Formation and Repair of Antitumor Antibiotic CC-1065-Induced DNA Adducts in the Adenine Phosphoribosyltransferase and Amplified Dihydrofolate Reductase Genes of Chinese Hamster Ovary Cells†

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ABSTRACT: CC-1065 is a potent antitumor antibiotic which bonds to duplex DNA specifically; the biological effects of the drug are presumably the consequences of its DNA interactions. In order to investigate the factors which may affect drug–DNA bonding in cells, a method using a thermal–alkaline treatment to induce phosphodiester bond breakage at the drug–DNA bonding sites and Southern DNA transfer–hybridization to quantify drug–DNA bonding at defined sequences in drug-treated cultured mammalian cells was developed. We have found that in vivo, in cultured Chinese hamster ovary (CHO) cells, CC-1065 bonds twice as efficiently in the highly amplified dihydrofolate reductase (DHFR) gene domains as in the nonamplified adenine phosphoribosyltransferase (APRT) gene domain. However, in vitro, in purified CHO cellular DNA, CC-1065 bonds equally to both the DHFR and APRT genes. We observed a significant degree of "gene-specific" preferential repair for drug–DNA adducts in the amplified DHFR gene domains, and it appears that this "gene-specific" repair reflects "transcribed-strand specific" repair. These results suggest that DNA amplification may affect drug–DNA adduct formation and transcription may affect its repair.

CC-1065 is an extremely potent antitumor antibiotic produced by *Streptomyces zelensis* (Hanka et al., 1978). This drug has been shown to be active against several experimental murine tumors in vivo (Neil et al., 1981) and about 100 times more potent than adriamycin against a broad spectrum of human tumors in cloning assays (Bhuyan et al., 1982). Structurally, CC-1065 consists of three repeated pyrroloindole subunits joined by amide bridges, resulting in a right-hand twisted, banana-shaped drug molecule (Figure 1). Only the exocyclic carbon atom of the cyclopropane ring of CC-1065 is able to form a covalent bond with DNA. Bonding occurs only at N3 of the adenine in duplex DNA, and only within certain sequence contexts. Molecular modeling shows that the CCC-1065–N3-adenine adduct lies within the minor groove of DNA, covering 3 bp 5′ and 1 bp 3′ of the modified adenine (Reynolds et al., 1986; Hurley et al., 1984).

Since CC-1065 selectively bonds only to DNA, all the biological effects of this drug may be attributed to its DNA bonding. The antitumor activity of this drug may reflect more efficient drug bonding to tumor cell DNA in general or to specific genes or regions in chromosomes which are critical for cell survival. A number of factors have been shown to affect chemical carcinogen–DNA alkylation; these include nucleosomal structure, nuclear matrix association, and the transcriptional activity of a gene [for a review, see Bohr et al. (1987)]. These factors may also affect drug–DNA adduction. Ample evidence has demonstrated that tumor and cancer cells may have different patterns of gene expression, especially oncogene expression, from normal cells (Erisman et al., 1965; Lawson et al., 1988; Leavitt & Kakunaga, 1980; Franza & Garrels, 1984; Bravo & Celis, 1980; Guillem et al., 1988; Slamon et al., 1984). Chromosome aberrations, including

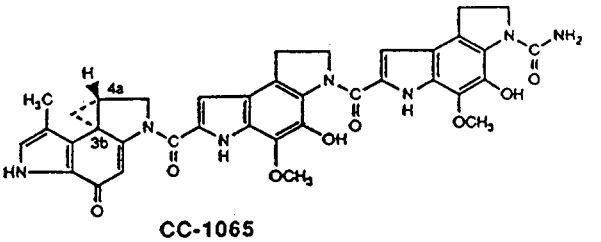

FIGURE 1: Chemical structure of CC-1065.

gene amplification and double-minute chromosomes, often occur in cancer and tumor cells, especially those in the stage of tumor progression (Yokota et al., 1986). The question of whether factors such as gene amplification and differential gene expression in tumor cells contribute to the greater susceptibility to CC-1065 is important for understanding the antitumor activity of this drug.

CC-1065–DNA adducts stabilize the DNA helix structure (Reynolds et al., 1986; Hurley et al., 1984); this is in marked contrast to ultraviolet light (UV) or bulky chemical carcinogen-induced DNA damage, which destabilizes the DNA helix structure (Friedberg, 1984). The manner in which DNA adducts are recognized and process by DNA repair systems may play an important role in the expression of drug-induced cytotoxic effects. Certain mechanisms for repairing DNA-stabilizing adducts may produce deleterious effects, such as the induction of DNA double-strand breaks. These effects may in turn contribute to antitumor activity; therefore, it is important to determine the mechanisms for repairing these drug–DNA adducts.

We need a better understanding of the factors which govern drug–DNA adductions in vivo and how fundamental gene activities such as transcription and amplification affect drug–DNA bonding and repair. It has been shown that thermal treatment induces phosphodiester bond breakage at CC-1065–

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DNA bonding sites (Hurley et al., 1984). In this paper, we report the use of a method involving thermal–alkaline treatment coupled with Southern DNA transfer–hybridization to examine the formation and the repair of CC-1065–DNA adducts in the coding and 3′-downstream noncoding regions of amplified DHFR gene domains, in the transcriptionally active APRT gene, and in total genomic DNA.

MATERIALS AND METHODS

Cell Culture and CC-1065 Treatment. The Chinese hamster ovary (CHO) cell line B-11 (Kaufman & Schimke, 1981) was grown in Hams' F-12 medium without glycine, hypoxanthine, and thymidine and supplemented with 10% fetal calf serum and 500 nM methotrexate to maintain the selection pressure for DHFR gene amplification. Fresh confluent cells were split 1 to 16 with fresh medium in 150-mm dishes. When the cells were grown to about 50–70% confluence, the monolayers were washed, and the medium was replaced with 15 mL of DPBS buffer (4.7 mM $MgCl_2$, 8.5 mM $CaCl_2$, 68 mM NaCl, 1.94 mM KCl, 1.07 mM KH_2PO_4, and 6.16 mM Na_2HPO_4, pH 7.4). Fresh stock solutions (14 μM) of CC-1065 in dimethyl sulfoxide were prepared immediately before treatment. Different amounts of CC-1065 were added to cell cultures in DPBS buffer (15 mL), and the cultures were incubated at 37 °C for 30 min. At the end of incubation, the DBPS buffer was removed, and the cells were washed 3 times with DBPS buffer to remove residual unreacted or noncovalently bound CC-1065. To determine the effect of drug concentration on drug–DNA bonding, the treated cells were lysed immediately after washing, and the DNA was isolated. To determine the repair kinetics, cells were incubated immediately after drug treatment in fresh growth medium containing 5-bromo-2′-deoxyuridine (10 μM) and 5-fluorodeoxyuridine (1 μM). After further incubation at 37 °C for 0, 2, 6, and 24 h, the cells were washed 3 times with DPBS buffer, and DNA was isolated.

DNA Isolation. For DNA isolation, cells were washed with 5 mL of DPBS buffer 3 times and lysed by incubation with lysing solution (0.5% SDS, 10 mM Tris, pH 7.8, 10 mM EDTA, and 10 mM NaCl) for 2 min. Although it has been reported that an additional washing of the drug-treated cells with 10% ethanol removes more of the unbonded drug in cultured African green monkey kidney cells (Zsido et al., 1991), we have found this additional treatment does not change the number of drugs bonding to DHFR and APRT genes significantly in CHO cells treated with CC-1065 ranging from 24 to 60 nM. The cell lysates were treated with proteinase K (100 μg/mL) at room temperature overnight. Nucleic acids were isolated after precipitation by the addition of sodium acetate (0.33 M) and 2.5 volumes of 95% ethanol and spooled on a glass rod. The precipitated DNAs were dissolved in 1× TE [10 mM Tris (pH 7.5)/1 mM EDTA] and precipitated by ethanol twice, followed by washing with 95% ethanol 3 times. The final pellet was dissolved in 1× TE. RNA contamination was reduced by treatment with RNase A (0.4 μg/mL) and RNase T1 (0.7 μg/mL) at 37 °C for 2 h. The RNase and residual proteins in the solution were removed by phenol extractions (3 times) followed by diethyl ether extractions (3 times), and the DNAs were ethanol-precipitated. The purified DNAs were then digested with restriction enzyme *Asp718* (1 unit/3 μg of DNA) at 37 °C overnight. The digestions were checked for completion by electrophoresis of samples on an agarose gel. Replicated and nonreplicated DNAs were separated by CsCl gradient centrifugation in the Ti 60.1 rotor, 37 krpm, for 64 h at 21 °C. For determining the relationship between drug concentration and DNA–adduct formation, total DNA isolated from treated cells was used. For determining the kinetics of adduct removal, only nonreplicated DNA was used. This precautionary step is important since a single round of semiconservative DNA replication will result in an apparent dilution of adducts per unit length of DNA by half. Significant levels of semiconservative DNA replication have been found after treatment with most DNA damaging agents (Cohn et al., 1984). We have found that 30% of the DNA isolated from B-11 cells treated with 60 nM CC-1065 replicated once during 24-h posttreatment incubation.

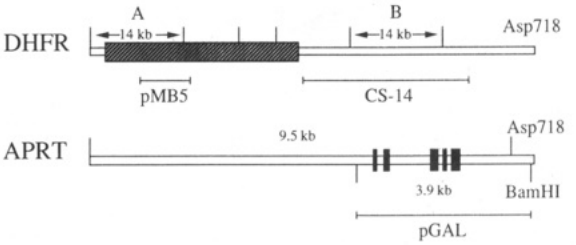

FIGURE 2: Restriction map of DHFR and APRT gene domains of CHO cells and the DNA used for probing.

Modifications of Purified DNA with CC-1065. To determine the sequence effects on drug–DNA adduct formation, DNA (90 μg) isolated from untreated B-11 cells as described above was modified with different amounts of CC-1065 (stock solution at concentration of 560 nM) at a final volume of 1.1 mL of 1× TE at 37 °C for 14 h. At the end of the incubation, the DNA was precipitated with ethanol, and unreacted drug was removed by repeat ethanol precipitation. The method for modifying ^{32}P end-labeled (*Msp*I–*Bst*NI) 117 bp fragments of M13 mp1 was the same as described previously (Tang et al., 1988).

Thermal–Alkaline Treatment. Purified DNAs were dissolved in 1× TE buffer. To induce DNA breakage at the CC-1065–DNA adduct site, 30 μL of concentrated formamide solution was added to 10 μL of DNA solution to reach final concentrations of 10 mM NaOH, 75% formamide, 2.5 mM Tris, and 0.25 mM EDTA, and the mixtures were heated at 90 °C for 20 min in an oil bath. After heat treatment, samples were quenched in an ice bath.

DNA Denaturation and Gel Electrophoresis. In preparation for electrophoresis, the thermal–alkaline-treated DNA (40 μL) was added with 60 μL of fresh deionized formamide solution. To denature DNA samples which were without thermal–alkaline treatment, a 90-μL aliquot of formamide was added to 10 μL of DNA solution, and the mixtures were incubated at 37 °C for 60 min. The DNA samples were then electrophoresed at 5 V/cm for 3 h in a preformed 0.5% agarose horizontal gel in 0.5× TBE buffer (25 mM Tris, pH 7.9, 25 mM borate, and 2.5 mM EDTA) with 0.5 μg/mL ethidium bromide. *Hin*dIII-digested λ DNA was electrophoresed in parallel as size markers. After electrophoresis, the DNA in the gels was depurinated according to the procedure of Maniatis et al. (1989), and denatured and transferred to a Zetabind membrane in 0.5 M NaOH and 0.6 M NaCl solution. The DNA in the membrane was subsequently hybridized with ^{32}P-labeled DNA or strand-specific riboprobes as described by Bohr et al. (1985) and Mellon et al. (1987). The *Asp718* restriction sites of the DHFR and APRT gene domains and the probing plasmid DNAs are shown in Figure 2.

Quantitation. Autoradiographs were scanned in a BioImage Analyzer with a 100 Visage whole-band analysis software program. The intensity of the full-length fragment was

normalized with an internal standard pBR322 band. The number of thermal–alkaline labile sites (TALS) was calculated by Poisson distribution equation:

$$P(0) = e^{-n}$$

where n is the average number of TALS per full-length DNA fragment. The ratio of the normalized intensity of the full-length fragment from the sample with thermal–alkaline treatment versus the sample without treatment is equal to $P(0)$. To quantify the number of TALS in total genomic DNA, the average molecular weight of sample DNAs separated by electrophoresis was calculated based on the DNA molecular standards and the method described previously (Tang & Patrick, 1977). In brief, the center of mass to represent the electrophoresed DNA profile was calculated on the basis of the equation $\rho = \sum_i F_i C_i / \sum_i C_i$, where C_i is the relative intensity of the ith fraction and F_i is the distance from the well to the ith fraction. The average molecular weight was then obtained by comparing ρ with the mobility of standard DNA markers. The number of TALS was then calculated on the basis of the equation TALS = $(M_0/M_t) - 1$, where M_0 and M_t are the average molecular weights of DNA without and with thermal–alkaline treatment, respectively.

RESULTS

Specificity of Thermal–Alkaline Treatment-Induced Strand Breakage in a CC-1065-Modified DNA Fragment. It has been found that CC-1065 covalently bonds at the N3 adenine with sequence selectivity (Hurley et al., 1984). Thermal–alkaline treatment leads to cleavage of the N-glycosidic linkage and subsequent strand breakage of the backbone at the 3′-side of the covalently modified adenine to leave a 5′-phosphate on the 3′-side of the break, and presumably a modified deoxyribose on the 5′-side is released. Thermal treatment has been used to determine the sequence specificity of CC-1065–DNA bonding (Hurley et al., 1984). In order to use this technique for quantification of CC-1065-DNA adducts, we first attempted to establish conditions under which maximal numbers of strand breaks are specifically induced at drug–DNA-adducted sites in a drug-modified DNA fragment, accompanied by minimal nonspecific breakage. 3′-End ^{32}P-labeled 117 bp fragments modified with CC-1065 were subjected to different time periods of thermal–alkaline treatment, and the reacted samples were separated by sequencing gel electrophoresis. The results in Figures 3 and 4 demonstrate that the number of thermal–alkaline-indued strand breaks at drug bonding sites increases as a function of reaction time, plateauing after 20 min, and that the induced strand breakage occurs specifically at CC-1065 bonding sites.

Quantification of CC-1065–DNA Adduct Formation at Defined Sequences in in Vitro-Modified DNA. Since the thermal–alkaline treatment specifically induces strand breakage at CC-1065-modified adenines, this method can be used to detect drug–adduct formation within defined DNA sequences. The approach we have used is similar to one we reported for quantifying aminofluorene–DNA adducts in the DHFR gene (Tang et al., 1989). DNA purified from CHO B-11 cells was modified with different concentrations of CC-1065, and the modified DNA was incubated under thermal–alkaline conditions to induce strand breakage at drug modification sites; this treatment also causes DNA denaturation. The resultant DNAs were then separated by electrophoresis, transferred to a cellulose membrane, and subsequently hybridized with ^{32}P-labeled probes. The number of

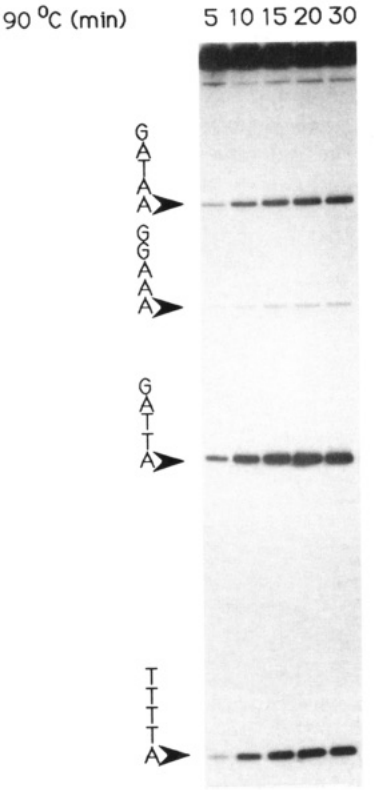

FIGURE 3: Sequence specificity of CC-1065-induced thermal-alkaline labile breakage and the time course of their formation. The 3′-end ^{32}P-labeled *Msp*I–*Bst*NI 117 bp M13mp1 fragments were modified with 28 nM CC-1065 and heated under alkaline conditions as described under Materials and Methods. The resultant DNA was electrophoresed in an 8% sequencing gel. The time periods of thermal–alkaline treatment are indicated on the top of the gel, and the thermal–alkaline treatment-induced bands and sequences are indicated at the left side. No detectable breaks were observed in the unmodified DNA fragments after 30 min of thermal–alkaline treatment.

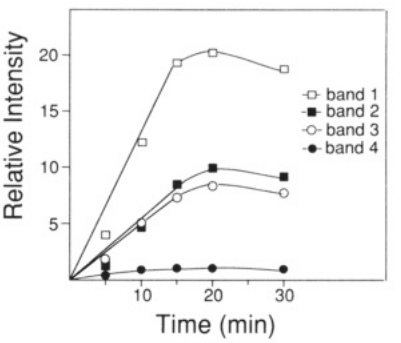

FIGURE 4: Kinetics of strand break induction in *Msp*I–*Bst*NI 117 bp M13mp1 DNA fragments modified with CC-1065. The thermal–alkaline-induced bands in Figure 3 were scanned, and the relative intensities of each band were plotted against time (□), Band 1 (-GATTA-); (■) band 2 (-GATAA-); (○) band 3 (-TTTTA-); (●) band 4 (-GGAAA-).

single-strand breaks (representing the number of adducts formed within a particular DNA fragment) can be calculated from the relative hybridization intensities based on the Poisson equation. Figures 5–7 show the results of our determination of CC-1065-induced thermal–alkaline-labile sites in bulk genomic DNA and in DHFR and APRT gene domains after in vitro treatment of genomic DNA with CC-1065. These results demonstrate that the number of thermal–alkaline labile sites (TALS) formed in coding and 3′-downstream noncoding regions of the DHFR gene domain, and the APRT gene domain, is the same as in bulk genomic DNA; the formation of TALS is proportional to drug concentration. The molar

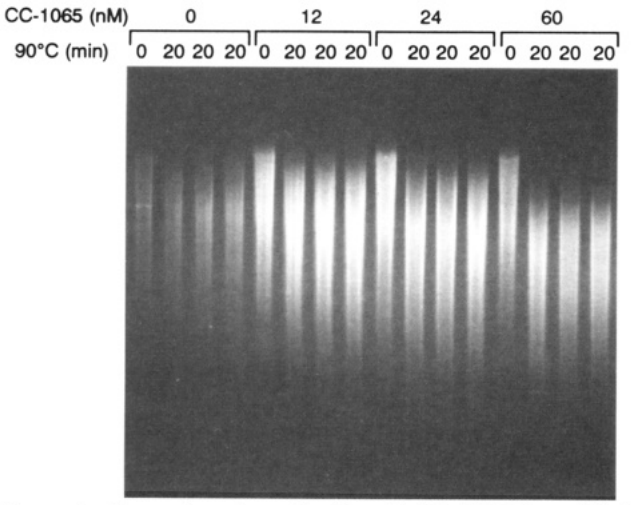

FIGURE 5: Electrophoresis results of purified B-11 DNA modified with different concentrations of CC-1065 with 20-min thermal–alkaline treatment. The details for DNA purification, restriction enzyme Asp718 digestion, drug modification, DNA denaturation, and the conditions of electrophoresis are described under Materials and Methods. The gel was stained with 0.5 μg/mL ethidium bromide.

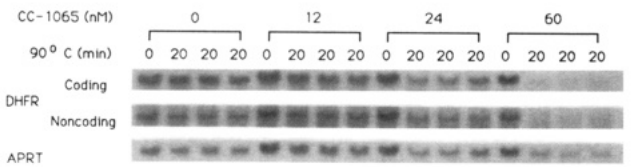

FIGURE 6: Detection of thermal–alkaline labile sites at defined sequences of purified B-11 DNA modified with different concentrations of CC-1065. The DNA shown in Figure 5 was transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled pMB5, cs-14, and pGAL sequentially for probing coding and 3′-downstram noncoding regions of the DHFR gene domain and the APRT gene domain, respectively. A typical autoradiograph is shown.

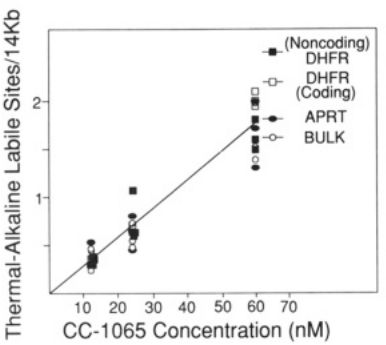

FIGURE 7: Formation of thermal–alkaline labile sites in bulk genomic DNA and the DHFR and APRT gene domains as a function of CC-1065 dose. The picture as shown in Figure 5 and the autoradiograph as shown in Figure 6 were scanned, and the number of breaks per 14 kb was calculated as described under Materials and Methods. The symbols (○), (□), (■), and (●) represent bulk genomic DNA, the coding region of the DHFR gene, 3′ downstream of the noncoding region of the DHFR gene, and the APRT gene domain, respectively.

ratios of drug/nucleotide under our reaction conditions ranged from 5.3×10^{-5} to 26×10^{-5}. Therefore, after 14-h incubation at 37 °C, most drug molecules were expected to be DNA-bound either covalently or noncovalently. The number of TALS we detected in drug-treated DNA is 48–67% of the number of CC-1065 molecules used in the reaction. Modifying calf thymus DNA under similar conditions as ours and using circular dichroism measurement Krueger and Prairie (1992) have shown that 59–64% of the DNA-associated CC-1065 are covalently bonded. Since only the covalently bonded drug

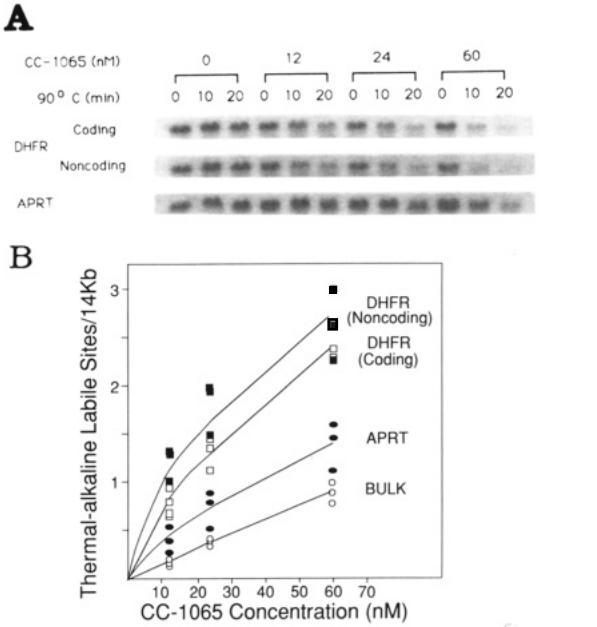

FIGURE 8: Detection of thermal–alkaline labile sites at defined sequences of DNA purified from B-11 cells following in vivo treatment with different concentrations of CC-1065. Methods for DNA separation, probing, and quantification are the same as described in Figures 5 and 7. (A) Typical autoradiograph; (B) quantification results of three experiments. (○) Bulk genomic DNA; (□) coding region of the DHFR gene; (■) 3′-downstream noncoding region of the DHFR gene; (●) APRT gene domain.

can induce a thermal-labile break, our results (Figures 3–7) suggest that the thermal–alkaline method permits quantitative detection of CC-1065–DNA adduct formation in defined DNA sequences.

Quantification of CC-1065–DNA Adducts Formed at Defined Sequences in Cellular DNA Isolated from Drug-Treated Cultured CHO Cells. Having demonstrated the validity of the thermal–alkaline method for quantification of CC-1065–DNA adduct formation in in vitro-modified DNAs, we then used this method to examine the genomic distribution of CC-1065–DNA adducts in drug-treated CHO B-11 cells. These cells, which are diploid for the APRT locus but have amplified the DHFR gene domain 50–100-fold (Kaufman & Schimke, 1981), permit comparison of the relative efficiencies of drug–DNA bonding within a single-copy gene locus or within a highly amplified gene array, to reactions in bulk genomic DNA. B-11 cells were treated with different concentrations of CC-1065, and the levels of drug–DNA adduct formation in bulk genomic DNA and the DHFR and APRT gene domains were determined. The results in Figure 8 show that, in each case, the numbers of adducts formed were proportional to drug concentration; however, the efficiencies of adduct formation in the three DNA categories were markedly different. In both coding and noncoding regions of DHFR, adduct formation appears to be twice as efficient as in the APRT gene domain, while in bulk genomic DNA it is somewhat lower than in the APRT gene domain. These results are in striking contrast to the levels of adduct formation detected in DNA modified in vitro, where no significant differences in the levels of drug–DNA adduct formation were observed in the APRT and DHFR genes or in bulk genomic DNA (Figure 7). Results from Figures 5–8 together suggest that the rather substantial difference in CC-1065 bonding between the amplified DHFR gene domain and the nonamplified APRT gene domain is not simply due to sequence differences but may instead be a consequence of differences

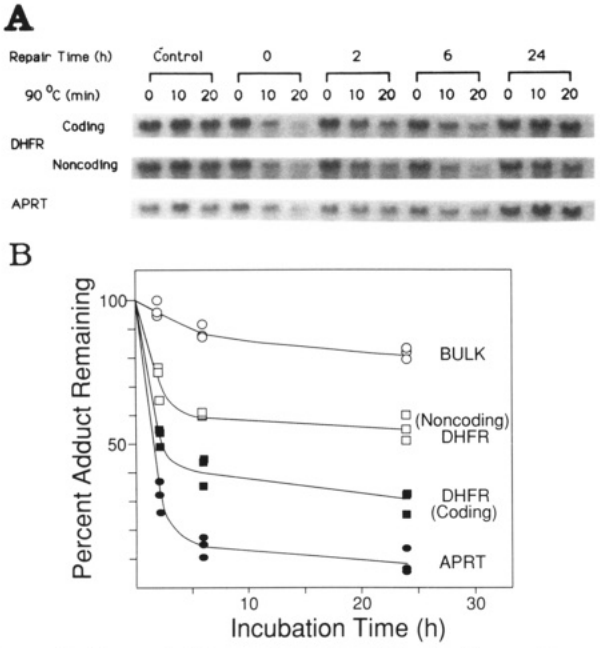

FIGURE 9: Removal of thermal–alkaline labile sites from coding and noncoding regions of DHFR and APRT gene domains of B-11 cells. DNA isolated from drug (60 nM)-treated cells with different postdrug incubation periods was analyzed as described in Figure 8. (A) Typical autoradiograph; (B) quantification results of three experiments. The symbols (○), (□), (■), and (●) represent bulk genomic DNA, the coding region of the DHFR gene, 5′ downstram of the noncoding region of the DHFR gene, and the APRT gene domain, respectively.

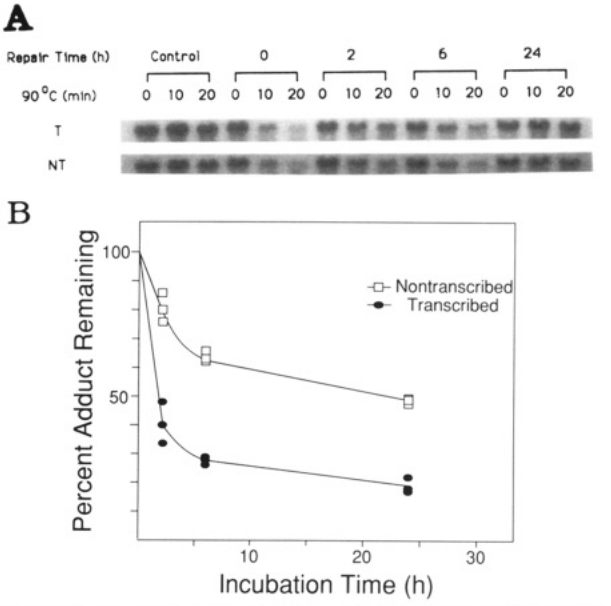

FIGURE 10: Removal of the thermal–alkaline labile sites from the transcribed strand and nontranscribed strand of the DHFR gene of CC-1065-treated B-11 cells. The same membranes used in Figure 9 were probed with DHFR strand-specific ^{32}P-labeled riboprobes; (A) is a typical autoradiograph result, and (B) is the quantification results of three experiments. (●) represents the transcribed strand, and (□) represents the nontranscribed strand.

in chromatin structure/conformation associated with gene amplification or may be due to different organization of DHFR and APRT genes in vivo. The higher level of CC-1065–DNA bonding in the APRT gene domain than in bulk genomic DNA may be a consequence of the transcriptional activity of the APRT gene.

Repair of CC-1065–DNA Adducts in the Amplified DHFR Gene Domain and the APRT Gene. It has been shown that certain types of DNA damage such as UV-induced photoproduct–cyclobutane pyrimidine dimers, and cisplatinum–DNA adducts, are repaired more efficiently in coding regions of the DHFR gene than in downstream, noncoding regions, and more efficiently in the transcribed strand than in the nontranscribed strand [for reviews, see Bohr et al. (1987) and Bohr (1991)]. However, evidence of such "gene-specific" preferential repair has not been observed for aminofluorene–DNA adducts and methylpurines (Tang et al., 1989; Scicchitano & Hanawalt, 1989, 1990). It has been postulated (Sancar & Tang, 1993) that DNA lesions which block transcription may trigger a highly efficient transcription repair coupling factor dependent process to remove damage, while those which do not block transcription may not. Although CC-1065–DNA adducts inhibit RNA synthesis, they also stabilize DNA helix structure; this effect is in marked contrast to cyclobutane pyrimidine dimers and aminofluorene–DNA adducts which destabilize DNA helix structures (Reynolds et al., 1986; Hurley et al., 1984; Friedberg, 1984). To determine whether the transcription process affects the repair of CC-1065–DNA adducts, the kinetics of repair of CC-1065 adducts in bulk genomic DNA and at the coding region and the 3′-downstream noncoding region of the DHFR gene domain and the APRT gene from CHO B-11 cells treated with 60 nM CC-1065 were determined. Figure 9 shows typical autoradiographic results. While relatively little repair (10–20%) was detected in bulk genomic DNA, substantial repair was observed in the DHFR and APRT gene domains. In the amplified DHFR domain, repair in the coding region was significantly faster and more efficient than in the downstream noncoding region; after 6 h of posttreatment incubation, 60% of drug–DNA adducts were repaired in the former while only 40% of adducts are repaired in the latter. In both regions of the DHFR gene domain, little or no additional repair was observed beyond 6 h of posttreatment incubation. In the nonamplified APRT gene domain, 85% of the drug–DNA adducts were repaired after 6-h posttreatment incubation, but, again, longer incubation times did not result in any further increases in adduct removal.

The higher efficiency of repair of CC-1065–DNA adducts in coding regions than in noncoding regions of the DHFR gene domain might reflect transcription-mediated repair on the transcribed strand of active genes, as has been observed in the case of UV-induced cyclobutane pyrimidine dimers (Mellon et al., 1987). Alternatively, it could represent "gene-specific repair" which does not necessarily involve transcribed-strand-specific preferential repair [an example would be the repair of (6-4) photoproducts (Bohr, 1991)]. To distinguish between these two possibilities, we have examined the formation and repair of CC-1065–DNA adducts in both the transcribed and the nontranscribed strand of the DHFR gene. The same membranes which were used for examining the formation and repair of drug–DNA adducts at the DHFR gene were probed with strand-specific ^{32}P-labeled riboprobes as described by Mellon et al. (1987). The results are shown in Figure 10. These results demonstrate that while CC-1065–DNA adducts are formed to the same extent in both the transcribed and nontranscribed strands (data not shown), repair is significantly faster and more efficient in the transcribed strand than in the nontranscribed strand; at 6 h of posttreatment incubation, 75% of the adducts were repaired in the transcribed strand, but only 32% of the adducts were repaired in the nontranscribed strand. These results demonstrate that the higher efficiency of repair in the coding region is due to a higher efficiency of adduct removal in the transcribed strand.

DISCUSSION

CC-1065 bonds specifically to DNA; thus, the biological effects of the drug must derive from drug–DNA interactions. The sequence selectivity and effects on DNA structure of CC-1065–DNA bonding have been studied extensively. It has been found that the binding affinity is determined by a five-base sequence (Hurley et al., 1984). Bonding causes DNA bending and winding (Lee et al., 1991), increases the DNA melting temperature and resistance to S1 nuclease digestion [for reviews, see Warpehoski and Hurley (1988) and Lee et al. (1991)], and also affects protein–DNA interactions even at some distance from the actual drug binding site (Hurley et al., 1987). The bulky drug–DNA adducts are expected to block DNA and RNA chain elongation processes, and, indeed, it has been shown that CC-1065 treatment inhibits DNA and RNA synthesis. However, it has also been suggested that the sequence selectivity of drug bonding, in combination with its unique structural consequences to DNA, may interfere with the proper interactions of some regulatory DNA binding proteins and result in inhibiting the initiation of DNA replication (Lee, 1990). With the same reasoning, one may propose that the drug–DNA binding could cause deregulation of transcription. Consistent with this notion is the finding that treatment of cells with low levels of the CC-1065 analog U71184 stimulates RNA synthesis and consequently induces unbalanced growth (Adams et al., 1988). These results suggest that CC-1065 and its analogs may not only inhibit expression of genes which are necessary for maintaining cell growth but also might induce the expression of some genes which otherwise are silent under normal growth regulation. Either of these effects on the regulation of gene expression may contribute to cell death.

The reason why CC-1065 bonds to amplified DFHR regions more efficiently than to the nonamplified APRT gene in CHO B-11 cells is unclear. Since this variation was not observed in purified DNA, these results suggest that the differences in drug bonding observed between amplified DHFR and APRT genes may be due to structural differences between these two genes in vivo or be due to an altered chromatin structure associated with gene amplification. Presently, we are unable to differentiate these two possibilities. The differential staining of highly aplified regions by DNA binding or intercalating agents such as methylene blue and eosin (ingredients in Giemsa staining solution) and ethidium bromide stain, which allows them to be visualized as homogeneous staining regions or HSRs (Cowell, 1982), suggests that the amplified DNA regions may have a different conformation or higher order organization than other regions of chromosomes. Such differences may affect not only drug–DNA bonding but also adduct removal by DNA repair processes. Consistent with this notion is the finding that the efficiency of repair for DNA adducts in multicopy ribosomal genes, which are arranged in tandem, amplified arrays, is much less than in that for the other active genes (Christians & Hanawalt, 1992).

There are two explanations for the results in Figure 9B, which appear to show that the repair of drug–DNA adducts in the nonamplified APRT gene domain is faster and more efficient than in the amplified DHFR gene domains. It is possible that gene amplification may affect the efficiency of repair; since we have found that the repair of CC-1065–DNA adducts in the DHFR gene is more efficient in the transcribed strand than in the nontranscribed strand, one explanation for the lower efficiency of repairing in the amplified DHFR gene compared to the nonamplified APRT gene is that not all amplified DHFR genes are transcriptionally active. On the other hand, since there are more adducts per unit length of DNA in the amplified DHFR gene domains than in the APRT gene domain (Figure 8B), this initial difference in damage, for whatever reasons, could affect both the kinetics and efficiency of drug–DNA removal. It is worth noting that the amounts of drug–DNA adducts removed in the amplified DHFR gene domains and the APRT gene domain are similar; there are 1.1, 1.4, and 1.5 adducts removed from the former during 2, 6, and 24 h of incubation, while there are 1, 1.2, and 1.3 drug–DNA adducts removed from the latter during the same time periods of incubation. At present, the effect of gene amplification on the removal of drug–DNA adducts remains unclear.

It has been recently found that DNA is often amplified in tumor cells, and the potency of tumor cells for tumorigenesis is related to its ability to amplify DNA (Otto et al., 1989; Tlsty et al., 1989, 1992). Using a sensitive comparative genomic hybridization technique, Kallioniemi et al. (1992) have found that multiple regions in the chromosomes of all the cancer cells tested are amplified. These findings raise the possibility that amplified DNAs are necessary for maintaining rapid cancer cell growth and/or their defense against the immune system. If CC-1065 bonds preferentially to amplified DNA regions, as we have observed in the amplified DHFR genes, then it is possible that this preferential bonding may contribute to its antitumor activity. The potential lower efficiency of repair of drug–DNA adducts in amplified gene domains versus nonamplified gene domains may enhance the specificity of the drug. Together, these two effects may cause tumor cells to be much more susceptible to drug-induced cytotoxicity.

Using T4 endonuclease V for cyclobutane pyrimidine dimer (CPD) detection, it has been found that cells preferentially repair CPD in the transcribed strand of active genes (Mellon et al., 1987); our results as well as those of Bohr et al. (1985) found no differences in CPD repair in amplified and nonamplified genes. However, we recently found that the repair of BPDE– and AF–DNA adducts, as detected by UvrABC nuclease, appears to be more efficient at a nonamplified DHFR gene locus than in amplified DHFR genes. Moreover, although there was 10–15% of transcribed-strand-specific preferential repair in amplified DHFR genes, no strand-specific preferential repair was observed for these kinds of adducts at a nonamplified DHFR gene (Tang et al., 1993). To reconcile these disparate observations, we propose that the transcription proces may have two distinct effects on DNA repair: (1) the so-called "transcription repair coupling factor" may facilitate the repair of DNA damage which blocks transcription. (2) As a consequence of the transcription process, the regions surrounding active genes may be "opened" and become more accessible to DNA repair enzymes and accessory proteins. This would most likely affect the repair of DNA damage indiscriminantly. If the relative "openness" of a chromosome region results in efficient repair of DNA damage in that region, this might obscure any effects of transcription repair coupling factor; thus, little of no "transcribed-strand-specific" preferential repair would be observed. On the other hand, if the relative "openness" of a chromosome region has little effect on the efficiency of repair, then the effect of "transcription repair coupling factor" on the repair would be more pronounced, and significant transcribed-strand-specific preferential repair would be observed. It is likely that for those types of DNA damage (such as CPD and CC-1065) which are repaired poorly in bulk genomic DNA, the "openness" that results from transcription will have less effect

on the recognition of these kinds of damage, and therefore require "transcription repair coupling factor" for their recognition. For those types of DNA damage which are repaired relatively efficiently in bulk DNA, recognition of the damage by repair enzymes probably does not require the "transcription repair coupling factor"; "openness" of the DNA region itself may sufficiently enhance accessibility to repair enzymes.

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