic DNA from each cell line with several different enzymes individually and in most double combinations. Restriction fragments used for further analysis are shown in Figure 1. The restriction patterns for all cell types were identical in the coding region but differed, as expected, in the nontranscribed spacer. For example, all the rDNA repeats in HT1080 cells but only about 80% of the GM38 repeats had an *Eco*RI site between fragments C and B; CHO B11 repeats lacked this site entirely. In HT1080 cells, about half the repeats had an approximately 2-kb insert just 3′ to the coding region, whereas only approximately 10% of the GM38 repeats and none of the CHO B11 repeats had such an insert. We do not know whether such variation existed between repeats within each cell or between cells in the population.

Induction of CPD in rDNA. The sequence and structure of rDNA have characteristics that might make the induction of UV damage different from that of other genes. Within an rDNA repeat, CPD induction in regions with very high G+C contents (Lewin, 1980) would be expected to be lower than average, whereas thymine-containing pyrimidine stretches (La Volpe et al., 1985) should be CPD induction hotspots. The high concentration of polymerases and topoisomerase I molecules (Zhang et al., 1988) associated with the transcribed region of active rDNA might also influence CPD induction.

The only information we obtained concerning CPD induction in CHO B11 cells is from the repair experiments described below. The CPD frequency in the CHO transcribed rDNA strand was only about 70% as high as in the nontranscribed strand (Table I). The CPD frequencies in both strands of the DHFR gene in CHO cells were about the same as in the nontranscribed rDNA strand, direct evidence that CPD induction in rDNA was not grossly unusual.

The human rDNA repeat is much better characterized than that of the hamster; therefore, we examined CPD induction in rDNA of human cells in more detail. We measured directly the frequencies of CPD induced by different doses of UV applied to cells or isolated DNA. Four *Hin*dIII–*Eco*RI restriction fragments of similar length encompass the transcribed region of the human rDNA repeat (fragments C, B, A, and D in Figure 1B). The frequency of CPDs for each UV dose in each of the four fragments was determined using nick-translated probes (Figure 2B). Results show the following: (1) The CPD induction in the rRNA genes (fragments B and A) was slightly lower than in the nontranscribed spacer (fragments C and D). (2) For each fragment, very similar CPD frequencies were observed whether we irradiated cells

(white bars in Figure 2B) or DNA purified from cells (stippled bars). (3) The CPD frequency observed in rDNA [3–5 CPDs $(10^6$ bp$)^{-1}$ $(J/m^2)^{-1}$] was within the range of that observed in the DHFR gene of a cell line, 293C18-MTX-r, amplified for the DHFR gene [average of 4.8 CPDs $(10^6$ bp$)^{-1}$ $(J/m^2)^{-1}$]. These results, in addition to confirming the results from CHO cells, show that proteins and chromatin structure do not influence CPD induction at the level at which we measure it.

The repair experiments described below addressed the issue of CPD induction at the level of DNA strands in human cells. We observed a strand bias much like that seen in CHO cells: in fragment C+B, on average only about 60% as many CPDs were induced by UV in the transcribed strand (white bar in Figure 2C) as in the nontranscribed strand (black bar). Furthermore, we observed CPD hotspots in the nontranscribed strand. In samples treated with TEV, discrete bands smaller than the full-length band were visible when membranes were probed for the rDNA nontranscribed strand (e.g., Figure 4B) but not when probed for the transcribed strand (e.g., Figure 4A). In the absence of CPD hotspots, TEV cleavage products appear as a smear, rather than discrete bands, below the full-length band. No CPD hotspots were seen in CHO rDNA (e.g., Figure 3A,B), so the clusters in human rDNA must be in a nonconserved region. The two prominent cleavage products were approximately 7 and 8 kb, about half the length of the complete 14-kb *Hin*dIII restriction fragment (fragment C+B in Figure 1B). Thus, the position of the pZH9 probe at the end of the fragment allows the placement of the CPD hotspots on the nontranscribed strand in the middle of the *Hin*dIII fragment, or just upstream of the transcriptional start site. Paradoxically, this region contains an abundance of potential CPD hotspots, thymine stretches, on the transcribed strand (Sylvester et al., 1989). We can account for neither this apparent discrepancy nor the exact cause of the CPD hotspots.

Repair of rDNA and DHFR in CHO B11 Cells. In cultured hamster cells, significant repair of CPDs has been observed only in the transcribed strand of pol II-transcribed genes; the nontranscribed strand and overall genome are repaired at very low levels (Bohr et al., 1985; Mellon et al., 1987). The preferential repair of the transcribed strand was shown to depend upon transcription by pol II (Christians & Hanawalt, 1992). We used the same assay to determine the rate and extent of removal of CPDs from the two strands of rDNA. Proliferating CHO B11 cells were irradiated with 20 J/m^2 254-nm UV. DNA isolated 0, 4, 8, and 24 h later was restricted with *Pst*I, electrophoretically fractionated, and hybridized with strand-specific probes made from pZH8 or pZH9 to detect the 20-kb rDNA restriction fragment (Figure 1A). No repair of rDNA was observed in CHO cells in either the transcribed (Figure 3A) or the nontranscribed (Figure 3B) strand; that is, the rDNA fragments in all samples exhibited substantial nicking by TEV. However, when the same membranes were hybridized with the DHFR probe pZH4, substantial repair of the transcribed strand of the DHFR gene was evident by 4 h (Figure 3C). Little repair was seen for the nontranscribed strand of the DHFR gene (Figure 3D). The entire experiment was repeated using 20 J/m^2 UV, and a third experiment was done using 10 J/m^2 UV. All three experiments gave consistent results: efficient removal of CPD from the transcribed strand of the DHFR gene, little repair of its nontranscribed strand, and no detectable repair of either strand of the ribosomal RNA genes (Table I). The data for DHFR are consistent with those reported previously for CHO cells (Mellon et al., 1987; Christians & Hanawalt, 1992).

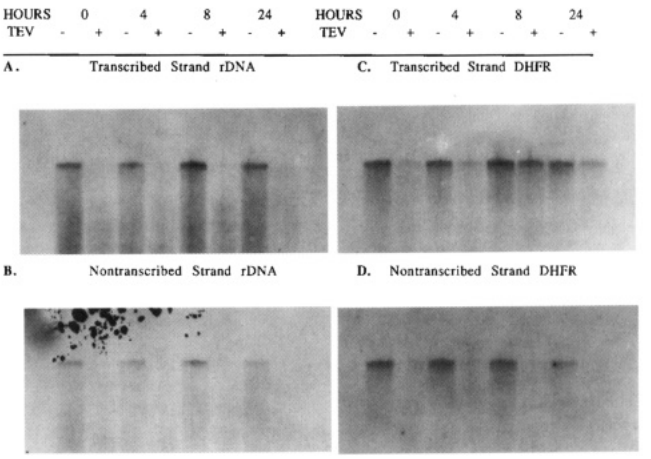

FIGURE 3: Strand-specific repair analysis of hamster rDNA and DHFR. Exponentially growing B11 cells were irradiated with 20 J/m^2 UV. DNA was isolated 0, 4, 8, and 24 h post-UV, restricted with *Pst*I, treated (+) or not treated (–) with TEV, fractionated on alkaline agarose gels, and transferred to nylon membranes. Autoradiography detected hybridization of strand-specific ^{32}P-labeled RNA probes. Probes made from pZH9 hybridized with the 20-kb *Pst*I rDNA fragment (panels A and B); the 12-kb DHFR *Pst*I fragment was detected with pZH4 probes (panels C and D).

Table I: Induction and Removal of CPD in CHO B11 Cells[a]

		transcribed strand		nontranscribed strand	
gene[b]	time (h)	initial CPD[c]	% CPD removed	initial CPD	% CPD removed
rDNA	0	3.1 ± 0.8		4.4 ± 0.8	
	4		<10		<10
	8		<10		<10
	24		<10		<10
DHFR	0	4.1 ± 0.1		4.4 ± 0	
	4		49 ± 7		9 ± 12
	8		80 ± 8		24 ± 21
	24		90 ± 15		4 ± 6

[a] Data are from three experiments (one using 10 J/m^2, and two using 20 J/m^2) and are expressed as the average ± the standard deviation. [b] rDNA fragment: 20-kb *Pst*I detected by pZH8 and pZH9. DHFR fragment: 12-kb *Pst*I detected by pZH4. [c] CPD $(10^6$ bp$)^{-1}$ $(J/m^2)^{-1}$.

Repair of rDNA in Human Cells. We performed similar experiments using proliferating HT1080 cells irradiated with 20 J/m^2 UV. We first measured repair in the 14-kb C+B *Hin*dIII fragment of rDNA (Figure 1B). By 8 h, both the transcribed (Figure 4A) and the nontranscribed (Figure 4B) rDNA strands showed significant CPD removal. The entire experiment was repeated twice using HT1080 cells. Consistent results were obtained for all three experiments: both rDNA strands were repaired at the same rate and to the same extent (Table II). We also tested whether gross changes in the rDNA transcription level affected the repair of rDNA. Growth arrest decreases the demand for rDNA; Tower and Sollner-Webb (1987) and Conconi et al. (1989) demonstrated that the cellular rDNA transcription level is concomitantly decreased in the systems they examined, cultured mouse cells. We analyzed the repair of the C+B *Hin*dIII rDNA fragment (Figure 1B) in proliferating and growth-arrested primary human GM38 cells following 20 J/m^2 UV (Table II). The repair of rDNA in proliferating GM38 cells was the same for both strands and was very similar to that observed in HT1080 cells. Growth-arrested cells showed essentially the same results. Taken together, these results demonstrated that while CPDs in rDNA were repaired to some degree in human cells, pol I transcription did not appear to influence repair. We also probed the pol II-transcribed DHFR gene but did not get reliable results due

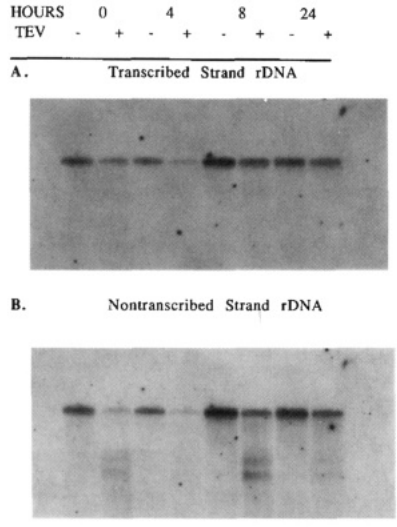

FIGURE 4: Strand-specific repair analysis of human HT1080 rDNA. Exponentially growing HT1080 cells were irradiated with 20 J/m^2 UV. DNA was isolated 0, 4, 8, and 24 h post-UV, restricted with *Hin*dIII, treated (+) or not treated (–) with TEV, fractionated on alkaline agarose gels, and transferred to nylon membranes. Autoradiography detected hybridization of strand-specific ^{32}P-labeled RNA probes made from pZH9 with the 14-kb *Hin*dIII fragment.

Table II: Repair in the Human *Hin*dIII C + B rDNA Fragment[a]

strand probed	time (h)	% CPD removed[b] proliferating HT1080 cells	proliferating GM38 cells	growth-arrested GM38 cells
transcribed	4	5 ± 6	14 ± 7	6 ± 8
	8	27 ± 17	23 ± 17	30 ± 16
	24	58 ± 4	50 ± 9	47 ± 18
nontranscribed	4	5 ± 6	10[c]	1 ± 0
	8	29 ± 1	16 ± 15	27 ± 13
	24	64 ± 13	52 ± 11	57[c]

[a] See restriction map (Figure 1B). [b] Data are from two experiments (GM38) or three (HT1080), all using 20 J/m^2, and are expressed as the average ± the standard deviation. [c] Only one determination.

to the weak signal for this single-copy gene in these cells. However, preferential repair of CPDs in both HT1080 and GM38 has been demonstrated previously. Bohr et al. (1986) found efficient repair of the DHFR gene in GM38 cells, Kantor et al. (1990) found similar results in the β-actin gene in the same cells, and similar results have also been observed in the DHFR gene in HT1080 cells.[2] Combining these results with ours for rDNA, we conclude that in human cells, as in CHO cells, the repair of CPDs is considerably faster in genes transcribed by pol II than in rDNA.

The finding of some repair in rDNA, but with no strand specificity and with different kinetics than previously observed in pol II-transcribed genes, prompted us to examine the repair of other parts of the human rDNA repeat. The membranes from the HT1080 experiments were hybridized with strand-specific probes made from pZH8 to detect the *Hin*dIII A+D fragment (Figure 1B). This restriction fragment showed repair rates and levels, as well as CPD frequencies, similar to those of its upstream neighbor (data not shown). Because more than half of each of the *Hin*dIII fragments is not transcribed, the possibility existed that the heterogeneity of transcription masked transcription-coupled repair. We tested this possibility by using double-stranded probes to analyze the repair of the four *Hin*dIII–*Eco*RI fragments (Figure 1B) in proliferating GM38 cells irradiated with 20 J/m^2 UV (Table III). Again,

[2] R. Hsu, C. Kane, and P. C. Hanawalt, unpublished results.

Table III: Repair of *Hin*dIII–*Eco*RI rDNA Fragments in Proliferating Human GM38 Cells (Double-Stranded Probes)

fragment[a]	time (h)	% CPD removed[b]
C	8	9
	24	53
B	8	0
	24	67
A	8	0
	24	23
D	8	18
	24	67

[a] See Figure 1B for fragment identification. [b] All data are from one experiment using 20 J/m^2 UV.

no obvious differences were seen, either among these small fragments or between the small fragments and the larger *Hin*dIII fragments. We did observe less repair in fragment A than in the rest, but the low CPD frequency (Figure 2) and the small fragment size made quantitation difficult.

DISCUSSION

We examined the repair of CPDs in the ribosomal RNA genes of cultured hamster and human cells. Although many aspects of rDNA are well understood, very little is known about the repair of these genes. We considered the possibility that the differences between rDNA and pol II-transcribed genes could influence repair. Transcription by pol I, the natural amplification of rDNA, its high rate of transcription, and its localization within the nucleolus are some of the factors that could cause the excision repair system to treat rDNA differently from pol II-transcribed genes or from the genome overall. We found no evidence for transcription-coupled repair of rDNA: repair was the same for both strands, in transcribed and nontranscribed restriction fragments, and in cells with greatly different rDNA transcriptional levels. Not only did rDNA lack transcription-coupled repair, but it appeared that both strands were repaired even more slowly than the genome overall.

The high G+C content of rDNA (Lewin, 1980) and a previous report, published only as an abstract for a conference, of 3–4-fold lower induction of CPD compared to cellular DNA (Rajagopalan et al., 1984) led us to expect a very low CPD frequency in rDNA. However, CHO cells showed similar CPD frequencies in both strands of the DHFR gene and the nontranscribed rDNA strand, and only 30% fewer CPDs in the transcribed rDNA strand (Table I). Human cells showed a similar strand bias in CPD induction in rDNA (Figure 2). The nucleotide sequence and the high concentration of transcription-associated proteins in rDNA are two factors that might cause differential CPD induction in the two strands of a gene. Two findings render unlikely the possibility that transcription-associated proteins shielded the transcribed rDNA strand from UV damage. First, we observed the same CPD frequency, with the same strand bias, in proliferating (high rDNA transcriptional activity) and growth-arrested (low activity) GM38 cells (Table II). Second, removal of chromatin-associated proteins by phenol extraction before UV irradiation did not significantly affect CPD induction (Figure 2). We conclude that the rDNA sequence accounts for the different CPD frequencies in the two strands. This conclusion is supported by our rough sequence analysis using the DNA Strider 1.0 program. The most likely sites of CPD induction, i.e., TT, TC, and CT (Gordon & Haseltine, 1982), are found 15% less frequently in the transcribed strand than in the nontranscribed strand of the human rDNA repeat (data not shown). The sequence also accounts for the slightly lower

CPD frequency in the transcribed portions of the repeat relative to the nontranscribed spacers (Figure 2). While the rRNA-encoding DNA is about 50% G+C, the transcribed spacers are about 80% G+C, averaging 62% G+C for the transcription unit (Gonzalez et al., 1990).

It is well established that Chinese hamster cells in culture remove CPDs from the overall genome at a very low level: e.g., Bohr et al. (1985) found that only about 15% of the TEV-sensitive sites were removed in 24 h after 5 J/m^2 UV, and Regan et al. (1990) found about 30% repair in 24 h after 15 J/m^2. Most CPD removal at early times post-UV seems to result from the repair of transcribed strands of active genes. In addition, survival after UV irradiation correlates well with the preferential repair of CPDs in the transcribed strand of expressed genes (Mellon et al., 1987; Lommel & Hanawalt, 1991). In contrast, we found no evidence of a relationship between repair and transcription in rDNA. We measured no repair of rDNA in CHO cells in 24 h after 10 or 20 J/m^2 UV, whereas the same experiments showed proficient repair of the DHFR gene. We propose that rDNA is treated like bulk DNA by the excision repair system. Vos and Wauthier (1991) reported similar findings for the repair of the psoralen monoadduct, another bulky lesion. Apparently the efficient repair of a large fraction of rDNA in CHO cells is not important for cell survival.

While cultured human cells display the transcription-coupled removal of CPDs from pol II-transcribed genes, they also have a slower, transcription-independent, repair mechanism that acts on the genome overall [for reviews, see Smith and Mellon (1990) and Terleth et al. (1991)]. Regan et al. (1978) reported that 50–55% of the CPDs induced by 20 J/m^2 UV were removed from human cells in 24 h; Mellon et al. (1986) found 69% removal in 24 h after 10 J/m^2. We found repair levels of 50–60% in human rDNA after 24 h (Table II), an extent similar to that of the genome overall. The strong relationship between the transcription and the repair of pol II-transcribed genes was not observed for pol I-transcribed genes: strand-specific repair was absent in rDNA, decreasing the rDNA transcription level did not affect repair, and repair was similar in transcribed and nontranscribed rDNA restriction fragments. These results are consistent with the idea that rDNA is repaired by the transcription-independent genomic repair system.

In *E. coli*, which also exhibits transcription-coupled repair (Mellon & Hanawalt, 1989), a transcription-repair coupling factor has been identified and shown to be the product of the *mfd* gene (Selby et al., 1991). Our results suggest that the mammalian counterpart of the transcription-coupling factor either does not interact with pol I or cannot access pol I in the nucleolus. A precedent for coupling factor/polymerase specificity exists: *E. coli*, but not T7, RNA polymerase directs transcription-coupled repair in the presence of the coupling factor (Selby & Sancar, 1993).

While the repair of rDNA in human cells is as extensive as that of the genome overall, the kinetics are different. The repair of rDNA is slower than that of the genome overall, suggesting that one or more factors are impeding the transcription-independent repair system in rDNA at early times after UV. The nucleolus may limit access of repair proteins to rDNA or may otherwise be a poor environment for repair reactions. Alternatively, the high level of pol I transcription in active repeats could actually inhibit repair. Trailing polymerases may stack up behind a polymerase blocked at a lesion, thereby preventing any backward slipping of the blocked polymerase and freezing it over the site of damage. In this way, the blocked polymerase could shield the lesion from repair enzymes. There is evidence that reverse polymerase movement is necessary for traversing transcriptional pause sites (Reines et al., 1992), but less is known about what happens when an RNA polymerase encounters a lesion, so this argument is speculative. However, this explanation for reduced repair of rDNA would suggest that repair of rDNA in growth-arrested cells should be more efficient than that in proliferating cells, whereas we found no difference (Table II).

We were concerned that the transcriptional heterogeneity of rDNA would confound our analysis. The *Pst*I fragment for hamster cells is composed of only about 50% transcribed sequence (Figure 1A), as is the human *Hin*dIII C+B fragment (Figure 1B). Additionally, even in rapidly growing tissue culture cells, only about half of the rDNA repeats are transcriptionally active (Haaf et al., 1991). Thus, only about 25% of the "transcribed strand" of the restriction fragments examined was actually transcribed. Nevertheless, we suspect that if a strong relationship between transcription and repair existed for hamster rDNA, we would have detected at least some repair. We addressed this concern directly in human cells in two ways. First, we examined the repair of rDNA in growth-arrested cells, in which the rDNA transcription rate has been shown to be reduced severalfold (Tower & Sollner-Webb, 1987; Conconi et al., 1989). Despite the much lower rDNA transcriptional level in growth-arrested cells, the repair of rDNA was essentially identical to that in proliferating cells. Second, we examined the repair of small rDNA restriction fragments, each of which is entirely either transcribed or not transcribed. Although the small size of these fragments made accurate quantitation difficult, results showed no obvious differences in the repair of the different fragments. Thus, with the system used, we were unable to detect any influence of transcription on the repair of rDNA.

Our finding of low repair of rDNA in human cells and no detectable repair in CHO cells poses a question: How is damage circumvented and the coding sequence conserved? One possible answer is that the repair of rDNA is localized in the small, highly conserved regions of the rDNA repeat, i.e., the 18S gene and portions of the 28S gene [see Gonzalez et al. (1990) and references cited therein]. The method we used would not have detected such highly localized repair unless it were confined to one DNA strand. A second possible explanation is that the damage is not circumvented. Rather, the redundancy of rDNA may be sufficient in most cases to provide enough undamaged templates for survival and thus allow tolerance of persisting damage. If this is true, somehow the undamaged templates must be selected and probably amplified for the next generation of the organism. Maybe efficient repair of rDNA does occur in the germline. In this respect, it is important to appreciate that the cell may not be a good model for the survival of the organism and the species. A third, and probable, explanation for our finding of low repair of rDNA is that unrepaired or mutated sequences might be removed from use via recombination. The high copy number of rDNA facilitates recombination. It has been speculated that a sequence in yeast rDNA (*HOT1*), which promotes recombination, might help maintain rDNA homogeneity (Voelkel-Meiman et al., 1987). Furthermore, theoretical modeling studies suggest that high copy number genes resist change because gene conversion is biased against mutant types (Birky & Walsh, 1992). Any or all of these mechanisms, due to the repetitive nature of rDNA, could lessen the need for repair. A final consideration is that there is very strong selection pressure acting on the primary sequence. This would

not be surprising given that rRNA has an active role in translation and may even be the catalytic component of the ribosome (Noller et al., 1992).

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