We examined repair of DNA strand breaks induced by the anti-cancer drug bleomycin in both Pol I and Pol II transcribed genes in permeabilized human fibroblasts. The majority of these breaks (>80%) are single strand breaks (SSBs) thought to be repaired by base excision repair enzymes. Repair was examined in each strand of a 7.2-kilobase fragment, completely within the Pol I transcribed region of ribosomal DNA (rDNA) and an 8.3-kilobase fragment completely within the Pol II transcribed region of the dihydrofolate reductase (DHFR) gene. Bleomycin dose-response studies revealed no bias for SSBs in either strand of the rDNA fragment. Furthermore, repair of SSBs is rapid (~80% resealed in 60 min in both the transcribed and nontranscribed strands of rDNA. Rapid repair of SSBs is also observed in both strands of the DHFR gene (~60% resealed in 60 min). In contrast, little (or no) repair of UV photodimers occurs in either strand of human rDNA, regardless of whether cells are confluent or actively growing. Thus, DNA lesions in human ribosomal genes may be more accessible to base excision repair enzymes than those involved in nucleotide excision repair.

It has been known for over 2 decades that two forms of DNA excision repair synthesis exist in human cells (Regan and Setlow, 1974). “Long patch” repair, induced by UV photodimers and bulky chemical adducts, involves removal and insertion of 20–30 nucleotides at the damage site (Th'ng and Walker, 1985; Dresler, 1985; Smith, 1987; DiGiuseppe and Dresler, 1989; DiGiuseppe et al., 1990; Sidik and Smerdon, 1990a), and human cell extracts contain an activity that cleaves on each side of a cyclobutane pyrimidine dimer (CPD) in positions 27–29 nucleotides apart (Huang et al., 1992). In contrast, “short patch” repair, induced by nonligatable strand breaks and some alkylated bases, involves removal and insertion of only a few nucleotides per DNA lesion (Regan and Setlow, 1974; DiGiuseppe and Dresler, 1989; DiGiuseppe et al., 1990; Sidik and Smerdon, 1990a).

Bleomycin is a radiomimetic antitumor agent that induces (primarily) strand breaks in chromosomal DNA (reviewed by Stubble and Kozarich, 1987) and Povirk and Finley Austen (1991); also see Bennett et al. (1993)). Unlike bulky DNA lesions, repair of these breaks is resistant to aphidicolin, and the average repair patch size is less than 6 nucleotides (DiGiuseppe and Dresler, 1989; DiGiuseppe et al., 1990; Sidik and Smerdon, 1990a). Bleomycin is a glycopeptidase that complexes with divalent cations (mainly Fe2+) and O2, and a portion of the molecule intercalates in DNA, particularly at NGC sequences (Kuwahara and Sugihara, 1988). It produces strand breaks or apurinic/apyrimidinic sites by abstracting hydrogen from C-4' of deoxyribose adjoining the guanidyl-3'-phosphate at the site of bleomycin-DNA intercalation (Stubble and Kozarich, 1987; Povirk and Finley Austen, 1991). At this point, the resulting free radical can partition into one of two alternate damage pathways. Upon addition of O2, a peroxy radical is formed, which decomposes to yield a strand break with 5'-phosphate and 3'-phosphoglycolate termini, with release of a base propenal. Alternatively, hydroxylation of the C-4' radical can occur opening the sugar ring and releasing the base. This pathway yields an apurinic-apyrimidinic site with a chemically modified sugar. In chromatin, however, many of these modified apurinic-apyrimidinic sites undergo spontaneous cleavage, probably by reaction with histone amine groups (Bennett et al., 1993). These strand breaks cannot be ligated directly and may be repaired via removal of the modified base (and a few adjacent bases) by a 3'→5' exonuclease and (short patch) repair synthesis (e.g., see Mosbaugh and Linn (1984)).

We analyzed repair of bleomycin-induced single strand breaks (SSBs) in the individual strands of Pol I-transcribed ribosomal genes (rDNA) and the Pol II-transcribed DHFR gene. Repair in the DHFR gene has been well characterized, and both preferential and strand-specific repair of UV-induced cyclobutane pyrimidine dimers (CPDs) occurs in this gene (reviewed in Friedberg et al., 1995). In contrast, repair of CPDs in either total rDNA of human and hamster cells (Christians and Hanawalt, 1993) or the transcriptionally active fraction of rDNA in mouse cells (Fritz and Smerdon, 1995) is very inefficient in both DNA strands. We report here that, in contrast to very inefficient repair of CPDs in rDNA, repair of bleomycin-induced SSBs occurs rapidly in both strands of a human 7.2-kb rDNA fragment and an 8.3-kb fragment in the DHFR gene (Fig. 1).

 MATERIALS AND METHODS

Chemicals—Bleomycin (Blenoxane) was a generous gift from Bristol Myers Laboratories (Syracuse, New York). L-α-Lysophosphatidylcholine (LPC) was purchased from Sigma.

Cell Culture—Human diploid fibroblasts (strain AG 1518) were split 1:3 and grown in culture as described previously (Smerdon et al., 1982). Cells were prelabelled with 5 nCi/ml [3H]deoxythymidine (50 Ci/mmol; DuPont NEN) for 1 week after splitting. Prelabeling medium was replaced with fresh medium, and the cells were grown an additional 2–3 weeks until confluent. The medium was changed every 8 days during this period. To obtain actively growing cells used in some experiments, cells were split 1:4 and harvested 3 days after splitting.
Briefly, cells were washed twice with ice-cold PBS (16 mM phosphate buffer, 7.2, 5 mM KCl, 135 mM NaCl). An 80 \mu M solution of LPC (dissolved in PBS and 1 mM CaCl2) was added to the cells, which were then kept on ice for 2 min. The effectiveness of cell permeabilization was determined in separate plates by the fraction of cells taking up trypan blue dye (Lorenz et al., 1988). The LPC solution was then carefully removed and replaced with repair mixture (35 mM HEPES, pH 7.4, 50 mM sucrose, 80 mM KCl, 5 mM MgCl2, 7.5 mM KH2PO4, 1 mM CaCl2, 5 mM ATP; see Sidik and Smerdon (1990a, 1990b)).

Bleomycin treatment was performed according to Sidik and Smerdon (1990a, 1990b). For repair experiments, cells were treated in three groups. Group 1 cells (no bleomycin) were made permeable by LPC treatment followed by addition of repair mixture containing 3 \mu M dNTPs. The cells were washed and harvested after a 30-min incubation at 37°C without bleomycin. Group 2 cells (damage, no repair) were made permeable by LPC treatment followed by addition of repair mixture containing 3 \mu M dNTPs. Then the cells were incubated for 30 min. Following this incubation, the bleomycin concentration was increased in the presence of dNTPs and various concentrations of bleomycin dissolved in 10 mM PIPES buffer. The cells were then harvested immediately, and DNA was prepared and separated on a 0.8% alkaline agarose gel (A) and 0.8% native agarose gel (B). The average strand breaks/fragment was determined using the Poisson expression \([-\ln(1-P)]\) and nontranscribed strand (○). Different symbols are for different experiments.

RESULTS

Bleomycin Dose-Response of rDNA—For many of these experiments, confluent human fibroblasts were reversibly permeabilized with LPC to improve transport of bleomycin into cells (Sidik and Smerdon, 1990b). Using this treatment, bleomycin dose-response measurements were performed to determine the level of strand breaks produced in the 7.2-kb EcoRI rDNA fragment. Total strand breaks were assayed by alkaline agarose gel electrophoresis (Fig. 2A, inset). As shown in Fig. 2, at bleomycin doses >1 \mu M, there is an approximately linear relationship between strand breaks in the rDNA fragment and bleomycin concentration. There is also a significant number of double strand breaks, or breaks on opposite strands.
in close proximity, at bleomycin doses >1 μg/ml, as measured by neutral gel electrophoresis (Fig. 2B, inset). These breaks account for <20% of the total breaks induced by bleomycin in the rDNA fragment (analysis not shown). Furthermore, there is no bias for bleomycin-induced strand breaks in either strand of this fragment (Fig. 2). A dose of 3 μg/ml was used for most repair experiments where 90–95% of the DNA fragments analyzed contain at least one single strand break.

Repair of Strand Breaks in the Ribosomal and DHFR Genes—For most repair experiments, permeabilized, confluent human fibroblasts were exposed to 3 μg/ml bleomycin. At this dose, the 7.2-kb rDNA fragment (Fig. 1A) contained 1.8–2.3 strand breaks in each strand, while the 8.3-kb DHFR fragment (Fig. 1B) contained 2.4–2.6 strand breaks in each strand. This dose was chosen to illustrate the dramatic change in strand breaks after even short repair times (see below). Also, as observed for the rDNA fragment, no bias was observed for strand breaks in either strand of the DHFR fragment (not shown).

Repair of bleomycin-induced strand breaks in both rDNA and DHFR displayed initial rapid kinetics (Fig. 3). This is illustrated by the rapid return of intact rDNA fragments after only 40 min of repair incubation (Fig. 3A, inset). At least 80% of the strand breaks are repaired in each strand of these genes after only 90 min (Fig. 3). This value approaches 100% after 24 h of repair incubation (data not shown). The “plateau” in repair data at about 80% for rDNA (Fig. 3A) most likely reflects a much slower repair of double strand breaks (Coquerelle et al., 1987), present at <20% the level of SSBs in rDNA (above). However, regardless of the reason for the plateau in repair kinetics, it is clear that most strand breaks are very rapidly repaired during the first hour in ribosomal genes.

It is important to note that the results in Fig. 3 represent repair in total ribosomal genes, which are present in several hundred copies in human cells (Long and Dawid, 1980). Using psoralen cross-linking to separate the transcriptionally active from the inactive fraction, we find that <30% of rDNA genes are in the active chromatin fraction in these cells (data not shown; see Fritz and Smerdon, 1995). Therefore, the results in Fig. 3 indicate that efficient repair occurs in each strand of both the active and inactive rDNA fractions.

Repair of UV-induced Damage in Human Ribosomal Genes—It has been established that rDNA transcription is greatly reduced in stationary cells (Sollner-Webb, 1986; Conconi et al., 1989). Therefore, we irradiated both confluent and actively growing human fibroblasts with 20 J/m² UV light (254 nm), and the newly replicated DNA in growing cells was separated from parental DNA using isopycnic centrifugation (see “Materials and Methods”). The yield of CPDs in each strand of the 7.2-kb rDNA fragment was measured using the method of Bohr et al. (1985). This assay measured the fraction of restriction fragments resistant to cutting by T4 endonuclease V (T4 endo V), which cleaves DNA strands specifically at CPD sites. As shown in Fig. 4, A and B, little change occurs in the fraction of T4 endo V-resistant rDNA fragment after 8 h of repair incubation, and there is only a small increase in this band after 24 h of repair. Indeed, quantitation of these results indicates that only 20–30% of the CPDs are removed from each strand after 24 h (Fig. 4, C and D), and repair was inefficient during each growth state. Thus, as previously found for rodent and human cells (Christians and Hanawalt, 1993; Fritz and Smerdon, 1995), repair of CPDs is inefficient in each strand of the ribosomal genes of human fibroblasts.

DISCUSSION

Our results indicate that bleomycin-induced SSBs in rDNA are repaired efficiently, but not in a strand-specific manner (Fig. 3). On the other hand, repair of UV-induced CPDs is very inefficient in each strand of rDNA in both growing and confluent human fibroblasts (Fig. 4). This latter observation agrees qualitatively with past studies on repair of total rDNA in human and hamster cells (Christians and Hanawalt, 1993, 1994). Recently, we have shown that inefficient repair of CPDs takes place even in the transcribed strand of the transcriptionally active fraction of ribosomal genes (Fritz and Smerdon, 1995). Thus, the rapid and extensive repair of bleomycin-induced SSBs is dramatically different from the repair of UV photodimers in rDNA.

The differences we observe between the kinetics of repair of SSBs induced by bleomycin and CPDs induced by UV light are striking. Stevnsner et al. (1993) also observed differences in repair efficiency in rDNA damaged with various agents in CHO cells. These authors reported inefficient repair of CPDs, and both monoadducts and diadducts induced by nitrogen mustard.

![Figure 3. Repair of bleomycin-induced strand breaks in rDNA and the DHFR gene of human cells.](image)
UV-induced CPDs and bleomycin-induced strand breaks are (presumably) repaired by different repair pathways (e.g., see Friedberg et al. (1995)). DNA polymerase inhibitor studies strongly support the involvement of polymerase $\beta$ (independent of polymerases $\alpha$ and $\delta/e$) in the repair of bleomycin-induced damage (DiGiuseppe and Dresler, 1989; DiGiuseppe et al., 1990; Sidik and Smerdon, 1990a). The nucleolus, where rDNA sequences are sequestered, could act as a barrier to the bulky enzyme complex associated with nucleotide excision repair (Friedberg et al., 1995), but allow access to the 39-kDa polymerase $\beta$ protein (Abbotts et al., 1988; Wilson et al. 1988). (Obviously, polymerases $\alpha$ and $\delta/e$ have access to these regions during S-phase; however, these cells represent a small fraction of the total population even in exponentially growing cultures.) Furthermore, because rDNA sequences are reiterated and quite highly conserved (Gonzales et al., 1990), one can speculate that recombinational repair (rather than nucleotide excision repair) of UV-induced CPDs (and perhaps other bulky DNA lesions) would offer a selective advantage in maintaining sequence integrity among the rDNA gene copies (see Christians and Hanawalt (1994)), perhaps involving a different mechanism than used in nonreiterated sequences (Ozenberger and Roeder, 1991).

As a control for our studies, we examined repair in the DHFR gene, which is transcribed by RNA Pol II. As stated earlier, transcription repair coupling appears to require an elongating Pol II complex (Leadon and Lawrence, 1991; Christians and Hanawalt, 1992), and we were concerned that DHFR might not be actively transcribed in confluent cells. However, Northern analyses with these human fibroblasts showed that equivalent amounts of DHFR mRNA are present in actively growing cells and in confluent cells in deep confluence (Fritz, 1994). Although these studies were not performed on permeabilized cells, the fact that these cells "recuperate" rapidly from LPC permeabilization (Lorenz et al., 1988) and go on to divide normally indicates that the DHFR gene is indeed transcribed following permeabilization. Furthermore, Leys and Kellems (1981) concluded that the relative rates of DHFR transcription in growing and resting cells is the same. We note that these authors observed a small increase of DHFR mRNA levels in growing cells, after long times (30 h) of growth stimulation, and concluded this increase is due to increased stability of the DHFR message in growing cells (Leys and Kellems, 1981). In addition, Venema et al. (1990) reported transcription-coupled repair of CPDs in the DHFR gene in confluent human fibroblasts, which (indirectly) suggests that transcription of DHFR occurs in confluent cells.

Finally, a previous study reported efficient repair of bleomycin-induced strand breaks in an amplified c-myc gene in human cells (Bianchi et al., 1990). However, as double strand probes were used in that study, it could not be determined if this gene from newly replicated DNA (see "Materials and Methods"). Parental DNA was treated with (or without) T4 endo V and separated on 0.8% alkaline agarose gels. The samples were transferred to nylon membranes and hybridized to a strand-specific pSP28S riboprobe. Following autoradiography, membranes were stripped and reprobed with the opposite strand. Panels show autoradiograms for the transcribed strand (TS) and nontranscribed strand (NTS) of actively growing cells (A) and confluent cells (B). Lower panels show the results of quantitative analysis of the autoradiograms in A and B. UV-induced CPDs/fragment of the transcribed (●) and nontranscribed (●) strands are shown for actively growing (E) and confluent (C) cells.
was repaired in a strand specific manner. Furthermore, Leadon and Cooper (1993) found more efficient repair of ionizing radiation-induced lesions in the transcribed strand of the active metallothionein IIA gene in both normal and xeroderma pigmentosum (complementation group A) human fibroblasts, while Cockayne’s syndrome (group B) cells yielded no strand specific repair of this gene. Since bleomycin is considered a radiomimetic agent (Povirk and Finley Austin (1991); although see Affentranger and Burkart (1995)), this report suggests that more efficient repair of bleomycin-induced strand breaks may also occur in the transcribed strand of DHFR. Although we observed only a slight difference between the repair efficiency of the transcribed and nontranscribed strands of the DHFR gene (Fig. 3), this distinction could easily be missed due to the rapid repair kinetics in each strand.

Acknowledgments—We thank Sylvia Hering for assistance with cell culture. Drs. José Sogo and Antonio Conconi for providing plasmid pSP72BS, Drs. C. Allen Smith, Lori Lommed, and Phillip Hanawalt for providing plasmids p2H-15 and p2H111, and Dr. R. Stephen Lloyd for providing purified T4 endo V. We also thank Drs. Antonio Conconi, David Springer, Jose Sogo, and Fritz Thoma for their critical reading of this manuscript.

REFERENCES
Abbotts, J., SenGupta, D. N., Zmudzka, B., Widen, S. G., Notario, V., and Wilson, S. H. (1988) Biochemistry 27, 901–909
Affentranger, M. I., and Burkard, W. (1995) J. Histochem. Cytochem. 43, 229–235
Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1988) Current Protocols in Molecular Biology, John Wiley and Sons, New York
Bennett, R. A. O., Swedlow, P. S., and Povirk, L. F. (1993) Biochemistry 32, 3188–3195
Bianchi, N. O., Bianchi, M. S., Lopez-Larraza, D., Alitalo, D., and de la Chapelle, A. (1996) Cancer Res. 56, 2379–2384
Bohr, V. A., and Okumoto, D. S. (1988) in DNA Repair: A Laboratory Manual of Research Procedures (Friedberg, E. C., and Hanawalt, P. C., eds.) pp. 347–366, Marcel Dekker, Inc., New York
Bohr, V. A., Smith, C. A., Okumoto, D. S., and Hanawalt, P. C. (1985) Cell 40, 359–369
Christians, F. C., and Hanawalt, P. C. (1992) Mutat. Res. 274, 93–101
Christians, F. C., and Hanawalt, P. C. (1993) Biochemistry 32, 10512–10520
Christians, F. C., and Hanawalt, P. C. (1994) Mutat. Res. 323, 179–187
Conconi, A., Widmer, R. M., Koller, T., and Sogo, J. M. (1989) Cell 57, 753–761
Coquerelle, T. M., Webezahn, K. F., and Lukce Huhle, C. (1987) Int. J. Radiat. Biol. 51, 209–218
DiGiuseppe, J. A., and Dresler, P. C. (1989) Biochemistry 28, 9515–9520
DiGiuseppe, J. A., Hunting, D. J., and Dresler, P. C. (1990) Carcinogenesis 11, 1021–1026
Dresler, P. C. (1985) Biochemistry 24, 6861–6869
Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, American Society for Microbiology Press, Washington, D. C.
Fritz, L. K. (1994) Differential DNA Repair in Mammalian Ribosomal Genes. Ph.D. thesis, Washington State University, Pullman, WA
Fritz, L. K., and Smerdon, M. J. (1995) Biochemistry 34, 13117–13124
Gonzales, I. L., Chambers, C., Gorski, J. L., Stambollian, D., Schmickel, R. D., and Sylvester, J. E. (1990) J. Mol. Biol. 212, 27–35
Huang, J. C., Svoboda, D. L. Reardon, J. T., and Sancar, A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3664–3668
Kuwahara, J., and Sugiura, Y. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2459–2463
Leadon, S. A., and Cooper, P. K. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10499–10503
Leadon, S. A., and Lawrence, D. A. (1991) Mutat. Res. 255, 67–78
Leys, E. O., and Dawid, I. B. (1980) Annu. Rev. Biochem. 49, 727–764
Lorenz, J. D., Watkins, J. F., and Smerdon, M. J. (1988) Mutat. Res. 193, 167–179
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Mosbaugh, D. W., and Linn, S. (1984) J. Biol. Chem. 259, 10247–10251
Murad, A. O., de Cock, J., Brown, D. W., and Smerdon, M. J. (1995) J. Biol. Chem. 270, 9494–9507
Ozenberger, B. A., and Roeder, G. S. (1991) Mol. Cell. Biol. 11, 1222–1231
Povirk, L. F., and Finley Austin, M. J. (1991) Mutat. Res. 257, 127–143
Regan, J. D., and Setlow, R. B. (1974) Cancer Res. 34, 3318–3325
Sidik, K., and Smerdon, M. J. (1990a) Biochemistry 29, 7501–7511
Sidik, K., and Smerdon, M. J. (1990b) Cancer Res. 50, 1613–1619
Smerdon, M. J., Lan, S. Y., Calza, R. E., and Reeves, R. (1982) J. Biol. Chem. 257, 13441–13447
Smith, C. A. (1987) J. Cell Sci. Suppl. 6, 225–241
Solinr-Webb, B., and Tower, J. (1986) Annu. Rev. Biochem. 55, 801–830
Stevnsner, T., May, A., Petersen, L. N., Larminat, F., Pirsel, M., and Bohr, V. A. (1993) Carcinogenesis 14, 1591–1596
Stubbe, J., and Kozarich, J. W. (1987) Chem. Rev. 87, 1107–1136
Thëng, J. P. H., and Walker, I. G. (1985) Mutat. Res. 165, 139–150
Venema, J., van Huffen, A., Natarajan, A. T., van Zeeland, A. A., and Mullenders, L. H. F. (1990) Nucleic Acids Res. 18, 443–444
Wauthier, E. L., Hanawalt, P. C., and Vos, J. M.-H. (1990) J. Cell. Biochem. 43, 173–183
Wilson, S. H., Abbotts, J., and Widen, S. (1988) Biochim. Biophys. Acta 949, 149–157
