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Delayed Cytotoxicity and Cleavage of Mitochondrial DNA in Ciprofloxacin-Treated Mammalian Cells

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SUMMARY

We have previously shown that 4-quinolone drugs cause a selective loss of mitochondrial DNA (mtDNA) from mouse L1210 leukemia cells. The loss in mtDNA was associated with a delayed loss in mitochondrial function. Here, we report that the 4-quinolone drug ciprofloxacin is cytotoxic to a variety of cultured mammalian cell lines at concentrations that deplete cells of mtDNA. The IC_{50} values for ciprofloxacin varied from 40 to 80 μg/ml depending on the cell line tested. Cytotoxicity required continuous exposure of cells to drug for 2–4 days, which corresponded to approximately three or four cell doublings. Shorter times of drug exposure did not cause significant cytotoxicity. In addition, cells became drug resistant when they were grown under conditions that bypassed the need for mitochondrial respiration. Resistance was not due to a decrease in cellular drug accumulation, suggesting that ciprofloxacin cytotoxicity is caused by the loss of mtDNA-encoded functions. Analysis of mtDNA from ciprofloxacin-treated cells revealed the presence of site-specific, double-stranded DNA breaks. Furthermore, exonuclease protection studies indicated that the 5′-, but not the 3′-, ends of the drug-induced DNA breaks were tightly associated with protein. These results suggest that ciprofloxacin may be causing cytotoxicity by interfering with a mitochondrial topoisomerase II-like activity, resulting in a loss of mtDNA.

The 4-quinolones are a class of antibacterial drugs that act by inhibiting the type II topoisomerase DNA gyrase (1–3). At high concentrations, these drugs have also been shown to reversibly inhibit the growth of cultured mammalian cells (4–7). The mechanism underlying this inhibition of mammalian cell growth is unknown. Several studies have suggested that 4-quinolones may act by interfering with *de novo* pyrimidine synthesis (7, 8). Other studies have found that high concentrations of 4-quinolones can inhibit respiration in isolated rat liver mitochondria, indicating that the drug may be interfering with the enzymes involved in energy metabolism (8–10). Although a similar inhibition of respiration was seen in drug-treated mammalian cells, the onset was delayed, suggesting that the loss in mitochondrial respiration was not caused by a direct inhibition of the enzymes involved in oxidative phosphorylation (6).

Studies have also suggested that 4-quinolones may interfere with cell growth by inhibiting mammalian mtDNA replication (6, 11). Castora *et al.* (11) found that the 4-quinolone drugs nalidixic acid and oxolinic acid inhibited mtDNA replication in isolated rat liver mitochondria. These investigators inferred that this effect might be mediated by the inhibition of a mitochondrial topoisomerase II activity related to the bacterial enzyme DNA gyrase.

We recently demonstrated that the 4-quinolone drugs nalidixic acid and ciprofloxacin cause a selective loss of mtDNA in drug-treated mammalian cells (6). The loss of mtDNA was associated with a decrease in mitochondrial respiration and an arrest in cell growth. These results suggested that inhibition of mammalian cell proliferation by 4-quinolone drugs might be caused by the selective depletion of mtDNA, resulting in compromised mitochondrial activity. We now report that ciprofloxacin causes a delayed cytotoxicity in cultured mammalian cells at concentrations that deplete cells of mtDNA. Cytotoxicity was prevented by culturing cells under conditions that bypassed the need for normal mitochondrial processes, suggesting that ciprofloxacin was acting by inhibiting a critical mitochondrial function or functions. Furthermore, the mtDNA from ciprofloxacin-treated cells was found to have protein-linked double-stranded DNA breaks, suggesting that mitochondria contain a topoisomerase II activity that is a target for ciprofloxacin.

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ABBREVIATIONS: mtDNA, mitochondrial DNA; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; α-MEM, α-minimal essential medium.

Materials and Methods

Reagents. Ciprofloxacin hydrochloride was a generous gift from Miles Laboratory (Naperville, IL). Concentrated drug stocks were prepared in water and filter sterilized before use. VM-26 [4′-demethyl-9-[4,6,-*O*-(2-thenylidene-β-D-glucopyranosyl)oxy]epipodophyllotoxin] was kindly provided by Bristol-Myers Squibb (Princeton, NJ), and concentrated stocks were prepared in dimethylsulfoxide. Glass beads (40 μM diameter) were obtained from Whatman. Proteinase K, RNase T1, and λ exonuclease were obtained from GIBCO BRL (Baltimore, MD). Exonuclease III, wheat germ topoisomerase I, and *Sac*I were purchased from Promega (Madison, WI). All other reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

DNA clones and labeled probes. The entire mouse mitochondrial genome, inserted into the *Sac*I site of pSP64, was obtained from William Hauswirth (University of Florida, Gainesville, FL) (12). The mouse mitochondrial subclone pMBS 2.1 contains the 2.1-kb *Bam*HI/*Sac*I fragment of mouse mtDNA (13) inserted into the *Bam*HI/*Sac*I site of pSP64. The human mtDNA clone pKB *Mbo*I 2.8 contains the 2.8-kb *Mbo*I fragment of mtDNA inserted into the *Bam*HI site of pBR322 and was obtained from David Clayton (Stanford University). These DNAs were labeled by nick translation using [α-^{32}P]dCTP as described previously (6).

Cell culture. B16 cells, a mouse melanoma cell line with a doubling time of 14 hr, were obtained from Tien-Wen Wiedmann (Stanford University). HeLa S3 cells are a human cervical carcinoma cell line with a doubling time of 24 hr; they were obtained from Bert Flanagan (University of Florida, Gainesville, FL). Unless stated otherwise, cells were maintained as subconfluent monolayer cultures in α-MEM (GIBCO BRL) supplemented with 10% fetal bovine serum at 37° in a 5% CO_2 atmosphere.

CHO cells were obtained from Warren Ross (University of Florida, Gainesville, FL); they have a doubling time of ~24 hr. CHO cells were maintained as subconfluent monolayers in complete DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum, 2× GIBCO MEM amino acids, 2× GIBCO MEM vitamins, 6 g/liter HEPES, 3.7 g/liter sodium bicarbonate, and 40 mg/liter L-proline at 37° under a humidified atmosphere containing 5% CO_2 (Table 1). Alternatively, cells were maintained in DMEM PUT (complete DMEM supplemented with 1 mM sodium pyruvate, 10 μg/ml uridine, and 10 μg/ml thymidine) or in α-MEM supplemented with 10% fetal bovine serum.

Cytotoxicity assay. Cytotoxicity was measured using a colony-forming assay as described previously (14). Briefly, logarithmically growing B16 or HeLa cells were detached from culture dishes by incubation with Puck's A saline (5 mM KCl, 138 mM NaCl, 4 mM $NaHCO_3$, pH 7.4, 5.6 mM glucose) supplemented with 0.02% (w/v) EDTA and counted. Cells were then seeded in triplicate into six-well culture plates at 300–10,000 cells/well for each drug concentration in a final volume of 1 ml of media. After an overnight incubation at 37°, the medium was removed by vacuum aspiration and replaced with 3 ml of fresh medium that contained the appropriate concentration of drug. In experiments in which the cells were allowed to recover in drug-free medium, the drug-containing medium was removed, and the cells were washed three times with 37° PBS (137 mM NaCl, 2.5 mM KCl, 1.5 m KH_2PO_4, 10 mmM $NaHPO_4$, pH 7.4) before the addition of 3 ml of fresh drug-free medium. Colonies were allowed to form for 7–10 days before staining with a 2% (w/v) solution of crystal violet in 95% ethanol. Colonies were counted, and the surviving fraction was expressed relative to the control, non-drug-treated cells. The IC_{50} values were determined from the cytotoxicity curves.

Drug accumulation assay. Cellular accumulation of ciprofloxacin was measured using a fluorometric assay (15). Briefly, CHO cells (1×10^7) were harvested and resuspended in 10 ml of culture medium at a density of 1×10^6 cells/ml. Drug was then added, and the cell suspension was incubated at 37° for 1 hr before placement of samples in an ice bath for 15 min. Cells were then pelleted at 1000 × *g* for 5 min at 4°, and the supernatants were discarded. The cell pellets were washed with ice-cold PBS (10 ml) two times before resuspension in 2 ml of PBS. Digitonin was then added to a final concentration of 100 μM (6.2 μl of a 4% w/v stock in absolute alcohol) to disrupt cell membranes. After 20 min at 20°, samples were centrifuged at 2500 rpm for 10 min, and the resulting supernatants from the digitonin-treated cells were analyzed for ciprofloxacin by fluorescence spectroscopy using an excitation wavelength of 335 nm and an emission wavelength of 415 nm. Values were corrected for nonspecific fluorescence by subtracting the fluorescent values obtained from the supernatants of non-drug-treated cells.

Isolation of cellular DNA. Total cellular DNA was isolated as described previously (6). The concentration of DNA was determined spectrofluorometrically (16), and equal amounts of DNA (10 μg) from each sample were analyzed by Southern blotting as described below. In some cases, the DNA was restricted with *Sac*I before Southern analysis.

Gel electrophoresis, Southern blotting, and autoradiography. DNA samples were combined with 0.1 volume of a solution containing 50% sucrose, 0.05% bromphenol blue, and 10 mM EDTA and loaded onto a 0.7% agarose horizontal gel in TBE buffer (90 mM Tris-borate, pH 8.3, 2 mM EDTA). Electrophoresis was done at 3 V/cm for 18 hr. DNA was then transferred onto a nitrocellulose filter and hybridized to nick-translated ^{32}P-labeled mtDNA probes as described by Sambrook *et al.* (17). The filter was autoradiographed at −70° using Kodak XAR 5 film and a Dupont Lightning-Plus intensifying screen (Wilmington, DE). Quantification of radiolabeled blots was done using a Betascope 603 Blot Analyzer (Betagen, Waltham, MA).

DNA unwinding assay. The DNA unwinding assay was done according to a modification of the procedure by Chen *et al.* (18). Reactions (30 μl) containing 50 mM Tris·HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.3 μg of supercoiled pMR2

TABLE 1
Growth media

	DMEM[a]	DMEM PUT[b]	α-MEM[a]	DMEM NG[a]
Glucose (g/liter)	4.5	4.5	1	None
Pyruvate (mM)	None	1	1	1
Glutamine (mM)	1	1	1	4
Thymidine (mM)	None	0.04	None	None
Uridine (mM)	None	0.04	None	10
Fetal calf serum	10%	10%	10%	None
Ultraser G[c]	−	−	−	+
Extra (2×)				
Amino acids	+	+	−	−
Vitamins	+	+	−	−

[a] Requires active mitochondria for growth.
[b] Supports respiratory-independent growth.
[c] Serum substitute from GIBCO BRL.

plasmid DNA (19), and 10 units of wheat germ topoisomerase I were incubated at 37° to relax the DNA. After 10 min, ciprofloxacin was added to the reactions, and the incubations were continued for an additional 20 min. Reactions were terminated by the addition of 10 μl of a solution containing 1% SDS, 40 mM EDTA, pH 8, 50% sucrose, 0.01% bromphenol blue, and 0.01% xylene cyanol. Samples were analyzed by electrophoresis in a 1% agarose gel containing 0.5 × TBE at 20 V for 4 hr. Gels were stained with ethidium bromide and photographed under UV light.

Isolation of protein-linked DNA using glass beads. Protein-linked DNA was isolated from cell extracts using a modification of the procedure described by Shin and Snapka (20). Briefly, HeLa cells (4×10^6) were pelleted and then resuspended in 100 μl of lysis buffer (20 mM Tris·HCl, pH 8.0, 1% SDS). After lysis, EDTA and PMSF were added to final concentrations of 25 and 1 mM, respectively. Lysates were then combined with 1 ml of PBB buffer (10 mM Tris·HCl, pH 8.0, 0.4 M guanidine-HCl, 0.01% sarkosyl, 0.3 M NaCl, 10 mM EDTA, 1 mM PMSF, 1 mM DTT) and 100 μl of a glass bead suspension in deionized water (50% w/v). Samples were incubated for 15 min at room temperature with intermittent mixing before the glass beads were pelleted at 3000 × *g* for 2 min. The pellets were washed two times with 1 ml of PBB and once with 1 ml of high salt buffer (10 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 3 M NaCl) at 37°. The DNA was then eluted by resuspension of the glass beads in elution buffer (10 mM Tris·HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.1% SDS, 500 μg/ml proteinase K). After an overnight incubation at 37°, the glass beads were pelleted, and the supernatant containing the eluted DNA was saved. DNA samples were then phenol extracted, ether extracted, and ethanol precipitated. The precipitated DNA was then resuspended in 100 μl of H_2O and used for the exonuclease studies described in the following section.

Exonuclease digestions of protein-linked DNA samples. Protein-linked DNA samples (100 μl) were divided into three equal aliquots. One aliquot served as a control, while the second aliquot was digested with 10 units of exonuclease III (1 unit produces 1 nmol of acid-soluble nucleotide from duplex DNA in 30 min at 37°) in 50 mM Tris·HCl, pH 8, 5 mM $MgCl_2$, and 1 mM DTT at 37° for 30 min. The third aliquot was digested with 1 unit of λ exonuclease (1 unit produces 10 nmol of acid-soluble nucleotide from duplex DNA in 30 min at 37°) in 67 mM potassium glycine, pH 9.4, 2.5 mM $MgCl_2$, and 50 μg/ml bovine serum albumin at 37° for 30 min. To ensure that the exonucleases were active, 100 pg of *Eco*RI restricted pMBS 2.1 plasmid DNA was included in the nuclease digestion reactions. Reactions were terminated by the addition of EDTA and SDS to final concentrations of 25 mM and 0.1%, respectively. RNase T1 (1 unit) was then added, and the samples were incubated at 37° for 1 hr. The mtDNA was then analyzed after electrophoresis and Southern blotting as described earlier.

Protein-linked DNA filter assay. Protein-linked DNA was measured using a glass filter binding assay as described by Shin and Snapka (20). This assay is based on the selective binding of protein-linked DNA to glass filters. Briefly, cellular DNA was labeled by incubation of cells in medium containing 0.5 μCi/ml [^{3}H]thymidine for 24 hr. The unincorporated label was then removed by washing the cells several times in PBS. Cells were then harvested and resuspended in fresh label-free medium at 1×10^6 cells/ml. Cells (1 ml) were then aliquoted into tubes and treated with drug at 37°. After 1 hr, cells were collected by centrifugation and lysed in 200 μl of 1% SDS, 20 mM Tris·HCl, pH 8.0, 25 mM EDTA, and 1 mM PMSF. The lysates were diluted with 5 ml of 37° PBB and filtered through GF/C glass-fiber filters (Whatman). Filters were washed two times with 3 ml of 37° PBB, dried, and then placed in a scintillation vial containing 5 ml of Scintiverse II (Fisher Scientific). The filter-bound ^{3}H-labeled DNA was measured by liquid scintillation counting and expressed as a fraction of the total labeled cellular DNA.

Results

Ciprofloxacin cytotoxicity. Ciprofloxacin, as well as other 4-quinolone drugs, has been shown to cause a reversible inhibition of cell growth (6, 7). However, subsequent studies in our laboratory have indicated that significant cytotoxicity occurred when cells were exposed to drug for longer periods of time. To further investigate this effect, mouse B16 melanoma cells were continuously exposed to various concentrations of ciprofloxacin for periods of ≤6 days. The drug-containing medium was then removed, and cells were incubated in drug-free medium for an additional 6 days to allow viable cells to grow into colonies. Exposure of B16 cells to ciprofloxacin for 24 hr had little affect on cell viability at drug concentrations of ≤120 μg/ml (Fig. 1). However, longer periods of drug exposure (2–6 days) caused a significant loss in cell viability at ciprofloxacin concentrations of >60 μg/ml. A >1.5 log cell kill was observed after a 6-day exposure of cells to 120 μg/ml ciprofloxacin.

Human HeLa cells also displayed a time-dependent loss in viability after exposure to ciprofloxacin (Fig. 2). In contrast to B16 cells, there was little change in cell viability after continuous drug exposures of ≤2 days. However, by 4 days, there was a dramatic drop in cell survival at drug concentrations of >60 μg/ml. Cell survival was reduced >2 logs when cells were exposed to 120 μg/ml ciprofloxacin for 8 days. It is interesting that HeLa cells required drug exposure for twice as long as B16 melanoma cells (4 days versus 2 days) to observe cytotoxicity. Although this could reflect any number of intrinsic differences between these two cell lines, it is striking that the doubling time of HeLa cells (24 hr) is almost twice that of B16 cells (14 hr), suggesting that ciprofloxacin cytotoxicity may be affected by the rate of cell division.

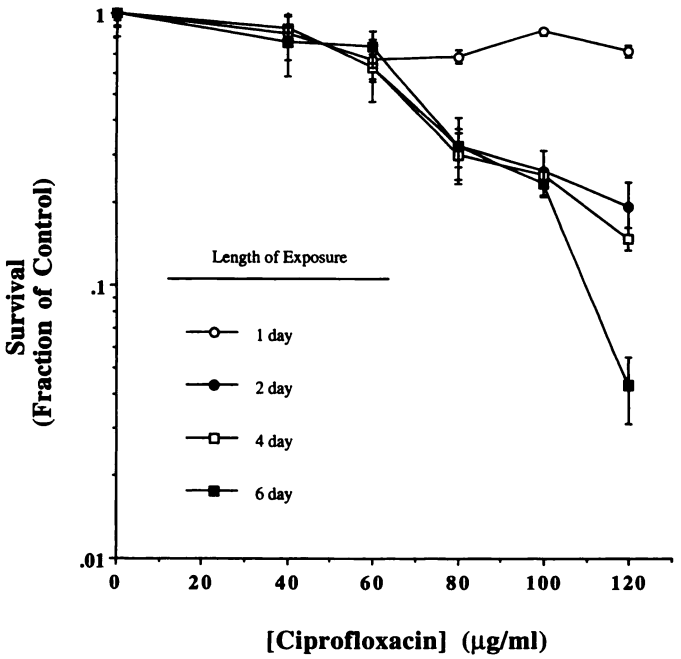

Fig. 1. Cytotoxicity of ciprofloxacin in mouse B16 melanoma cells. Mouse B16 melanoma cells were continuously exposed to various concentrations of drug for 1, 2, 4, or 6 days, and the cytotoxicity was assessed using a clonogenic assay as described in Materials and Methods. Samples were performed in triplicate, and the data are plotted as fraction ± standard error of control.

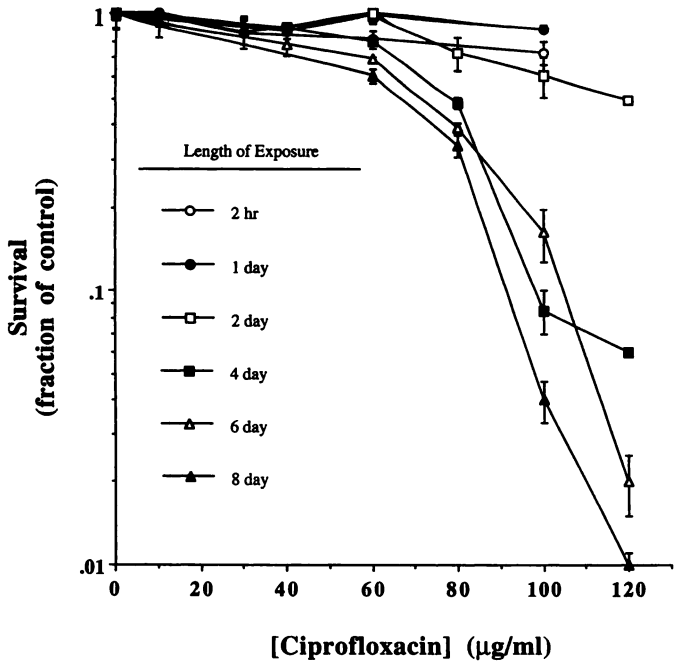

Fig. 2. Cytotoxicity of ciprofloxacin in human HeLa cells. HeLa cells were continuously exposed to various concentrations of drug for 2 hr or 1, 2, 4, 6, or 8 days, and the cytotoxicity was assessed using a clonogenic assay as described in Materials and Methods. Samples were performed in triplicate, and the data were plotted as fraction ± standard error of control.

Effect of culture medium on ciprofloxacin cytotoxicity. We previously demonstrated that ciprofloxacin induces a selective depletion of mtDNA in mammalian cells. The depletion of mtDNA preceded a decrease in mitochondrial respiration and cell growth, suggesting that mtDNA was a primary target of drug action (6). Studies have recently shown that some cultured mammalian and avian cells can survive in the absence of mtDNA-encoded functions if the growth medium is supplemented with pyrimidines, pyruvate, and elevated concentrations of glucose (21–23). Cells deficient in mtDNA rely exclusively on glycolysis for energy. In addition, they are auxotrophic for pyrimidines because *de novo* pyrimidine synthesis is coupled to active mitochondrial electron transport. The apparent requirement for pyruvate is unclear but may relate to its role in glycolysis or as a precursor for the cellular synthesis of alanine and ketone bodies.

To determine whether mitochondria might be a cytotoxic target for ciprofloxacin, we compared the survival of ciprofloxacin-treated CHO cells that were grown under normal and respiration-independent conditions. Cells grown in DMEM, which contains glucose but no pyruvate or pyrimidines, require mitochondrial activity for survival (Table 1). DMEM PUT is identical to DMEM except that it is supplemented with pyruvate and pyrimidines, enabling cell growth in the absence of mitochondrial function. α-MEM is similar to DMEM except that it contains pyruvate and has a lower concentration of glucose. The choice of CHO cells for this experiment was based on previous studies that showed that these cells could survive in the absence of mitochondrial respiration (23). CHO cells grown in either DMEM or α-MEM displayed a dose-dependent sensitivity to ciprofloxacin with a >95% loss in viability at 120 μg/ml (Fig. 3). In comparison, drug had little affect on the viability of CHO cells grown in DMEM PUT, indicating that ciprofloxacin is not cytotoxic when cells are grown under conditions that do not require mitochondrial respiratory function.

Cellular accumulation of ciprofloxacin in resistant cells. The high level of drug resistance seen in CHO cells grown under respiration-independent conditions suggests that ciprofloxacin is causing cytotoxicity by interfering with mitochondrial function or functions. However, it is also possible that resistance is due to decreased accumulation of drug

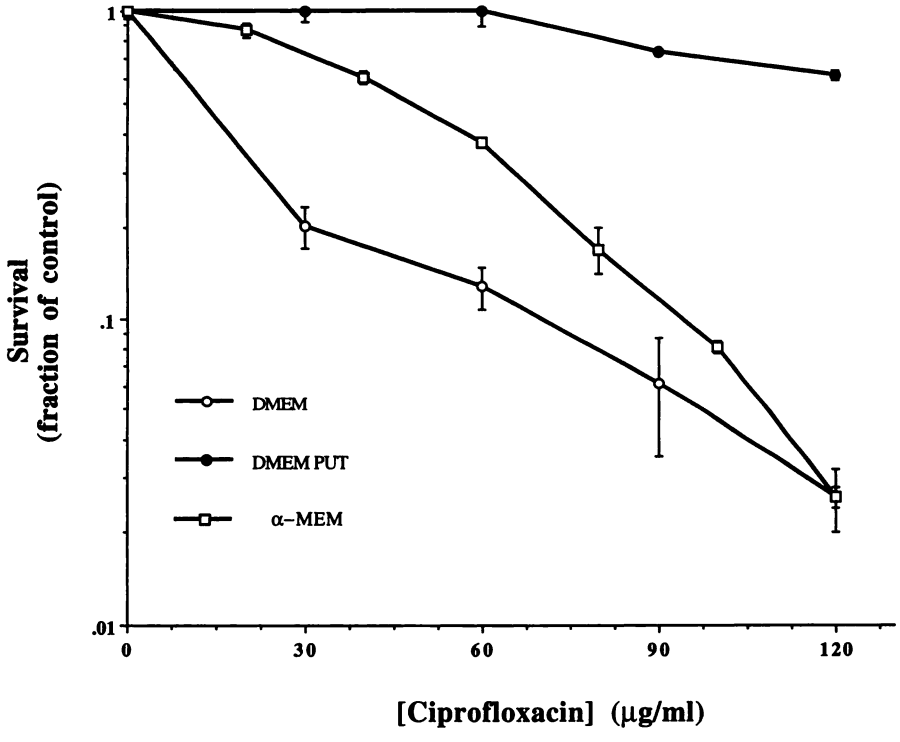

Fig. 3. Cytotoxicity of ciprofloxacin in CHO cells grown under respiration-dependent or -independent conditions. CHO cells maintained in DMEM, DMEM PUT, or α-MEM were continuously exposed to various concentrations of ciprofloxacin. After 4 days, the drug was washed out, and cytotoxicity assessed using a clonogenic assay as described in Materials and Methods. Samples were done in triplicate, and the data were plotted as fraction ± standard error of control.

in cells maintained in DMEM PUT. To test this possibility, CHO cells grown in either α-MEM or DMEM PUT were exposed to 100 μg/ml ciprofloxacin for 1 hr, and the level of drug accumulation was measured by fluorescence spectroscopy. We previously determined that the accumulation of ciprofloxacin reaches steady state within 30 min in CHO cells (data not shown). CHO cells maintained in α-MEM accumulated drug concentrations of ~0.25 fg/cell (Fig. 4). Surprisingly, CHO cells grown in DMEM PUT accumulated a higher level of ciprofloxacin (0.55 fg/cell) than cells grown in α-MEM. Therefore, even though growth in DMEM PUT altered the accumulation of ciprofloxacin, it produced an increase, rather than a decrease, in the amount of drug accumulated inside CHO cells. The reason for the increase in drug accumulation seen in cells grown in DMEM PUT is unclear. However, these results indicate that the resistance to ciprofloxacin observed in cells grown in DMEM PUT was not due to reduced intracellular accumulation of ciprofloxacin.

Ciprofloxacin induces cleavage of mtDNA. *In vitro* and *in vivo* studies have shown that mammalian mtDNA replication is inhibited by 4-quinolone drugs (11, 6). It has been suggested that the drug-induced decrease in mtDNA replication may be caused by inhibition of a bacterial-like mitochondrial topoisomerase II enzyme (11). Topoisomerase II enzymes regulate DNA topology by catalyzing the movement of DNA segments through reversible double-stranded breaks in the DNA phosphodiester backbone (24, 25). An unusual feature of the enzyme-mediated DNA breakage/reunion reaction is the formation of a transient covalent intermediate between the subunits of the topoisomerase II enzyme and the 5′-ends of the broken strands of DNA, which has been termed the cleavable complex (26). Exposure of the cleavable complex to a protein denaturant (i.e., detergent or alkali) irreversibly traps this covalent intermediate, resulting in the formation of site-specific protein-linked double-stranded DNA breaks. 4-Quinolone drugs have been shown to stabilize the cleavable complex formed by bacterial topoisomerase II, leading to an increase in the number of protein-linked chromosomal breaks in bacterial cells treated with a detergent (1–3). To determine whether mammalian mitochondria contained ciprofloxacin-sensitive topoisomerase II cleavage activity, drug-treated mouse L1210 cells were lysed with SDS to irreversibly trap cleavable complexes present in the mtDNA. Total cellular DNA was then isolated and restricted with *Sac*I before electrophoresis in an agarose gel. The DNA in the gel was then blotted onto a nitrocellulose filter and hybridized to a DNA probe homologous to the region immediately adjacent to the *Sac*I restriction site in mtDNA (Fig. 5, *top*). With this indirect end-labeling approach (27, 28), the distance (d) of any drug-induced cleavage site can be mapped relative to the *Sac*I restriction site in the mtDNA. Samples from control, non-drug-treated cells showed little cleavage of the mtDNA (Fig. 5, *middle*, *lane A*). However, there was a significant increase in the cleavage of mtDNA into discrete-size DNA fragments at drug concentrations of >0.125 μg/ml with maximal cleavage occurring at 1–3 μg/ml. Cleavage sites mapped within both tRNA- and protein-coding genes in the mtDNA (Fig. 5, *bottom*). Surprisingly, mtDNA cleavage was lost as the concentration of ciprofloxacin was increased to >3 μg/ml. A similar dose-response curve was observed for mtDNA cleavage in drug-treated CHO cells (Fig. 6). However, the peak of mtDNA cleavage in CHO cells occurred at somewhat higher drug concentrations (25 μg/ml). The higher drug concentrations required to induce cleavage of mtDNA in CHO cells is most likely due to the considerably shorter time of drug exposure (CHO cells were exposed to drug for 15 min versus 60 min for mouse L1210 cells), resulting in significantly less drug accumulation in CHO cells. In support of this notion, we found that the drug concentrations needed to induce cleavage of CHO mtDNA are considerably lower (6 μg/ml) during a 30-min drug exposure (data not shown).

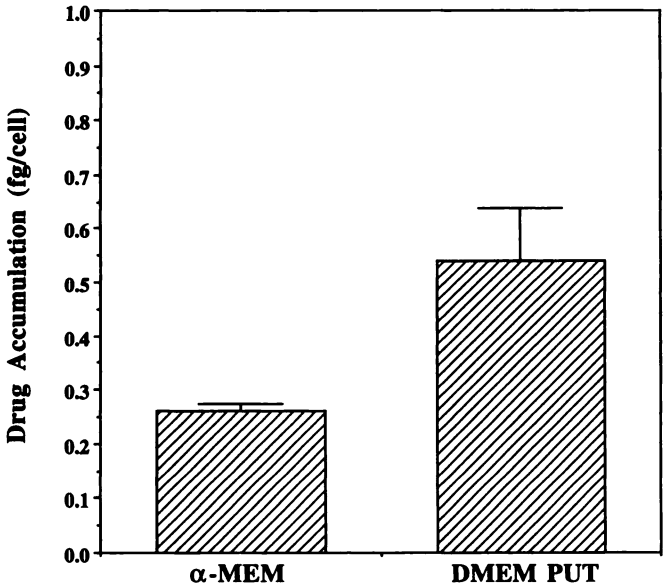

Fig. 4. Ciprofloxacin accumulation in CHO cells. CHO cells grown in α-MEM or DMEM PUT were incubated with 100 μg/ml ciprofloxacin for 1 hr, and the cellular accumulation of drug was measured using a fluorometric assay as described in Materials and Methods. Data are expressed as fg/cell ± standard error.

The apparent decrease in mtDNA cleavage at higher drug concentrations is reminiscent of the effect of DNA intercalating anticancer drugs on nuclear topoisomerase II enzymes (29, 30). Intercalating anticancer drugs such as 2-methyl-9-hydroxyellipticinium and Adriamycin have been shown to stimulate topoisomerase II cleavage at low concentrations but inhibit cleavage at high drug concentrations. Inhibition at higher drug concentrations is thought to result from destabilization of the topoisomerase II/DNA cleavable complex due to extensive intercalation and unwinding of the DNA double helix (29–31). It has been reported that the 4-quinolone drugs nalidixic acid and norfloxacin cause unwinding of DNA in the presence of magnesium (32, 33). However, in contrast to intercalating drugs, unwinding of DNA by 4-quinolones is thought to arise through an interaction of drug with single-stranded regions of DNA (2, 33). Not all 4-quinolones seem to promote DNA unwinding. In contrast to nalidixic acid and norfloxacin, the fluoroquinolones CP-67,804 and CP-115,953 cause no significant unwinding of DNA at drug concentrations of ≤150 μM (34). Therefore, to determine whether ciprofloxacin unwinds DNA, supercoiled pMR2 plasmid DNA was relaxed with purified wheat germ topoisomerase I. After 10 min, ciprofloxacin was added to the reactions, and the incubations were continued for an additional 20 min. The reactions were then terminated with SDS, and the DNA

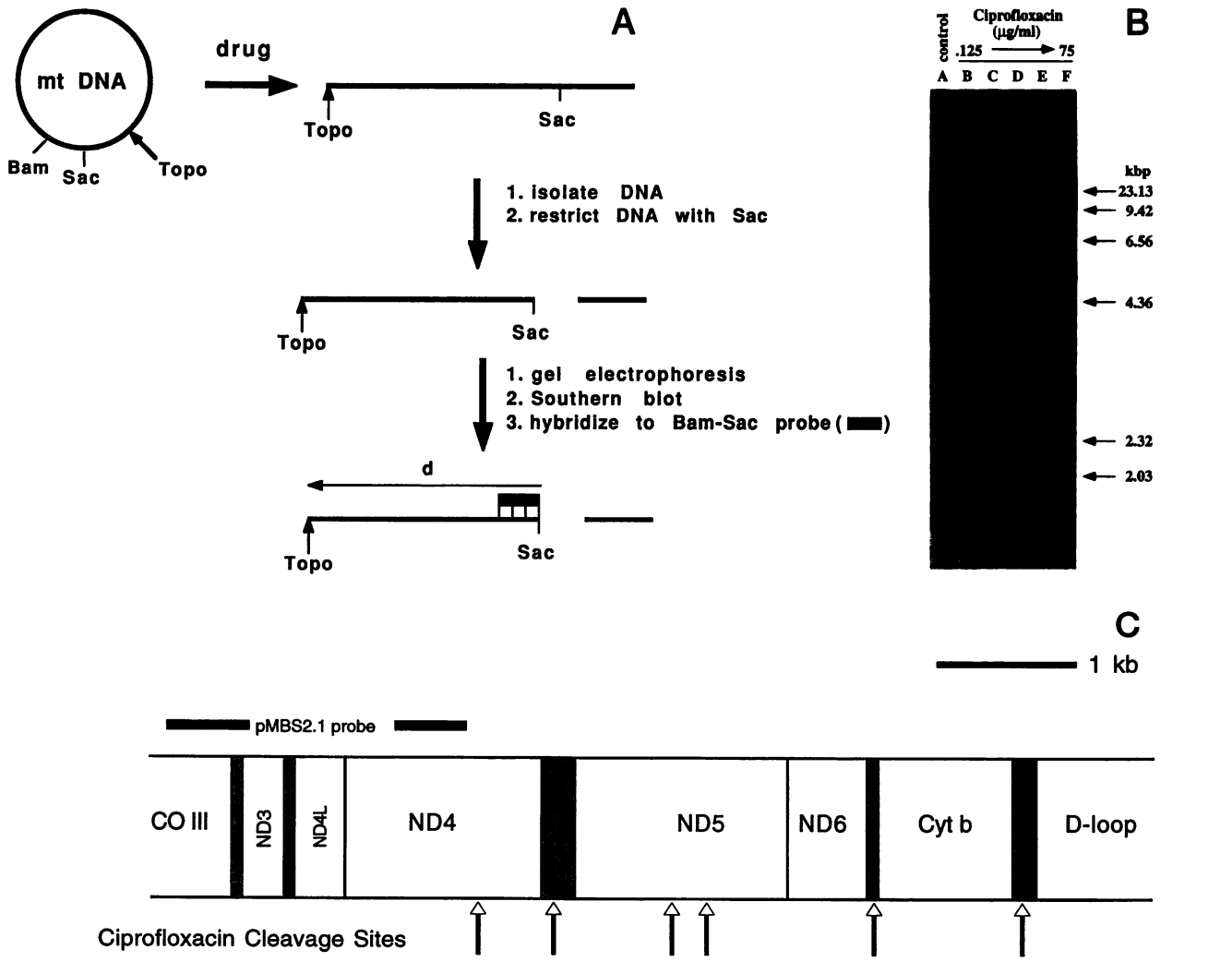

Fig. 5. Ciprofloxacin induces mtDNA strand breaks. *Top*, indirect end-labeling procedure used to map drug-induced cleavage sites in mtDNA (see text for details). *Middle*, Southern blot analysis of DNA from drug-treated L1210 cells. L1210 cells at a density of 2×10^5 cells/ml were exposed to various concentrations of ciprofloxacin for 1 hr, and the total cellular DNA was purified as described in Materials and Methods. After restriction of the DNA with *Sac*I, the samples were electrophoresed through a 0.7% agarose gel for 18 hr at 70 V and Southern blotted. The mtDNA was detected by hybridization to labeled mouse pMBS2.1 probe and is representative of three experiments. *Lanes A–F*, 0, 0.125, 0.600, 3.0, 15, and 75 μg/ml ciprofloxacin. *Arrows and numbers on the right*, positions and sizes (in kilobase-pairs) of λ *Hin*dII DNA markers. *Bottom*, approximate positions of drug-induced cleavage sites (*arrows*) on mouse mtDNA. *Shaded regions*, tRNA genes. The protein-coding genes depicted are cytochrome *c* oxidase subunit III (*CO III*), cytochrome *b* (*Cyt b*), and NADH dehydrogenase subunits 3, 4L, 4, 5, and 6 (*ND3*, *ND4L*, *ND4*, *ND5*, and *ND6*, respectively). *D-loop*, displacement-loop region that contains the origin for heavy-strand mtDNA synthesis and promoters for light- and heavy-strand transcription (39).

was analyzed by gel electrophoresis (Fig. 7). Untreated pMR2 DNA (Fig. 7, *lane F*) displayed two prominent bands representing supercoiled (faster migrating band) and nicked circular relaxed (slower migrating band) pMR2 DNA, respectively. After treatment with topoisomerase I, supercoiled pMR2 DNA was converted to a relaxed closed circular form that comigrated with nicked circular pMR2 DNA (Fig. 7, *lane A*). However, the addition of ciprofloxacin (33–300 μg/ml) after the initial incubation with topoisomerase I caused an increase in the electrophoretic migration of pMR2 DNA back toward highly supercoiled pMR2 DNA, indicating that ciprofloxacin causes significant unwinding of the DNA double helix. This effect may account for the inhibition of cleavage seen at concentrations of ciprofloxacin of >3 μg/ml and is consistent with other reports that 4-quinolones can cause DNA unwinding (32, 33). However, in contrast to other 4-quinolones, unwinding of DNA by ciprofloxacin did not require magnesium, nor did magnesium stimulate unwinding of the DNA by drug (data not shown).

The minimum concentration of ciprofloxacin required to unwind DNA *in vitro* (>33 μg/ml) was ~2-3-fold higher than the concentration of drug required to inhibit mtDNA cleavage in mouse L1210 cells (15 μg/ml), suggesting that these two events may be unrelated. However, previous studies have shown that the intracellular concentration of ciprofloxacin in mammalian cells is 4–7-fold higher than the extracellular concentration of drug (35). Therefore, an extracellular concentration of 15 μg/ml (the concentration at which mtDNA cleavage was abolished in mouse L1210 cells) would potentially result in intracellular concentrations of 60–100 μg/ml, concentrations that we have found cause significant DNA unwinding *in vitro*.

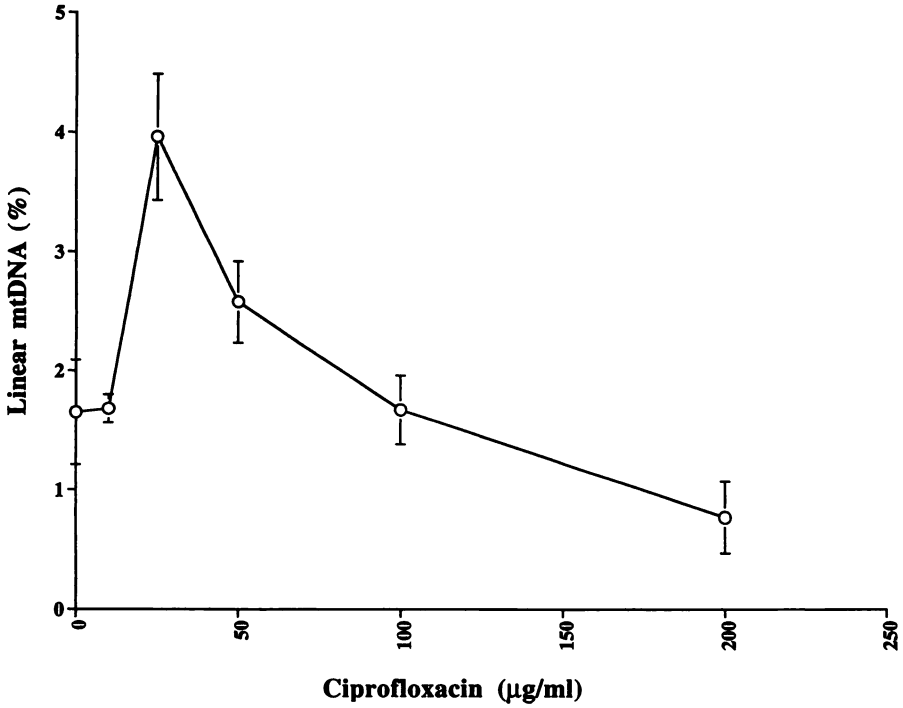

Fig. 6. Ciprofloxacin-induced cleavage of mtDNA in CHO cells. Logarithmically growing CHO cells maintained in DMEM-NG (see Table 1) were treated with various concentrations of ciprofloxacin for 15 min, and mtDNA cleavage was determined by Southern blotting as described in Materials and Methods. The linearized mtDNA *band* was quantified using a Betascope blot analyzer as described previously (6) and is expressed as percentage of the total mtDNA signal. Values represent the mean ± standard error of triplicate samples.

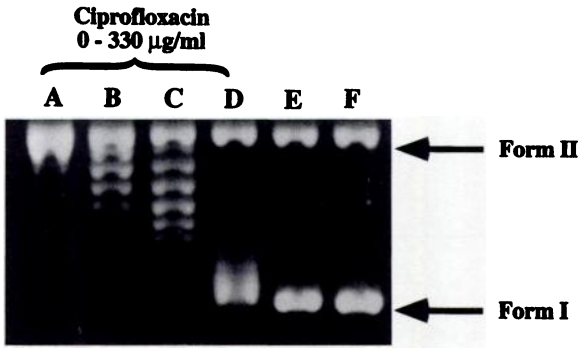

Fig. 7. Ciprofloxacin unwinds DNA. DNA unwinding was assayed as described in Materials and Methods. *Lanes A–D*, pMR2 plasmid DNA incubated with topoisomerase I in the presence of 0, 33, 100, or 330 μg/ml ciprofloxacin. *Lanes E and F*, no enzyme controls containing pMR2 DNA incubated in the absence or presence of 330 μg/ml ciprofloxacin, respectively. *Arrows*, positions of form I (highly supercoiled) and form II (nicked circular) forms of pMR2 plasmid DNA.

Ciprofloxacin-induced breaks in mtDNA are protein linked. Cleavage of DNA by topoisomerase II enzymes results in the covalent attachment of the protein subunits to the 5′-ends of the broken DNA strands via a phosphotyrosine bond (1, 2, 24, 25). DNA covalently linked to protein can be readily isolated by binding to glass beads (20). Glass has been shown to bind protein, but not DNA, in the presence of 0.4 M guanidine-HCl and 0.3 M NaCl. This procedure was used to isolate protein-linked DNA from ciprofloxacin-treated HeLa cells. The protein-linked DNA fraction was then split into three equal aliquots that were treated with exonuclease III, λ exonuclease, or no enzyme before Southern analysis of the mtDNA. The non-exonuclease-treated DNA contained both linear and nicked circular forms of mtDNA but did not contain closed circular supercoiled mtDNA (Fig. 8, *lane A*), suggesting that ciprofloxacin induces single- as well as double-stranded protein-linked breaks in the mtDNA. Treatment of the protein-linked DNA with exonuclease III (Fig. 8, *lane B*),

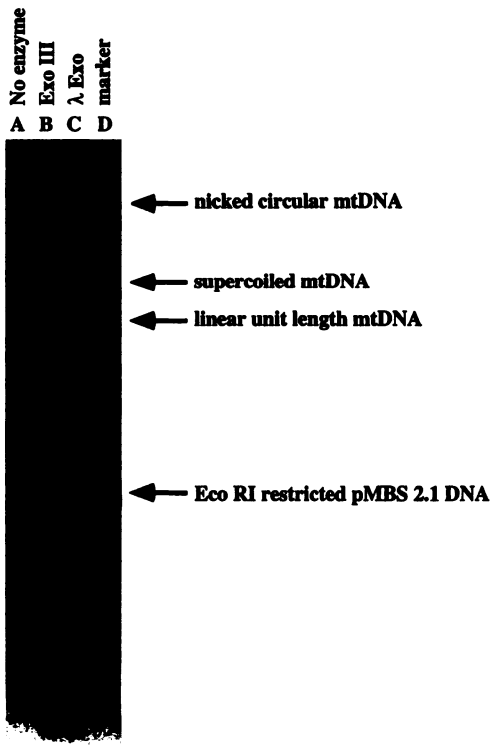

Fig. 8. Digestion of protein-linked mtDNA with λ exonuclease or exonuclease III. Protein-linked mtDNA was isolated from HeLa cells treated with 1 μg/ml ciprofloxacin for 1.5 hr using the glass bead method and analyzed by Southern blotting as described in Materials and Methods. Before Southern analysis, the sample was split into three equal aliquots. *Lane A*, untreated DNA; *lane B*, DNA was digested with 10 units of exonuclease III; *lane C*, DNA was digested with 1 unit of λ exonuclease. *Eco*RI restricted pMBS 2.1 plasmid DNA (100 pg) was included in the exonuclease reactions analyzed (*lanes B and C*) to confirm that the exonucleases were active. *Lane D*, 100 pg of *Eco*RI restricted pMBS 2.1 DNA only. The results are representative of an experiment that was performed in triplicate. *Arrows*, positions of nicked circular, supercoiled, and linear mtDNA as well as linear pMBS 2.1 plasmid DNA.

an enzyme that specifically degrades double-stranded DNA containing free 3′-ends, caused a loss of the mtDNA signal. In contrast, the mtDNA signal was unchanged after digestion by λ exonuclease, an enzyme that specifically acts on duplex DNA containing free 5′-ends (Fig. 8, *lane C*). As an internal control, *Eco*RI restricted pMBS 2.1 DNA was included in the exonuclease reactions to monitor the level of enzyme activity. The pMBS 2.1 DNA was degraded >95% in reactions containing either exonuclease III or λ exonuclease, indicating that both enzymes were highly active. These results strongly suggest that the ciprofloxacin-induced breaks in mtDNA contain protein bound at the 5′-, but not 3′-, ends of the DNA.

Stabilization of nuclear protein/DNA complexes by ciprofloxacin. Neutral elution studies have shown that ciprofloxacin can induce double-stranded breaks in nuclear DNA that are reversible on drug washout, suggesting the presence of a drug-sensitive nuclear topoisomerase II activity (36). However, these studies did not assess whether these breaks were protein linked. To address this issue, nuclear cleavable-complex formation was measured in CHO cells using a glass filter binding assay (20). After a 1-hr treatment with VM-26, a specific inhibitor of nuclear type II topoisomerases, or ciprofloxacin, cells were lysed, and the level of protein-linked DNA was measured (Fig. 9). As expected, VM-26 produced a dose-dependent increase in the amount of protein-linked DNA (Fig. 9A). In contrast, concentrations of ciprofloxacin of ≤100 μg/ml caused no detectable increase in protein-linked DNA (Fig. 9B). However, a low but significant increase in protein-linked DNA was detected at 300 μg/ml

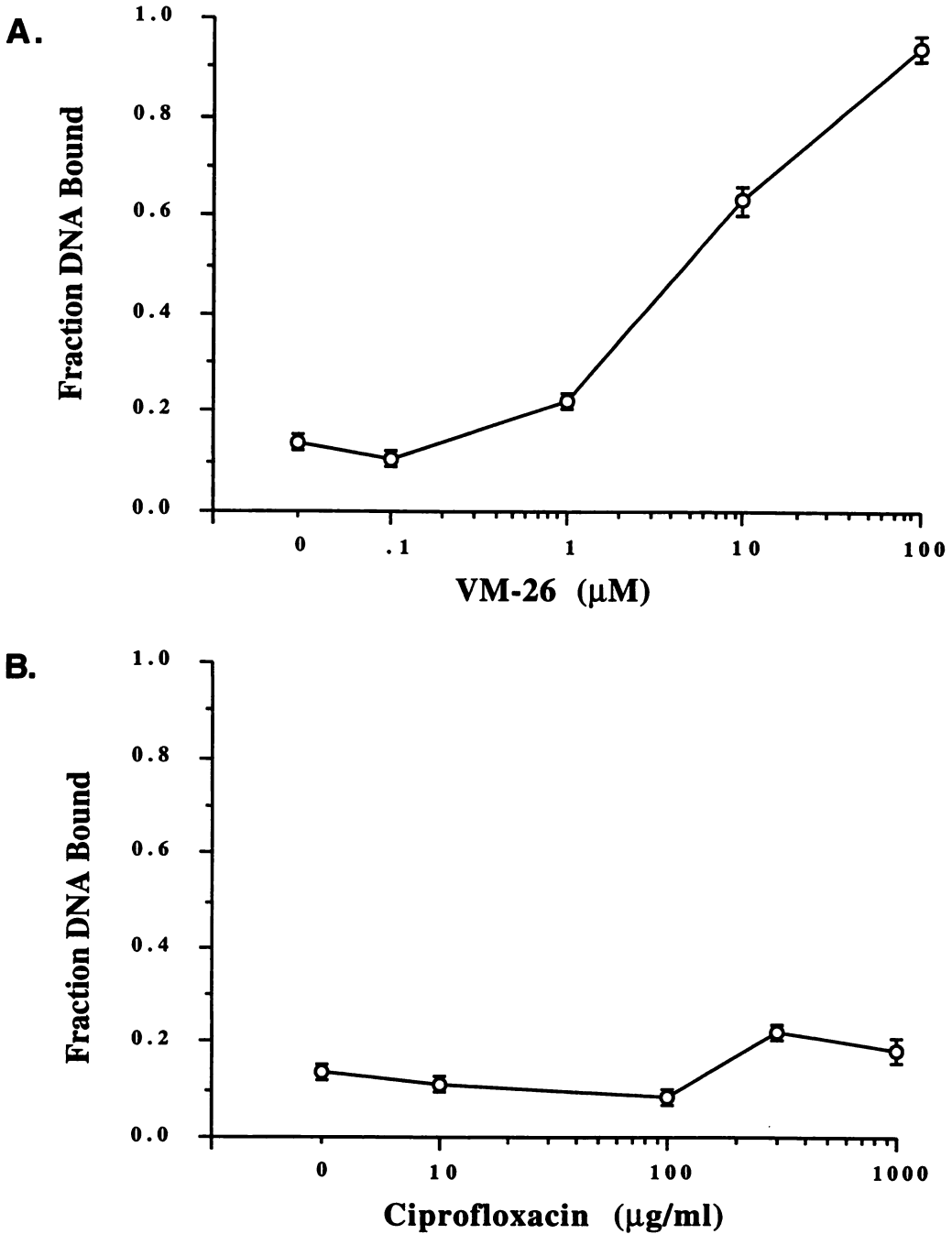

Fig. 9. Nuclear cleavable complex formation in CHO cells exposed to VM-26 or ciprofloxacin. The level of protein-linked DNA was measured in CHO cells maintained in DMEM using the protein-linked filter assay as described in Materials and Methods. Values represent the mean ± standard error of triplicate samples. A, Cells treated with VM-26. B, Cells treated with ciprofloxacin.

ciprofloxacin, suggesting that ciprofloxacin has some affect on nuclear topoisomerase II.

Discussion

Although ciprofloxacin has been shown to inhibit mammalian cell growth, the mechanism underlying this effect has yet to be elucidated (4–8). Several studies have suggested that ciprofloxacin may be inhibiting growth by interfering with *de novo* pyrimidine biosynthesis (7). However, cells could not be rescued by the addition of uridine to the growth medium, suggesting that an additional pathway or pathways were involved in growth arrest. Other studies have suggested that ciprofloxacin may be targeting nuclear topoisomerase II. This is supported by the finding that ciprofloxacin induces reversible double-stranded breaks in nuclear DNA (7, 36). However, it was unclear whether these drug-induced DNA breaks were protein linked. We detected a small but significant level of nuclear protein-linked DNA complexes at very high concentrations of ciprofloxacin (>100 μg/ml), suggesting that the drug may have some affect on a nuclear topoisomerase II. However, these concentrations of drug were considerably higher than those required to cause cytotoxicity, suggesting another target of action. Also, in contrast to drugs that are known to target nuclear topoisomerase II, ciprofloxacin does not seem to cause nuclear DNA mutations or rearrangements, nor does it cause a G2 arrest in the cell cycle (6, 8, 26). The kinetics of cell killing by ciprofloxacin are also different from those of nuclear topoisomerase II-acting drugs. The current results indicate that ciprofloxacin is not cytotoxic unless cells are continuously exposed to drug for a minimum of three or four cell doublings. In comparison, drugs that target nuclear topoisomerase II trigger an apoptotic type of cell killing, even after a short 2-hr drug exposure (37, 38).

Another possibility is that the growth inhibitory and cytotoxic effects of ciprofloxacin are caused by the inhibition of an essential mitochondrial function or functions. This is supported by the following observations: First, treatment of mammalian cells with ciprofloxacin results in a selective depletion of mtDNA, leading to a decrease in mitochondrial respiration (6). These mitochondrial events precede the drug-induced loss in cell growth and viability (Ref. 6 and current results). Second, cells become resistant to ciprofloxacin when they are grown under conditions that do not require mtDNA-encoded functions. Third, ciprofloxacin induces the formation of site-specific, protein-linked breaks in mtDNA, indicating the presence of a drug-sensitive mitochondrial topoisomerase II-like activity.

Cultured mammalian cells typically contain 10^3–10^4 copies of mtDNA (39). Studies using selective inhibitors of mtDNA replication suggest that this copy number is in great excess of that required to maintain cell growth and viability. Exposure of cultured human cells to ethidium bromide has been shown to cause a 2-fold dilution of mtDNA after each cell division (40, 41). However, no effect on cell growth was observed for three or four cell doublings, during which there was a >80% loss of the mtDNA. These results suggested that cells require only 10–20% of their normal complement of mtDNA to sustain growth. Analogous results have been obtained for other inhibitors of mtDNA synthesis, including 2′,3′-dideoxycytidine (a putative inhibitor of mitochondrial γ DNA polymerase; 42), the polyamine analogs *N*1,*N*12-bis(ethyl)spermine and methylglyoxal bis(guanylhydrazone) (43–45), and the bisintercalating drug dequalinium (46). Ciprofloxacin causes a similar delayed loss in mammalian cell growth and viability. Neither cell growth nor viability seems to be affected until cells have undergone three or four cell doublings in the presence of ciprofloxacin (Ref. 6 and current results). During this time span, the content of mtDNA decreases >90%, suggesting that drug is causing a loss in cell growth and viability by interfering with mtDNA replication.

The role of mtDNA as a target for drug action is also consistent with the observed affect of ciprofloxacin on *de novo* pyrimidine synthesis (7). Treatment of human lymphocytes with ciprofloxacin has been reported to cause a delayed inhibition of *de novo* pyrimidine synthesis that paralleled the loss in cell growth. This effect could not be attributed to a direct inhibition of the enzymes involved in pyrimidine biosynthesis (8, 47). Furthermore, the arrest of cell growth by ciprofloxacin could not be reversed by supplementing the culture medium with exogenous pyrimidines, suggesting that additional cellular pathways were being affected (7). Interestingly, one of the enzymes involved in *de novo* pyrimidine synthesis, dihydro-orotate dehydrogenase, is located on the outer surface of the inner mitochondrial membrane. This enzyme catalyzes the oxidation of dihydro-orotate to orotate, a reaction that is coupled to the reduction of components in the mitochondrial respiratory chain (48). A loss in respiratory function, due to the depletion of mtDNA, would therefore be expected to uncouple and block *de novo* pyrimidine biosynthesis. Although a blockade in *de novo* pyrimidine synthesis can be overcome by the addition of exogenous pyrimidines (7), additional nutrients are required to accommodate a loss in mtDNA-encoded functions (21, 22). In addition to pyrimidines, cells lacking mtDNA have been found to require pyruvate and high levels of glucose to sustain growth (21, 22). Our studies showed that cells grown under these conditions are resistant to ciprofloxacin, further implicating mtDNA as a drug target.

In vitro studies in rat liver mitochondria have suggested that mammalian mtDNA replication is inhibited by 4-quinolones. Castora *et al.* (11) reported that the 4-quinolone drugs nalidixic acid and oxolinic acid preferentially inhibited the incorporation of labeled nucleotides into elongation and termination intermediates of mtDNA replication. No effect was seen on the formation of 7–9 S initiation (D-loop) intermediates in mtDNA synthesis. In contrast, a more recent study reported that ciprofloxacin had no apparent affect on the incorporation of labeled nucleotides into rat mtDNA (10). However, the experimental approach used in this latter study would not have detected a selective affect of drug on a specific stage or stages in mtDNA replication because the relative incorporation of label into the different replication intermediates (initiation, elongation, termination) was not measured.

4-Quinolones have also been shown to interfere with mtDNA replication and gene expression in fungi (49–53). Genetic and biochemical studies in the yeast *Saccharomyces cerevisiae* have demonstrated that nalidixic acid selectively blocks mitochondrial gene expression and replication, resulting in the induction of mitochondrial petite mutants (respiratory deficient mutants caused by deletions or point mutations in the mtDNA). In addition, the fluoroquinolone

ofloxacin has recently been shown to induce petite mutants containing deletions in their mtDNA (54).

Inhibition of mtDNA replication by 4-quinolone drugs would provide an explanation for the selective depletion of mtDNA from mammalian cells. It has been hypothesized that 4-quinolones interfere with mtDNA synthesis by inhibiting a bacteria-like topoisomerase II activity similar to that of DNA gyrase (11). In support of this hypothesis, we found that the 4-quinolone drug ciprofloxacin stimulates the formation of site-specific double-stranded breaks in mtDNA. Furthermore, exonuclease protection studies indicate that the 5′-ends of these breaks are linked to protein, strongly suggesting that mitochondria contain a drug-sensitive topoisomerase II-like activity. Inhibition of such an activity would be consistent with *in vitro* replication studies showing that 4-quinolones inhibit elongation and supercoiling of mtDNA, processes that are known to be facilitated by topoisomerase II enzymes (1, 3, 25, 55). However, it is also plausible that ciprofloxacin acts by interfering with the function of a different protein or process that is critical to mtDNA replication.

A puzzling aspect of our studies was that cytotoxicity and the depletion of mtDNA occurred at drug concentrations that were 10–20-fold higher than those that stimulated cleavage of mtDNA. The reason for this discrepancy is unclear. However, *in vitro* studies have shown that inhibition of DNA gyrase catalytic activity requires ciprofloxacin concentrations that are ~10-fold higher than those that stimulate DNA cleavage (56, 57). This raises the issue that depletion of mtDNA may be caused by inhibition of topoisomerase II catalytic activity rather than by a conversion of the drug-stabilized enzyme/DNA cleavable complex into a lethal lesion in the mtDNA (1, 26). A similar scenario has recently been proposed to explain the action of fluoroquinolones on *Escherichia coli* topoisomerase IV, a newly discovered bacterial type II topoisomerase that shares significant homology with DNA gyrase (58, 59).

Although ciprofloxacin induced maximal mtDNA cleavage at very low drug concentrations (0.3–3 μg/ml), cleavage was inhibited at higher concentrations (≥10 μg/ml). The mechanism underlying this inhibition is unclear. However, a similar observation has been made for DNA intercalating anticancer drugs that target nuclear topoisomerase II (29, 30). Inhibition of cleavage by high concentrations of these drugs has been attributed to destabilization of the topoisomerase II/DNA cleavable complex due to an extensive unwinding of the DNA double helix by these intercalating agents. Ciprofloxacin, as well as several other 4-quinolone drugs, can cause significant unwinding of DNA (Refs. 32 and 33 and current results). However, evidence suggests that unwinding is probably mediated through an interaction of drug with single-stranded DNA rather than by a mechanism involving DNA intercalation (2). Interestingly, an unusual feature of mtDNA replication is the formation of large single-stranded replication intermediates (39). Binding of drug to these single-stranded regions may become saturated at high drug concentrations and directly interfere with the DNA binding and/or cleavage activity of the putative mitochondrial topoisomerase II. Alternatively, high drug concentrations may affect some other mitochondrial process that causes an indirect inhibition of mtDNA cleavage.

Lin and Castora (60) recently showed that a partially purified topoisomerase II activity from calf thymus mitochondria is inhibited by the nuclear topoisomerase II inhibitors VM-26 and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide. Whether this activity is related to the putative ciprofloxacin-sensitive topoisomerase II-like activity is unclear. However, preliminary studies in our laboratory indicate that treatment of mammalian cells with VM-26 also induces cleavage of mtDNA (data not shown), suggesting that the mitochondrial enzyme may be related to the topoisomerase II activity detected by Lin and Castora (60). The possibility also exists that the putative ciprofloxacin-sensitive mitochondrial topoisomerase is related to the nuclear α or β isoform of topoisomerase II (55). However, if the mitochondrial enzyme is encoded for by the α or β topoisomerase II gene, why should ciprofloxacin have a selective effect on mtDNA? Possibly, there is a preferential accumulation of drug into the mitochondrial compartment, or perhaps basic differences in the biochemical environment present in mitochondria contribute to the selective action of drug on the mitochondrial enzyme. Future studies should help to clarify these issues.

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