

# Analysis of DNA damage and repair in murine leukemia L1210 cells using a quantitative polymerase chain reaction assay

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## ABSTRACT

The polymerase chain reaction (PCR) represents an alternative to the current methods for investigating DNA damage and repair in specific genomic segments. In theory, any DNA lesion which blocks *Taq* polymerase can be measured by this assay. We used quantitative PCR (QPCR) to determine the lesion frequencies produced by cisplatin and ultraviolet light (UV) in a 2.3 kilobase (kb) segment of mitochondrial DNA and a 2.6 kb segment of the *DHFR* gene in mouse leukemia L1210 cells. The frequency of UV-induced lesions increased linearly with dose, and was 0.58 lesions/10 kb/10 J/m<sup>2</sup> in the mitochondrial DNA, and 0.37 lesions/10 kb/10 J/m<sup>2</sup> in the *DHFR* gene. With cisplatin, the lesion frequency also increased linearly with dose, and was 0.17 lesions/10 kb/10 μM in the *DHFR* gene, and 0.07 lesions/10 kb/10 μM in mitochondrial DNA. This result is contrary to that of Murata *et al.*, 1990 (1), in which mitochondrial DNA received greater cisplatin damage than did nuclear DNA. Using PCR to measure the repair of UV-induced lesions in the *DHFR* gene segment, we observed that less than 10% of the lesions were removed by 4 h, but over 70% of the lesions were removed by 8 h. Repair of 43% of UV-induced lesions in mitochondrial DNA was also observed during a 24 h period.

## INTRODUCTION

There is now a large amount of evidence which indicates that the repair of specific DNA lesions does not occur equally throughout the entire genome, but varies in accordance with such factors as chromatin structure, sequence context and transcriptional activity (2). For example, ultraviolet (UV)-induced pyrimidine dimers are removed from actively transcribed genes more efficiently than from nontranscribed regions of the genome (3-7). This gene-specific repair is apparently due to the rapid repair of the transcribed strand of certain actively expressed genes (8-10). In addition, there is evidence that (6-4) photoproducts (11), cisplatin-DNA adducts (12), aflatoxin B1 induced lesions

(6) and lesions induced by some alkylating agents (13,14) are also removed in a gene-specific manner. Such observations may reflect the varying accessibility of different genomic regions to DNA damaging agents, the varying accessibility of these genomic regions to DNA repair proteins and/or the coupling of DNA repair with other cellular processes such as transcription (2,15,16).

Preferential repair of DNA damage in actively transcribed genes was initially identified using a Southern hybridization based assay employing T4 endonuclease V, which specifically cleaves DNA at pyrimidine dimer sites (17). While this methodology has proven useful in the identification and study of gene-specific repair, it also has certain limitations, including the requirements for relatively large amounts of DNA, for specific restriction sites flanking the region to be studied, and most importantly, for a lesion specific endonuclease to incise near the damaged nucleotide. Because of this latter requirement, most of the investigations using this assay have focused on the repair of UV-induced pyrimidine dimers, recognized by T4 endonuclease V. In order to use the Southern hybridization assay to investigate the repair of other lesions in specific genomic regions, some investigators (12,13,18) have used the *Escherichia coli* UvrABC nuclease complex (19). However, this approach has certain limitations. Since the UvrABC complex only produces stoichiometric incisions, the use of this enzyme complex for single-copy genes is difficult (due to the large amount of DNA). Furthermore, for quantitative purposes, it is necessary to measure the UvrABC incision efficiency for each lesion studied.

A new approach to investigate DNA damage and repair in specific genomic regions using the polymerase chain reaction (PCR) has recently been reported (20). This assay is based on the fact that many DNA lesions can block the *Taq* polymerase, and thereby result in a decrease in amplification of a damaged DNA segment compared to the amplification of the same segment in a nondamaged template. Poisson analysis is then used to determine the lesion frequency. A quantitative PCR assay thus has the potential to measure the lesion frequency and subsequent repair of any DNA damaging agent which blocks *Taq* polymerase (or other polymerases used in PCR) in any DNA segment for

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which flanking sequences are known. The initial investigation of Govan *et al* (20), however, did not fully demonstrate the power of the assay because of the small size of the segments being amplified (<450 bp). This limited the sensitivity of the assay, and thus made necessary the use of extremely large doses of UV (1–24 kJ/m<sup>2</sup>) which greatly exceeded the range generally considered to be biologically relevant (17). In order to measure DNA damage and repair in cells exposed to lower doses of DNA damaging agents, we have refined the quantitative PCR (QPCR) assay by examining DNA damage in kilobase gene segments. The present study reports the lesion frequencies produced by more physiologically relevant doses of UV and the anticancer drug *cis*-diamminedichloroplatinum (II) (cisplatin) in the dihydrofolate reductase (*DHFR*) gene and mitochondrial DNA of murine leukemia L1210 cells. In addition, the initial rates of repair of UV-induced damage in the *DHFR* gene and mitochondrial DNA are also presented.

## MATERIALS AND METHODS

### Cell cultures

The murine leukemia cell line, L1210, used in this study was obtained from Dr. Miles Hacker, Department of Pharmacology, UVM. L1210 cells grow in suspension, and were cultured at 37°C in McCoy's 5A medium (Gibco, Grand Island, NY) supplemented with 5% heat inactivated horse serum (Hazelton, Lenexa, KS).

### Ultraviolet (UV) treatment of cells

L1210 cells were grown to a concentration of 1–1.5 × 10<sup>6</sup> cells/ml, pelleted, washed once with ice-cold sterile phosphate-buffered saline (PBS) and resuspended in PBS at 2 × 10<sup>7</sup> cells/ml. Cells were added (1 × 10<sup>7</sup> cells in 0.5 ml) to 100 mm tissue culture plates and spread over the complete surface. These cells were then irradiated with a 254 nm Sylvania G8T5 germicidal lamp at an incident dose rate of 1 J/m<sup>2</sup>/sec for various time periods. Following irradiation, the cells were recovered from the plates by multiple washings with PBS. All cells irradiated by the same UV dose were pooled, and then pelleted. Following removal of the liquid, the cells were either frozen for subsequent DNA preparations, or incubated in fresh culture medium to allow for a period of DNA repair (4 and 8 h for the *DHFR* gene; 4, 8, and 24 h for mitochondrial DNA). After this time, the cells were recovered, washed with ice-cold PBS and frozen for subsequent analysis.

### Cisplatin (*cis*-DDP) treatment of cells

L1210 cells were grown to a concentration of 1–1.5 × 10<sup>6</sup> cells/ml, pelleted, washed once with ice-cold sterile PBS and resuspended at a concentration of 1 × 10<sup>7</sup> cells/ml in fresh, unsupplemented McCoy's medium (lacking horse serum). An equal volume of unsupplemented McCoy's medium containing various doses of cisplatin (kindly provided by Dr. Nick Farrell, Department of Chemistry, UVM) was added to the cell suspension, and the cells were then incubated at 37°C for 2 h. Following incubation, the cells were pelleted, washed twice with ice-cold sterile PBS and frozen for later DNA preparations.

### Isolation of genomic DNA and restriction enzyme digests

Frozen L1210 cell pellets were thawed at room temperature and resuspended in 3 ml lysis buffer (2% SDS, 0.5 M NaCl, 0.05 M EDTA, 0.05 M TRIS HCL (pH 7.5)) containing 200 µg/ml

proteinase K (Boehringer Mannheim, Indianapolis, IN). After overnight incubation at 37°C, the lysates were extracted twice by phenol:chloroform:isoamyl alcohol (PCI) (25:24:1 v/v/v), and once with chloroform:isoamyl alcohol (24:1 v/v). The aqueous phase was transferred to a sterile 15 ml Corex tube, adjusted to 300 mM sodium acetate and then total genomic DNA was precipitated by addition of 2 vols of ice-cold 95% ethanol. Following transfer by pipet tip to a microcentrifuge tube, the genomic DNA was washed in 70% ethanol, lyophilized and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). To reduce the viscosity of the DNA solution, the DNA was subsequently incubated with either 3–5 U/µg DNA of restriction endonuclease *BAMHI* (BRL, Gaithersburg, MD) at 37°C overnight for PCR, or with 3–5 U/µg of restriction endonuclease *EcoRI* (BRL, Gaithersburg, MD) for Southern analysis. A PCI extraction was then performed, and the DNA was precipitated in 2 vols of 95% ethanol, washed with 70% ethanol, lyophilized and resuspended in TE buffer. The concentration of DNA was determined by fluorometry (Model 450, Sequoia-Turner, Mountain View, CA) using Hoechst 33258 dye (Sigma, St. Louis, MO).

### Amplification of genomic and mitochondrial DNA by PCR

The oligonucleotide primers used in this study, shown below, were prepared on a Model 391 PCR-mate DNA Synthesizer (IBI, Foster City, CA). A 2.3 kb segment of mitochondrial DNA (21) was amplified using the Mito 1 and Mito 2 primers, and a 2.6 kb segment of the *DHFR* gene (22) was amplified using the *DHFR* 1 and *DHFR* 2 primers (Figure 1). In order to confirm that the desired *DHFR* gene segment or mitochondrial DNA segment was amplified, after Southern transfer, amplification products were probed with radiolabeled oligonucleotides specific for the 5' region of the mouse *DHFR* gene (American Type Culture Collection, Rockville, MD), or for the mouse mitochondrial genome (pAM1, obtained from D. Clayton, Stanford University; ref 23).

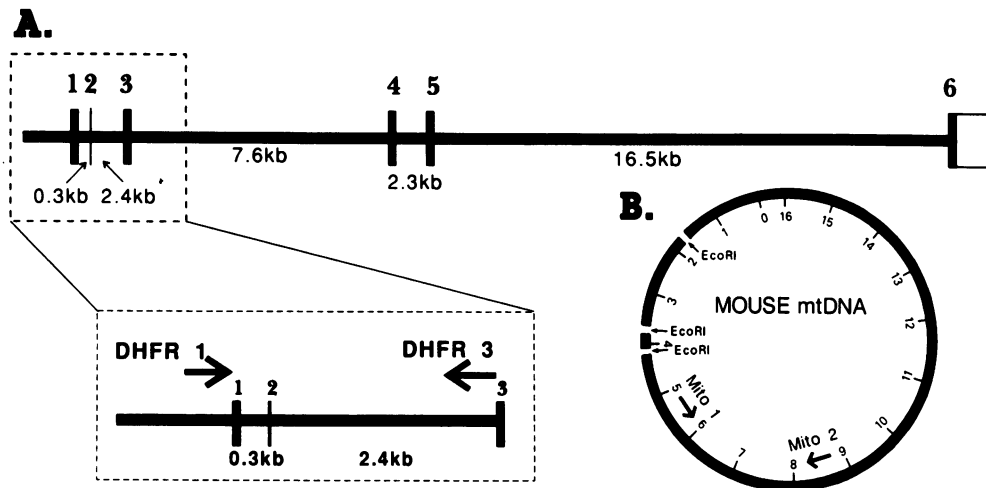
–Mito 1: 5'GCAGGAACAGGATGAACAGTCT, derived from the sense strand.

–Mito 2: 5'TGGAGACGGTTGTTGATTAGGC, derived from the antisense strand.

–*DHFR* 1: 5'CTAAGCTGCGCAAGTGGTAC, derived from the sense strand.

–*DHFR* 2: 5'GTGGAGAACAGGTTCTAATCC, derived from the antisense strand.

All amplification reactions, based on the methodology of Mullis and Faloona (24), were carried out in 0.5 ml microcentrifuge tubes in a final volume of either 50 or 100 µl, overlaid with mineral oil. Each reaction consisted of 500 ng of total genomic DNA, and contained the two appropriate primers (1 µM each), the 4 deoxynucleotides (200 µM each), 2 µCi [<sup>32</sup>P] dCTP (3000 Ci/mmol), 2.5 Units of *Taq* DNA polymerase (Promega Corporation, Madison, WI) and the supplied buffer (final concentration of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl<sub>2</sub>, 0.01% (w/v) gelatin and 0.1% TritonX-100). Amplification was accomplished using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CN). For the amplification of mitochondrial DNA, samples underwent 10 cycles of template denaturation at 94°C for 1 min, primer-template annealing at 55°C for 2 min and primer extension at 72°C for 2 min. For the amplification of genomic DNA, samples underwent an initial denaturation at 94°C for 4 min, followed by 25 cycles of template denaturation at 94°C for 1 min, primer-template annealing at



**Figure 1.** Map of the mouse dihydrofolate reductase (*DHFR*) gene (A) and mitochondrial genome (B). The primer locations for PCR are indicated by the labeled arrows (DHFR 1 & 3, Mito 1 & 2). The amplified segment of the *DHFR* gene is 2.6 kilobases (kb), and the amplified segment of the mitochondrial genome is 2.3 kb. For the *DHFR* gene, the exons are designated by numerals above the line. For the mitochondrial genome, the numbers in the interior of the circular map represent kilobases.

49°C for 1 min and primer extension at 72°C for 2 min. At the end of either 10 or 23 cycles, the extension reaction was continued for 7 additional min.

Following amplification, the mineral oil was removed with chloroform, and the radiolabeled amplification products were separated from unincorporated [<sup>32</sup>P] dCTP by electrophoresis in a 4.0% non-denaturing polyacrylamide gel, or in a 1.0% vertical agarose gel. Polyacrylamide gels were dried on a slab gel drier (HaakeBuchler, Saddle Brook, NJ), and agarose gels were dried on gelbond paper (FMC Bioproducts, Rockland, ME) at 57°C overnight, for autoradiography and/or quantitative analysis.

#### Quantification of DNA lesions using PCR

Amplification products were radioactively labeled by incorporation of [<sup>32</sup>P] dCTP during PCR (25). Radiolabeled amplification products were quantified from acrylamide or agarose gels using a Betascope 603 Blot Analyzer (Betagen, Waltham, MA). The amount of amplification was represented by the amount of radioactivity (in cpm) in the amplification product minus the background radioactivity. In order to measure the effect of DNA damage on amplification, the amount (cpm) of amplification product from genomic DNA which had received damage was divided by the amount (cpm) of amplification product from genomic DNA which was not damaged. This gave the fraction of nondamaged templates at a given dose of DNA damaging agent. The QPCR assay determines the number of lesions per strand, in which both strands are measured simultaneously. Thus, the assay measures the average lesion frequency per strand for the two template strands in the genomic segment of interest. Assuming a random distribution of lesions, the Poisson equation ( $e^{-s}$  where  $s$  = lesion frequency) was then used to calculate the lesion frequency per genomic strand.

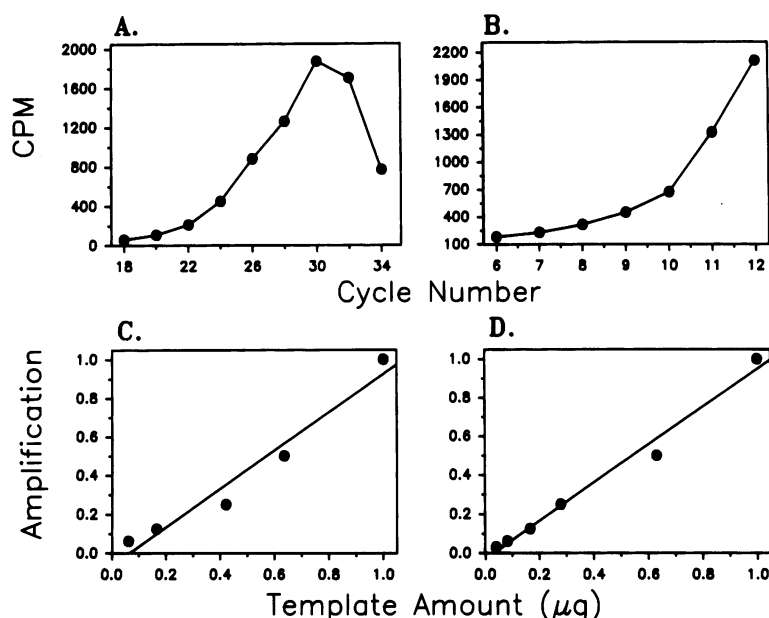
$s = -\ln A_D/A_0$  = lesion frequency/strand, where,  $A_0$  = amount of amplification (cpm) produced from a given amount of nondamaged DNA template, and  $A_D$  = amount of amplification (cpm) produced from a given amount of damaged DNA template (damaged by a particular dose of DNA damaging agent), so that  $A_D/A_0$  = fraction of nondamaged templates at a given dose (the zero class).

#### Enzyme assays for southern analysis

**UvrABC reactions.** The UvrA, UvrB and UvrC subunits were purified from *Escherichia coli* strain CH296 (containing the plasmids pUNC45, pUNC211 and pDR3274, respectively) essentially as described by Sancar and coworkers (26). These strains were generously supplied by A. Sancar, University of North Carolina.

A typical reaction (40  $\mu$ l) contained 1  $\mu$ g of DNA, and was carried out in 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl<sub>2</sub>, 5 mM DTT and 250  $\mu$ g/ml BSA. As required, reactions contained 2 mM ATP, 3.4 pmol UvrA, 15 pmol UvrB and 15 pmol UvrC. Reactions were incubated for 30 min at 37°C, and then Proteinase K and SDS were added to a final concentration of 1.9  $\mu$ g/ml and 0.4%, respectively. Digestion at 37°C was carried out for 60 min, followed by 2 h of microdialysis against TE (10 mM Tris-HCl, 1 mM EDTA). Samples were denatured at 65°C for 10 min by adding prewarmed 10 $\times$  running dye (to give a final concentration of 2.5% Ficoll, 1 mM EDTA, 0.025% bromocresol green, 0.05 M NaOH (added just before use) and 0.1% SDS), and then electrophoresed on a 0.55% alkaline agarose gels for 16–20 h at 90 mAmps and 35–45 volts. Final concentration of gel and running buffer was 30 mM NaOH, 1 mM EDTA. After electrophoresis, DNA was Southern transferred to Genatran 45 nylon (Plasco, Woburn, MA) and probed with the radiolabeled mitochondrial genome (pAM1, obtained from D. Clayton, Stanford University; ref 23). The probe was prepared using a random-oligolabeling kit (Pharmacia, Piscataway, NJ) and purified by gel filtration using mini-spin columns (Worthington, Freehold, NJ).

**T4 Endonuclease V reactions.** Endonuclease V from T4-phage was partially purified from the *E. coli* strain AB2480, which carried the over producing plasmid, pTac-denV (27) (obtained from Drs. K. Valerie and J. deRiel, Temple University). This reagent grade protein does not contain any nonspecific nucleases. The enzyme activity ranged from 10<sup>10</sup>–10<sup>11</sup> nicks per min/ $\mu$ l when assayed in a 25  $\mu$ l reaction containing superhelical PM2 DNA (500 ng) with 2–3 pyrimidine dimers per molecule. A typical reaction (40  $\mu$ l) contained 1  $\mu$ g of DNA, 10 mM Tris-



**Figure 2.** The cycle range for the *DHFR* gene segment (A) and mitochondrial DNA segment (B) in which the amount of amplification product increased exponentially with increasing cycle number. PCR amplification of the *DHFR* gene segment at 25 cycles (C) or mitochondrial DNA segment at 10 cycles (D) with 1.0, 0.5, 0.25, 0.125 or 0.0625  $\mu\text{g}$  of total genomic DNA. For these plots (C and D), the amount of amplification produced with 1  $\mu\text{g}$  of template DNA = 1.0.

HCl (pH 7.5), 10 mM EDTA, 100 mM NaCl, 1 mg/ml BSA (BRL, Bethesda, MD) and T4-endonuclease V (8  $\mu\text{l}$ ). The reaction was incubated for 30 min at 37°.

**Endonuclease III reactions.** Endonuclease III was a generous gift of Dr. Y.W.Kow (Dept. of Microbiology and Molecular Genetics, UVM), and was prepared as described previously (28,29). Typical reactions contained endonuclease III (240 ng), 10 mM Tris (pH 7.5), 1 mM EDTA, 100 mM KCl and DNA (1  $\mu\text{g}$ ). As a positive control, genomic DNA was treated with osmium tetroxide (0.04%, 65°C for 5–15 min), which produced thymine glycols (30).

#### Quantification of ESS sites by Southern analysis

Lesion frequencies were determined by the amount of full length (14 kb) *EcoRI* restriction fragment remaining following digestion with one of the three damage specific endonucleases, compared to a no enzyme control. The amount of radioactivity representing the full length 14 kb band was quantified by radioimaging on a Betascope 603 Blot analyzer (Betagen). The number of endonuclease sensitive sites (ESS) was determined using the Poisson distribution and the fraction of the 14 kb band remaining after enzyme digestion, where the number of ESS (N) =  $-\ln$  (fraction of cpm remaining).

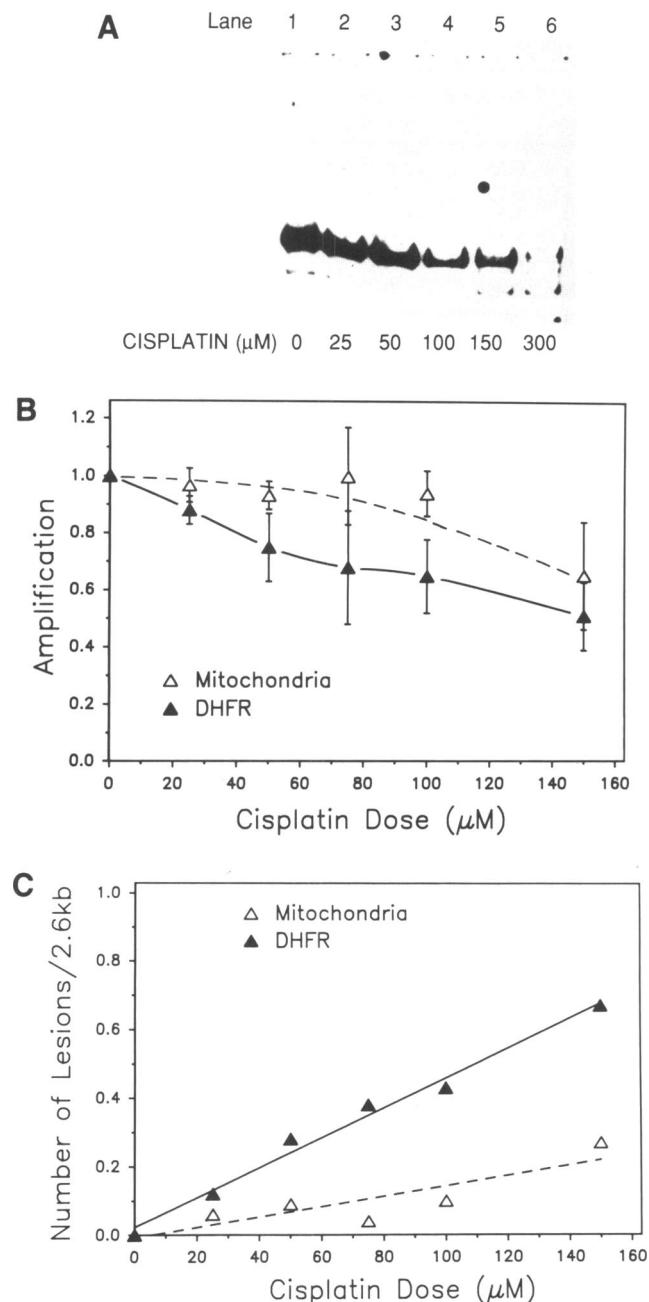
## RESULTS

### Quantitative PCR

In order to use PCR to quantify lesion frequencies, it is absolutely necessary that the only limiting component for the amplification of a given DNA segment be the amount of nondamaged template. In other words, any decreased amplification of a damaged DNA template (compared to the same amount of nondamaged template) must be due to the blocking of *Taq* polymerase by DNA lesions,

and not the exhaustion of some critical reagent. For this to be the case, at a given template concentration, amplifications must be performed at a cycle number that is within the exponential phase of the PCR (31–35). This insures that other components of the reaction, such as dNTPs, primers, or *Taq* polymerase, are not limiting. For every primer set, therefore, an important parameter that must be empirically determined for a given template concentration is the cycle range in which the amplification product increases exponentially with increasing cycle number. For the single copy *DHFR* gene segment, using 500 ng of total genomic DNA, this was between 18 and 30 cycles (Figure 2A), and for the multicopy mitochondrial DNA segment, using 500 ng of total genomic DNA, this was between 6 and 12 cycles (Figure 2B). Subsequently, all amplifications of mitochondrial DNA were performed at 10 cycles, and all amplifications of the *DHFR* gene segment were performed at 25 cycles.

To further insure that the limiting factor for amplification was nondamaged template availability, the amount of nondamaged template was titrated from 0.0625  $\mu\text{g}$  to 1.0  $\mu\text{g}$  to determine if the amplification signal was linearly related to the amount of nondamaged template. Figures 2C and 2D show that this is the case for both the 2.6 kb *DHFR* gene segment at 25 cycles, and the 2.3 kb mitochondrial DNA segment at 10 cycles. Thus, decreasing the template concentration by half decreases the amplification signal by 50%. This was used as a control in subsequent experiments to account for thermal cyclers variations (32,36). An additional experiment (data not shown) further demonstrates that QPCR is dependent not only on cycle number, but also on the amount of template DNA. In this experiment, a dose dependent decrease in amplification (with increasing dose of UV) of the *DHFR* gene segment at 25 cycles was not observed when using 2  $\mu\text{g}$  total genomic DNA in the reaction mixture, but was observed when using 500 ng. At the higher template



**Figure 3.** Cisplatin dose response. (A) Autoradiogram of a cisplatin dose response (0, 25, 50, 100, 150 or 300  $\mu\text{M}$ ) for the amplification of the *DHFR* gene segment. The radiolabeled amplification products at each dose were separated from unincorporated [ $^{32}\text{P}$ ] dCTP on a 1.0% vertical agarose gel. (B) Amplification of the *DHFR* gene segment and mitochondrial DNA segment versus cisplatin dose ( $\mu\text{M}$ ). The amplification level represents the amount of amplification (in cpm) from a damaged DNA template at each cisplatin dose divided by the amount of amplification from a nondamaged template (0  $\mu\text{M}$  cisplatin). The data were derived from 2 independent experiments, and represent the mean of 3 PCR amplifications of the *DHFR* gene segment, and 4 PCR amplifications of the mitochondrial DNA segment,  $\pm$  S.E. The standard error ranged from 0.06 to 0.19 in mitochondrial DNA, and 0.05 to 0.20 in the *DHFR* gene. (C) Lesion frequencies in the *DHFR* gene segment and the mitochondrial DNA segment as a function of cisplatin dose ( $\mu\text{M}$ ). The lesion frequency for mitochondrial DNA has been normalized to a 2.6 kb segment. Based on the slope of the best fit line, the lesion frequency was 0.17 lesions/10 kb/10  $\mu\text{M}$  in the *DHFR* gene segment, and 0.066 lesions/10 kb/10  $\mu\text{M}$  in the mitochondrial DNA segment. (NOTE: Although the QPCR assay measures the decrease in a particular DNA fragment with increasing dose of DNA damaging agent, the assay actually simultaneously measures the lesion frequency for each strand. Thus, the data is reported as lesion frequency/2.6 kb segment).

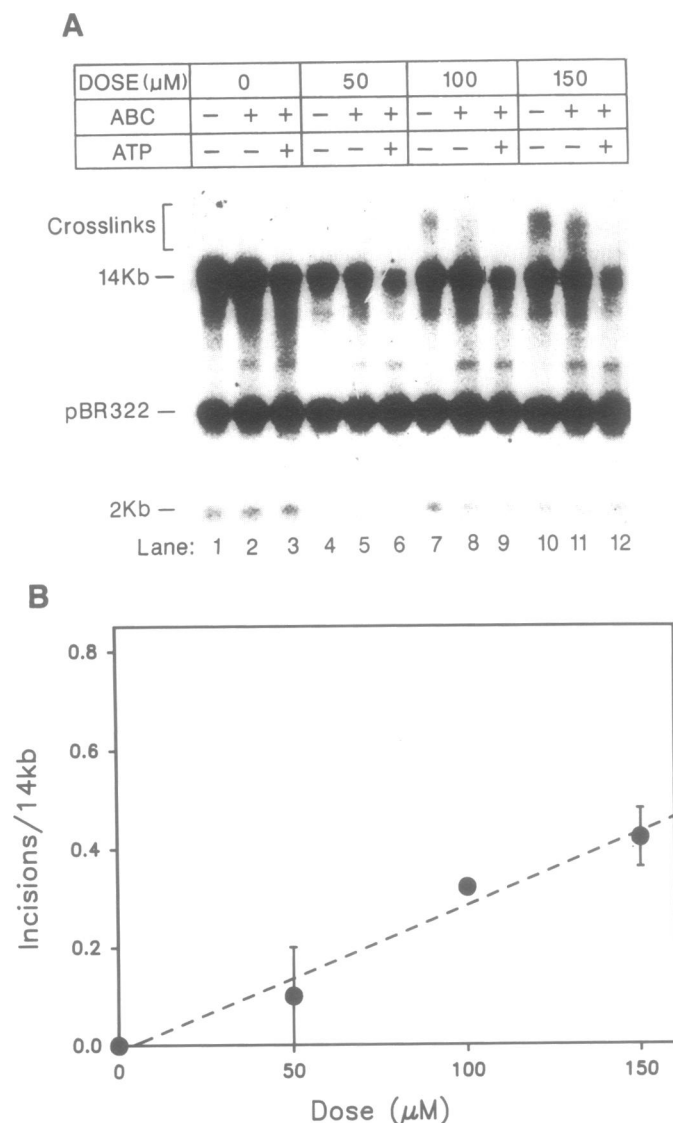
amount, the plateau phase of PCR had been reached by 25 cycles, and QPCR conditions were no longer operative.

#### Determining the frequency of cisplatin-DNA adducts and UV-induced photoproducts

**Cisplatin adducts.** Once the correct cycle range for QPCR had been established, experiments were conducted to determine lesion frequencies at various doses of cisplatin and UV. A typical experiment involved the amplification of the same amount of total genomic DNA (500 ng) isolated from cells exposed to increasing doses of DNA damaging agent (Figure 3A). With cisplatin, amplification decreased in a dose dependent manner from 0–150  $\mu\text{M}$  in the *DHFR* gene (Figure 3B). The cisplatin-DNA lesion frequency increased linearly with dose, and when normalized, was 0.17 lesions/10 kb/10  $\mu\text{M}$  cisplatin (Figure 3C). In the mitochondrial DNA segment, however, cisplatin did not produce a significant decrease in amplification until over 100  $\mu\text{M}$  (Figure 3B), and the lesion frequency when normalized, 0.066 lesions/10 kb/10  $\mu\text{M}$  cisplatin, was less than for the *DHFR* gene (Figure 3C). This result is contrary to an earlier finding in human malignant melanoma cells which showed mitochondrial DNA (when exposed to cisplatin) to receive more damage than nuclear DNA (1).

Validation of the QPCR gene-specific repair assay required that the sensitivity and detection limits match or exceed those observed with Southern hybridization based repair assays. We sought to directly compare both methods using the same damaged DNA. To this end, we have adapted the gene specific repair assay of Bohr and Hanawalt (3,4,11) for the quantification of DNA lesion frequencies in the mitochondrial genome. This approach uses total cellular DNA, making unnecessary the purification of mitochondrial DNA, and is dependent upon the quantitative conversion of DNA lesions into strand breaks by a damage specific endonuclease. Analysis of the DNA by electrophoresis on alkaline agarose gels, blotting to a nylon membrane and visualization by a radiolabeled mitochondrial probe allowed direct quantification of mitochondrial DNA lesions (see Materials and Methods). *EcoRI* digestion of the mouse mitochondrial genome results in three DNA fragments of 14.0 kb, 2.0 kb and 0.2 kb in length. All analyses were conducted on the 14.0 kb band.

To quantify the yield of cisplatin-DNA lesions, the UvrABC nuclease was used. This repair enzyme recognizes over 20 different types of DNA lesion (19), and has recently been used to monitor the repair of N-acetoxyaminofluorene adducts and cisplatin-DNA adducts in the *DHFR* gene of CHO cells (12,37). Data displayed in Figure 4 demonstrates that the UvrABC nuclease can detect a dose dependent increase in mitochondrial DNA adducts from cells treated with increasing doses of cisplatin (0–150  $\mu\text{M}$ ). It is interesting to note that at doses of 100  $\mu\text{M}$  or above, there is a population of DNA which migrates more slowly than the 14.0 kb fragment. This DNA might represent DNA interstrand cross-links or incomplete restriction enzyme digestion due to the high number of cisplatin-DNA adducts. In order to rule out this second alternative, the DNA was treated with cyanide prior to loading on the gel. Extensive cyanide treatment reverses cisplatin intra- and inter-strand cross-links, and was found to completely reverse this DNA band. Thus, this DNA band represents interstrand cross-links induced by the cisplatin (data not shown). Data from both assays are compiled in Table I. The QPCR assay is clearly detecting 3–5 fold more cisplatin lesions than the UvrABC Southern hybridization based assay.



**Figure 4.** Quantification of cisplatin-DNA adducts by Southern Analysis. Mouse L1210 cells were treated with 0, 50, 100, and 150  $\mu\text{M}$  of cisplatin for 2 hours at 37°C. (A) Radioactive image of a Southern blot. A typical blot was scanned for radioactivity using a Betascope 603 blot analyzer (Betagen). The radioactivity associated with the 14 kb band was quantified and used to determine the average number of cisplatin adducts/14 kb. (B) Data is displayed as the mean of two independent experiments.

**UV photoproducts.** QPCR was used to assay the number of UV-induced photoproducts in both the 2.3 kb mitochondrial DNA segment and the 2.6 kb *DHFR* gene segment. In both segments, UV-induced damage (0–80  $\text{J}/\text{m}^2$ ) decreased amplification in a dose dependent manner (Figure 5A). The calculated lesion frequency increased linearly with dose for both amplified segments, though slightly less for the *DHFR* segment. Based on the slope of this increase, the normalized lesion frequency was 0.58 lesions/10 kb/10  $\text{J}/\text{m}^2$  in the mitochondrial DNA segment, and 0.37 lesions/10 kb/10  $\text{J}/\text{m}^2$  in the *DHFR* gene segment (Figure 5B).

Southern hybridization analysis was also used to quantify the yield of UV-induced lesions in the mitochondrial genome following exposure to UV light (0–40  $\text{J}/\text{m}^2$ ). To do so, three

**Table 1.** Comparison of mitochondrial DNA lesions determined by Southern analysis and QPCR

Damage (Dose)	Southern <sup>a</sup>	PCR <sup>b</sup>	PCR <sup>c</sup> (normalized)	PCR/Southern <sup>d</sup>
UV (10 $\text{J}/\text{m}^2$ )	0.55	0.23	1.40	2.55
UV (20 $\text{J}/\text{m}^2$ )	0.60	0.40	2.43	4.05
UV (40 $\text{J}/\text{m}^2$ )	1.31	0.59	3.59	2.74
DDP <sup>e</sup> (50 $\mu\text{M}$ )	0.10	0.09	0.54	5.4
DDP (100 $\mu\text{M}$ )	0.32	0.1	0.61	1.9
DDP (150 $\mu\text{M}$ )	0.42	0.27	1.64	3.9

<sup>a</sup> = lesions/ 14 kb

<sup>b</sup> = lesions/ 2.3 kb

<sup>c</sup> = lesions/ 14 kb

<sup>d</sup> = mean = 3.42

<sup>e</sup> = DDP = cis-diaminodichloroplatinum, (cisplatin)

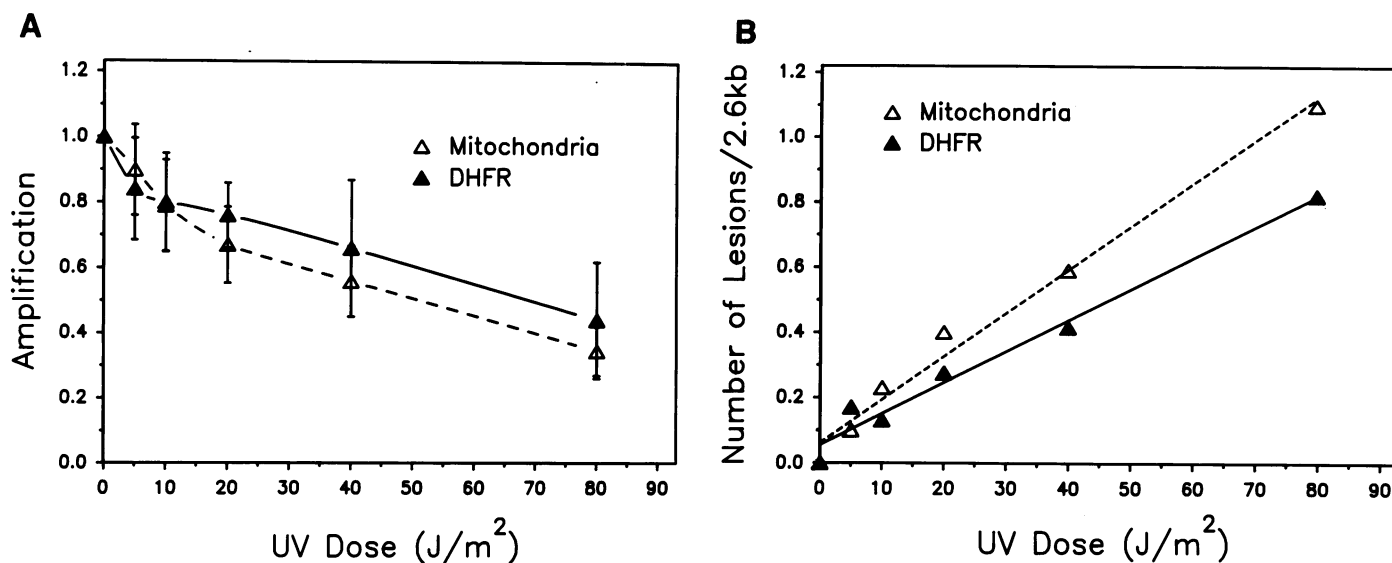
These data assume that PCR data gives lesion frequency per strand.

separate repair endonucleases were used: the pyrimidine dimer specific endonuclease V from phage T4, pyrimidine hydrate specific endonuclease III from *E. coli* and the *E. coli* UvrABC nuclease complex. As shown in Figure 6, both T4-endonuclease V and the UvrABC nuclease gave similar dose responses. While we consistently observed endonuclease III sites from cells which had only been damaged with UV, there was no dose dependent increase in these lesions. A direct comparison of QPCR and Southern hybridization analysis is displayed in Table I. As can be seen, the QPCR assay detects a greater frequency of DNA lesions than the Southern based assay.

#### Measurement of DNA repair

DNA repair in the *DHFR* gene segment was measured as the return of amplification signal at 4 and 8 hrs after irradiation with 40  $\text{J}/\text{m}^2$  UV (Figures 7A and 7B). In these experiments, L1210 cells were irradiated, and then cells were either immediately removed and lysed (to measure the initial lesion frequency), or the cells were cultured for 4 or 8 h and then lysed (to allow for a period of DNA repair). At time 0, the reduction in amplification (to about 60% that of an undamaged template) and the initial lesion frequency produced by 40  $\text{J}/\text{m}^2$  agreed well with our previous experiments at the same dose (approximately 0.5 lesions/segment). At 4 h after irradiation, the amplification signal had only returned 5–10%. By 8 h after irradiation, however, the amplification signal returned approximately 70% to that of an undamaged template. The lesion frequencies calculated at these times were 0.55 lesions/2.6 kb segment at time 0, 0.52 lesions/2.6 kb segment at 4 h and 0.15 lesions/2.6 kb segment at 8 h. This corresponded to the repair of over 70% of the lesions in the 8 h period.

Following UV irradiation at a dose of 20  $\text{J}/\text{m}^2$ , DNA repair in the mitochondrial DNA was measured as the return of amplification signal at 4, 8 or 24 h after treatment. The initial lesion frequency was determined as above, and was 0.45 lesions/2.3 kb segment at 20  $\text{J}/\text{m}^2$ . At 4 h after irradiation the amplification signal did not significantly change. However, an increase in the signal was seen at 8 and 24 h after irradiation. The lesion frequencies calculated for these times were 0.46 lesions/2.3 kb segment at 4 h, 0.34 lesions/2.3 kb segment at 8 h and 0.25 lesions/2.3 kb segment at 24 h (Figure 8). This would indicate that approximately 23% of the lesions were removed by 8 h, and 43% of the lesions were removed by 24 h. Similar results were observed by Southern hybridization analysis



**Figure 5.** UV dose response. (A) Amplification of the *DHFR* gene segment and mitochondrial DNA segment versus UV dose (0, 5, 10, 20, 40 and 80 J/m<sup>2</sup>). The amplification level represents the amount of amplification (in cpm) from a damaged DNA template at each UV dose divided by the amount of amplification from a nondamaged template (0 J/m<sup>2</sup>). The data were derived from 2 independent experiments, and represent the mean of 5 PCR amplifications of the *DHFR* gene segment, and 6 PCR amplifications of the mitochondrial DNA segment,  $\pm$  S.E. The standard error ranged from 0.12 to 0.16 in mitochondrial DNA, and 0.10 to 0.2 in the *DHFR* gene. (B) Lesion frequencies in the *DHFR* gene segment and mitochondrial DNA segment as a function of UV dose (0, 5, 10, 20, 40 and 80 J/m<sup>2</sup>). The lesion frequency for mitochondrial DNA has been normalized to a 2.6 kb segment. Based on the slope of the best fit line, the lesion frequency was 0.37 lesions/10 kb/10 J/m<sup>2</sup> in the *DHFR* gene segment, and 0.58 lesions/10 kb/10 J/m<sup>2</sup> in the mitochondrial DNA segment (See note for Figure 3).

using the UvrABC nuclease complex, but not T4-endonuclease V (data not shown). Thus, it appears that while pyrimidine dimers are not repaired, some fraction of UV-induced photoproducts might be repaired from the mitochondrial DNA.

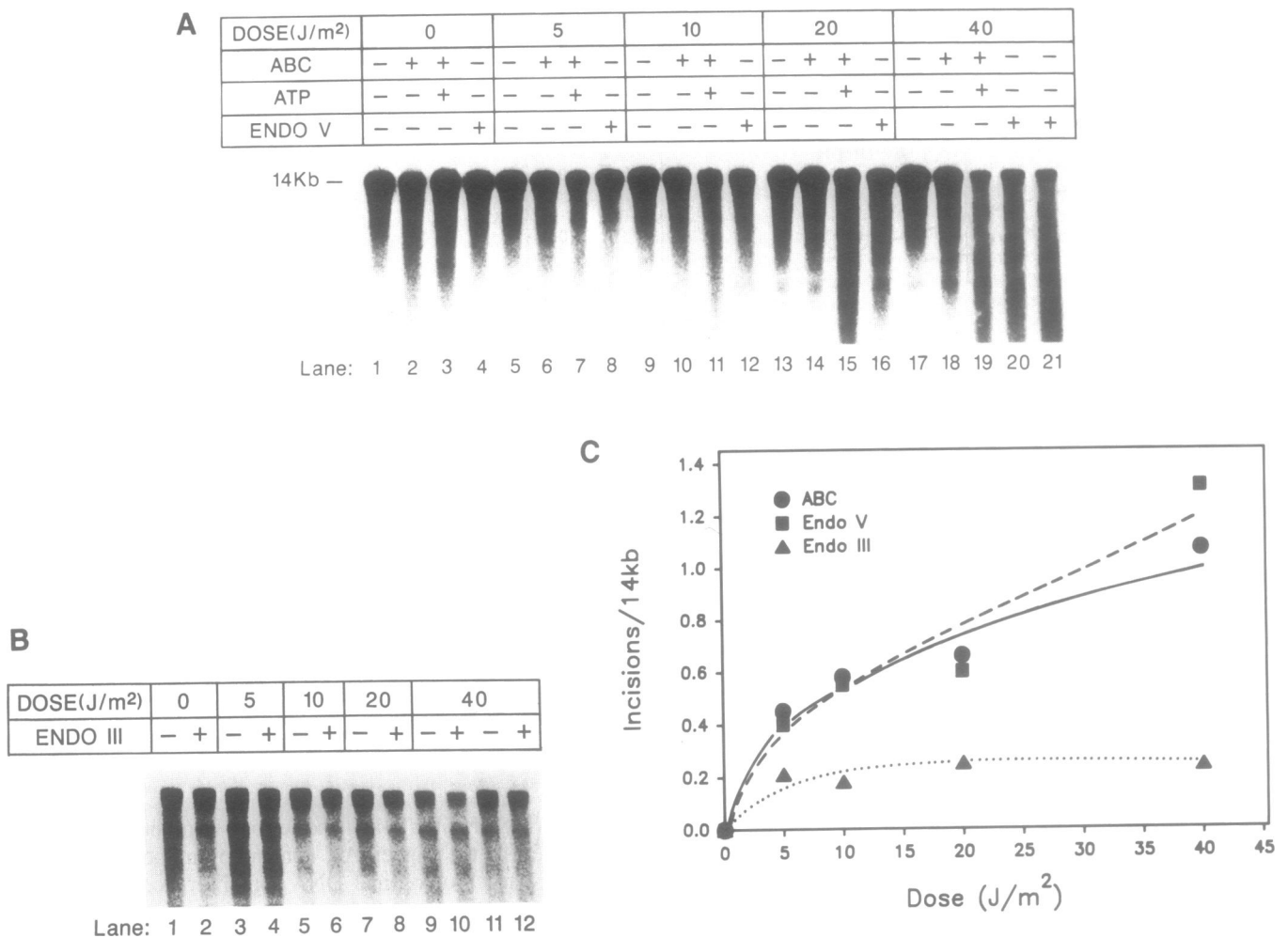
## DISCUSSION

The ability of a particular DNA lesion to stop *Taq* polymerase is the basis of a QPCR assay measuring DNA damage and repair. Such a lesion effectively removes the strand it resides in from the available template pool for PCR. Theoretically, a single lesion should be sufficient to do this, though not every DNA damaging agent will necessarily produce lesions which are blocks to *Taq* polymerase. For example, recent evidence indicates that the oxidative DNA lesion, thymine glycol, does not block *Taq* polymerase during PCR (Ilénye and Van Houten, unpublished observations).

It has previously been demonstrated, however, that both cisplatin-DNA adducts (38) and UV-induced photoproducts do block *Taq* polymerase (39). In addition, we have confirmed this for UV-induced photoproducts by primer extension experiments using M13 RF and mitochondrial DNA templates (data not shown). Bohr *et al.*, using the Southern based hybridization assay with T4 endonuclease V after UV irradiation, calculated a lesion frequency of 0.6 pyrimidine dimer sites/10 kb/10 J/m<sup>2</sup> in the amplified *DHFR* gene of CHO cells, and 0.7 lesions/10 kb/10 J/m<sup>2</sup> in the DNA overall (3). Madhani *et al.* (4), using the same assay in mouse fibroblasts, determined a lesion frequency of 0.47 lesions/10 kb/10 J/m<sup>2</sup>. Our QPCR calculated UV-induced lesion frequencies of 0.58 lesions/10 kb/10 J/m<sup>2</sup> in mitochondrial DNA and 0.37/10 kb/10 J/m<sup>2</sup> in the *DHFR* gene closely agree with these data. The slightly higher frequency (approximately

1.5 fold higher) of UV-induced lesions in mitochondrial DNA might reflect the higher AT content of mitochondrial DNA compared to chromosomal DNA, or differences in chromatin structure between the two locations which result in differences in shielding (40). The slightly lower lesion frequencies in the *DHFR* gene observed in our data may reflect the inherent screening effects which occur when cells are irradiated in suspension (mouse L1210), as compared to cells irradiated as monolayers (CHO), or the possibility that the *Taq* polymerase may occasionally bypass a lesion in a certain sequence context.

In order to investigate this second possibility, we examined the lesion frequencies in the mitochondria following UV or cisplatin damage using Southern analysis. This allowed us to directly compare the sensitivities of QPCR and the Southern hybridization based assay for the same lesions. Table 1 shows the lesion frequencies in mitochondrial DNA determined by Southern analysis (with either the UvrABC nuclease complex or T4 endonuclease V) compared to those determined by QPCR at several doses of UV. When normalized to a 14 kb fragment, the QPCR assay measures a greater number of lesions at a given dose of UV light than the Southern analysis does with either the UvrABC nuclease complex or T4 endonuclease V. If *Taq* polymerase were bypassing a significant number of lesions, one might expect the opposite result. For cisplatin-DNA adducts, the QPCR assay is 3–5 times more sensitive than the UvrABC nuclease complex. This difference may be due to the fact that the UvrABC nuclease complex does not incise efficiently at every lesion (12,18), though these lesions may still act as blocks to *Taq* polymerase. Jones *et al.* (12), in their investigation of the gene-specific repair of cisplatin-DNA adducts in CHO cells, estimated that the UvrABC nuclease complex only incised 30–40% of cisplatin-DNA adducts. Thus, this comparison of the lesion frequencies determined by the two methods



**Figure 6.** Quantification of UV-induced photoproducts by Southern Analysis. Mouse L1210 cells were irradiated with a dose of either 0, 5, 10, 20, and 40 J/m<sup>2</sup>, and then digested with either the UvrABC nuclease (ABC), T4-endonuclease V (endoV), (Panel A) or endonuclease III (endoIII), (Panel B). (A and B) Radioactive image of a Southern blot. A typical blot was scanned for radioactivity using a Betascope 603 blot analyzer (Betagen). The radioactivity associated with the 14 kb band was quantified and used to determine the average number of UV photoproducts/14 kb. (B) Data is the mean of two-three independent experiments. For clarity the S.E. of 10–15% was not shown. For panel B, lanes 9 & 10 contain half the amount of DNA as the other lanes. (C) The number of endonuclease sensitive sites were calculated as described in the Materials and Methods. The number of UvrABC nuclease sites are those incisions which have been corrected by subtracting both the UvrABC minus ATP breaks and the amount of incision observed for control DNA with UvrABC plus ATP.

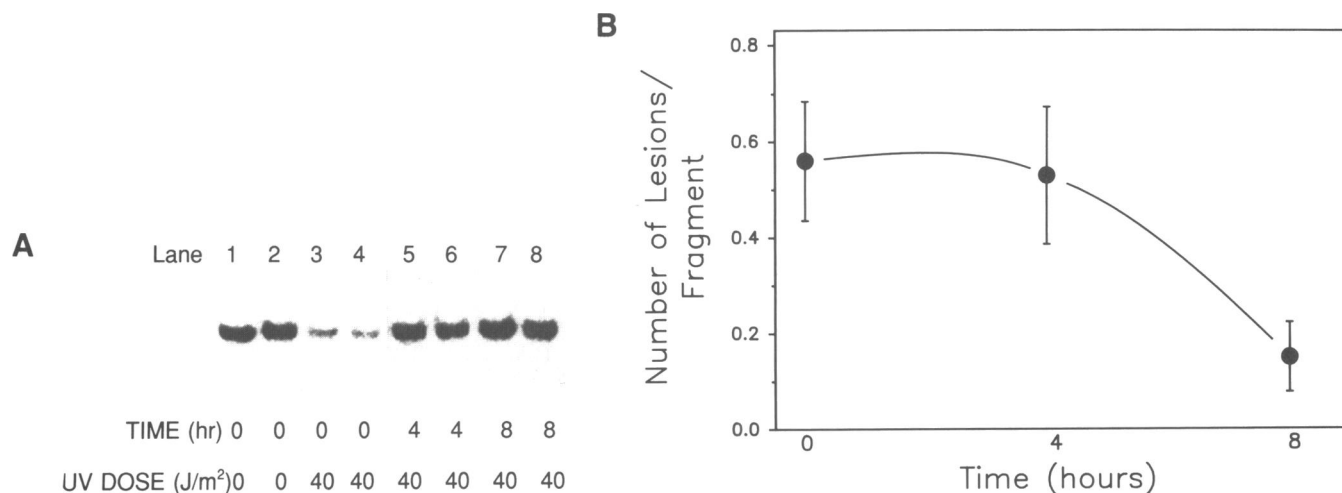
demonstrates that the sensitivity of QPCR is at least 3 to 5 times greater than that with UvrABC and Southern analysis.

Besides the nuclear DNA, another cellular target for DNA damaging agents is the mitochondrial genome. It has previously been reported that mitochondrial DNA sustains greater levels of damage than nuclear DNA when both are exposed to certain chemicals (41–46). Recently, in human malignant melanoma cells, higher levels of cisplatin-DNA adducts were observed in the mitochondrial DNA than in the chromosomal DNA (1). The results presented here show the opposite (Figure 3C). The lesion frequency was 0.17 lesions/10 kb/10 μM in the *DHFR* gene, and 0.066 lesion/10 kb/10 μM in the mitochondrial DNA. This difference in the level of mitochondrial damage between the study of Murata *et al.* (1) and ours could be due to the differential uptake of cisplatin by the mitochondria of the two cell types from the two species. Variations in the uptake of the cationic fluorochrome rhodamine 123, which localizes specifically in the mitochondria of living cells, have been observed between different cell types and between cells within the same cell population (46,47). This variation has been attributed to

heterogeneity in mitochondrial membrane potential, which is believed to be the driving force in the specific uptake of rhodamine 123 by the mitochondria (46–48). L1210 cells have a fairly low mitochondrial membrane potential, and this might account for the lower levels of mitochondrial DNA cisplatin damage observed in our study.

Several studies have demonstrated that UV-induced pyrimidine dimers in transcriptionally active genes are preferentially repaired over those in inactive regions of the genome (2–11). Madhani *et al.* (4) observed in mouse cells that, while 85% of pyrimidine dimers were removed from the active *c-abl* gene within 24 h, only 22% of dimers were removed from the inactive *c-mos* gene within the same time period. In this present study, the rate of repair of the active *DHFR* gene segment, less than 10% at 4 h, but approximately 73% at 8 h, is consistent with those results. After an initial slow period of repair before 4 h, there was a faster period between 4 and 8 h. Because these time periods were considerably less than the cell doubling time (12–14 h), and little if any DNA replication occurs following 40 J/m<sup>2</sup> UV irradiation (data not shown), it is unlikely that this decrease in lesion



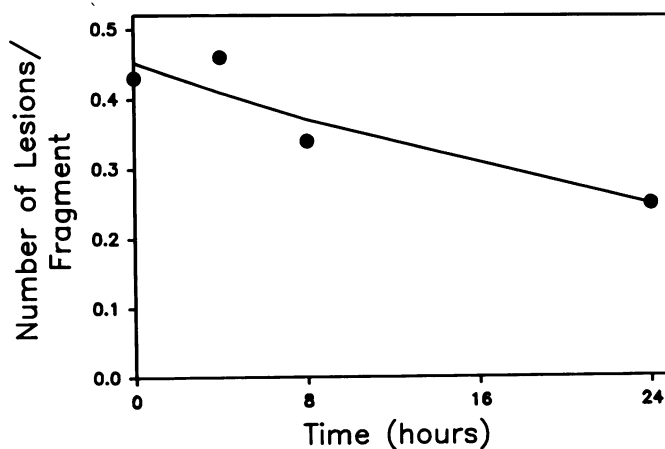


**Figure 7.** Repair of UV photoproducts. (A) Betascope image showing the return of PCR amplification signal in the *DHFR* gene segment after UV irradiation. L1210 cells were exposed to a UV dose of 40 J/m<sup>2</sup>. After exposure, cells were either immediately removed for DNA isolation (0 h), or cells were cultured an additional 4 or 8 h (before DNA isolation) to allow for a time of DNA repair. PCR reactions were performed as described in Materials and Methods. In the particular experiment shown here for illustration, the level of amplification at 4 h is higher than the usual (the mean at 4 h being an increase of approximately 5–10% that of the 0 time period). (B) Formation and repair of UV-induced lesions from a dose of 40 J/m<sup>2</sup> in the *DHFR* gene segment. The data represent the mean of 4 PCR amplifications  $\pm$  S.E.. The standard error ranged from 0.06 to 0.08.

frequency is due to dilution from DNA replication. The initial low level of lesion removal during the first 4 h could be due to transient, enzymatically induced breaks which occur in DNA during repair. Such breaks would represent absolute blocks to *Taq* polymerase during PCR. Subsequent repair of these breaks could result in the observed period of faster repair between 4 and 8 h.

In the mitochondrial DNA, over 40% of the UV-induced lesions which block *Taq* polymerase were removed by 24 h after irradiation with 20 J/m<sup>2</sup> (Figure 8). Although past investigations have shown that little or no excision repair occurs in mitochondrial DNA (49,50), recent evidence suggests that some types of lesions are repaired by other pathways (51,52). An alternative explanation for the repair observed in our study might be that mitochondrial replication over the 24 h time period caused us to overestimate the amount of repair. The replicated mitochondrial DNA would increase the amount of nondamaged template available for PCR and thus increase the amplification signal with time. To investigate this possibility, we undertook studies with bromodeoxyuridine labeled DNA, and separated nonreplicated from replicated DNA on CsCl gradients. Preliminary results indicate that approximately 30% of the lesions which block *Taq* polymerase are removed by 48 h (data not shown).

This present study has demonstrated that a QPCR assay can be used to measure DNA damage and repair in specific genomic sequences following exposure to DNA damaging agents. The speed and ease of PCR, and the fact that it needs neither a large amount of template DNA (0.25–0.50  $\mu$ g) nor a specific endonuclease to recognize lesions, makes it a potentially powerful assay in both basic research and clinical settings, which can now be used in many situations as an alternative to current methods. The observed measurements of lesion frequencies and repair levels are consistent with previous studies using Southern hybridization based assays. For example, while this present study was nearing completion, Jennerwein and Eastman (53) reported



**Figure 8.** Formation and repair of UV-induced lesions from a dose of 40 J/m<sup>2</sup> in mitochondrial DNA. UV irradiation, DNA isolation, PCR amplification, quantification and calculations of lesion frequencies were performed as described in Materials and Methods. The data represent the mean of 2 PCR amplifications.

cisplatin-DNA lesion frequencies determined by quantitative PCR analysis in the adenine phosphoribosyltransferase gene of CHO cells with sensitivity equal to that of other available methods. We are currently using this assay to study the repair of cisplatin-DNA adducts in both cisplatin sensitive and resistant mouse cell lines, and future research will be directed at improving the sensitivity of the assay.

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