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A polymerase chain reaction-based method to detect cisplatin adducts in specific genes

M.M.Jennerwein and A.Eastman
Department of Pharmacology, Dartmouth Medical School, Hanover, NH 03756, USA

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ABSTRACT

Every bulky lesion in DNA can potentially inhibit the Taq DNA polymerase and thereby decrease the amplification produced in the polymerase chain reaction. We investigated the feasibility of using this inhibition to quantify DNA lesions produced by the anticancer drug cisplatin. Products were detected by electrophoresis followed by ethidium bromide staining. Quantitation was obtained by including [^{32}P]dCTP in the amplification reaction and subsequently assessing the incorporated radioactivity. Hamster genomic DNA was platinated *in vitro* to defined levels and amplified with primers that produce either a 150, 750 or 2,000 base pair fragment. The degree of inhibition of PCR agreed with the predicted level of DNA platination in each size of fragment, suggesting that the polymerase was inhibited by every cisplatin-induced lesion. This method was used to detect cisplatin-induced lesions in the adenine phosphoribosyltransferase gene of CHO cells. Cells were incubated with 0 – 125 μM cisplatin for 2 h, the DNA was purified and subjected to PCR. A significant decrease in amplification of the 2 kbp fragment was observed in DNA from cells incubated with cisplatin at 75 μM. The degree of inhibition agreed closely with the amount of DNA damage in the overall genome as measured by atomic absorption. No change was detected in amplification of the 150 base fragment which can therefore be used to normalize data for any variations between DNA samples. This assay has the same sensitivity as other methods currently used for the analysis of gene-specific damage. The advantage of this assay is that it obviates the need for specific endonuclease complexes to recognize and cleave DNA adducts as previously required when analyzing damage in specific genomic sequences.

INTRODUCTION

Studies correlating DNA damage with cytotoxicity have generally assumed homogeneity in the distribution of damage. Various studies on carcinogens, however, have shown that DNA damage can occur in a non-random manner (1). Furthermore, DNA repair can also vary throughout the genome. It has been shown that actively transcribed genes can be repaired more efficiently than untranscribed regions (1). This is particularly true for UV-induced DNA damage. The formation of pyrimidine dimers in specific genes can be measured using the dimer-specific enzyme T4 endonuclease V. This enzyme introduces a single strand break at the site of the damage. DNA from UV-irradiated cells is digested with an appropriate restriction endonuclease, cleaved with T4 endonuclease V, denatured and electrophoresed. Southern hybridization is used to identify the amount of full length single-strand DNA of any gene of interest: the intensity of hybridization reflects the amount of undamaged DNA. To measure gene specific damage induced by other agents, particularly those that produce bulky lesions in DNA, it is necessary to use the *E. coli* uvrABC excinuclease complex that recognizes and incises at a broad spectrum of DNA lesions (2).

Cisplatin is a major anticancer drug that elicits its cytotoxicity as a consequence of producing bifunctional lesions in DNA; these lesions are predominantly DNA intrastrand cross-links but DNA interstrand cross-links are also formed (3). We have previously established that mouse L1210 cells resistant to cisplatin exhibit enhanced DNA repair (4,5). Enhanced DNA repair has subsequently been observed in human ovarian carcinoma cells (6). Recent work has also shown that cisplatin lesions may be repaired preferentially from transcribed regions of the genome (7), and furthermore, that this may contribute to the altered repair observed in resistant cells (8). The detection of cisplatin lesions in specific genes has required the use of the uvrABC excinuclease complex although it appears to cleave at less than 30% of the cisplatin lesions (7). The polymerase chain reaction can be used as an alternative approach to measuring gene-specific damage and has the advantage of not requiring specific damage-recognition endonucleases. To date, this method has only been reported for the measurment of UV-induced lesions (9), but the sensitivity was far inferior to that for the T4 endonuclease V-dependent method. Here we describe the development of this method to detect cisplatin lesions in DNA and demonstrate that it has at least as good sensitivity as the uvrABC excinuclease-dependent method. The advantage of this method is that it requires no specific enzymes to recognize and cleave DNA adducts, and therefore will facilitate studies in many different laboratories.

METHODS

Cell culture

Chinese hamster ovary (CHO) cells were grown as described previously (10). Cells (10^7) were plated in 160 mm culture dishes. The following day the media was replaced with serum-free medium containing the required concentration of cisplatin.

The cells were incubated at 37°C for 2 h, harvested by scraping and washed twice with phosphate buffered saline. DNA was purified by cell lysis, protease digestion and phenol extraction (11). After ethanol precipitation, the purified DNA was redissolved in 10 mM tris/1 mM EDTA and digested overnight with Kpn I (BRL Life Technologies) in the suppliers recommended buffer. This digestion reduces the viscosity of the DNA without cleaving the sequences to be amplified. DNA was reprecipitated with ethanol, dissolved in water and the concentration was determined by fluorimetry using Hoechst 33258 dye (12). The samples were diluted to 25 μg/ml and the concentration was confirmed by fluorimetry. The level of DNA platination was also determined by flameless atomic absorption spectrometry.

In vitro platination of DNA

Undamaged DNA was purified from CHO cells and then platinated to defined levels as previously described (13).

Polymerase chain reaction

The following primers derived from the hamster adenine phosphoribosyltransferase gene were synthesized. The numbers refer to the position in the published sequence (14). The designation 'F' represents the forward primer derived from the sense strand, and 'R' represents the reverse primer derived from the antisense strand. 331F: GTTCCCGGAC TGGTATGACC. 1620F: GGTCAATACT ATTCACTCAA 2194F: CAGCTACAGG CTGAGGTGGT. 2350R: GTTCAACCGT AGATGCTGAG. 2370R: TTTGGTAAGG CTGAGCCACT. Amplification of a 2 kbp fragment was obtained with primers 331F and 2370R; the 750 bp was obtained with 1620F and 2370R; and the 150 bp fragment was obtained with 2194F and 2350R.

A typical 50 μl reaction contained 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, 1 μM of each primer, 0.5μl AmpliTaq (Perkin-Elmer Cetus) and 500 ng DNA. The amplification protocols varied with the specific sequence to be amplified and are detailed in Table 1. In all cases the initial denaturation was at 94°C for 4 min. Amplification was usually for 23 cycles followed by an extra annealing and extension at 72°C. To confirm that the extent of amplification remained directly dependent upon the amount of amplifiable sequence, a control reaction with a two fold dilution of undamaged DNA (C/2) was included in most experiments. In all cases this produced an amplification that was half of the normal dilution of undamaged DNA.

The reaction products was separated on a 1% (2 kbp), 1.2% (750 bp) or 2% (150 bp) agarose gel and the products visualized by ethidium bromide staining. The molecular weight standards used were either BstE II digested lambda DNA or Msp I digested pBR322. For quantitative analysis, 2 μCi α[^{32}P]-dCTP was included in the reaction mixture, 25 μl of the reaction mixture was separated by electrophoresis, the gel front containing unincorporated radioactivity was cut off, the gel was washed twice with water for 20 min to remove background radiation, then the gel was dried and autoradiographed for 12–24 h. Quantitation was performed on a Betascope 603 Blot Analyzer (Betagen, Waltham, MA).

Table 1. Amplification protocols used in the current experiments

Fragment size	Time in seconds		
	denaturation 94°C	annealing 55°C	extension 72°C
2,000 bp	90	60	120
750 bp	70	60	90
150 bp	60	60	60

RESULTS

To quantify DNA damage by PCR, it is essential that the substrate DNA is the limiting component in the reaction. Initial experiments were designed to determine the number of cycles that could provide quantitative amplification. For these experiments, the 150 bp fragment was amplified for 20–40 cycles and the products were detected by electrophoresis followed by staining with ethidium bromide (Fig. 1). The amplification signal increased from almost undetectable at 20 cycles to maximum at 30 cycles with no further increase up to 40 cycles. Up to 26 cycles, the amount of product appeared to double with each additional cycle. In addition, many other unexpected bands were observed at and above 26 cycles. Similar results were observed with the 2 kbp fragment except that the product decreased beyond 29 cycles presumably due to continued denaturation of the product without reannealing (not shown). It was further shown that the amount of product increased linearly with DNA concentration between 100–500 ng (not shown). Based on these results, subsequent experiments were performed with 500 ng DNA and for 23 cycles of amplification. In many experiments, one reaction was performed with 250 ng undamaged DNA (C/2) to confirm that the amplification obtained was 50% of that obtained with 500 ng undamaged DNA.

Experiments were designed to determine whether the amount of amplification of a specific DNA fragment was directly related to the level of DNA platination. DNA was purified from undamaged CHO cells and platinated to defined levels. This DNA was then subjected to PCR using primers spanning a 150 bp, 750 bp or 2 kbp fragment of the adenine phosphoribosyltransferase gene. Amplification was detected by ethidium bromide staining. Amplification of the 2 kbp fragment was clearly inhibited by DNA damage: at a level of 5 Pt/10 kb, which corresponds to 1 Pt adduct every 2 kb, the amplified fragment was barely visible (Fig. 2). Amplification of the 750 bp fragment was barely visible when platinated to 10 Pt/10 kb, whereas amplification of the 150 bp fragment was only slightly reduced at 40 Pt/10 kb. These results demonstrate that the sensitivity for detection of DNA damage is related to the size of the fragment amplified.

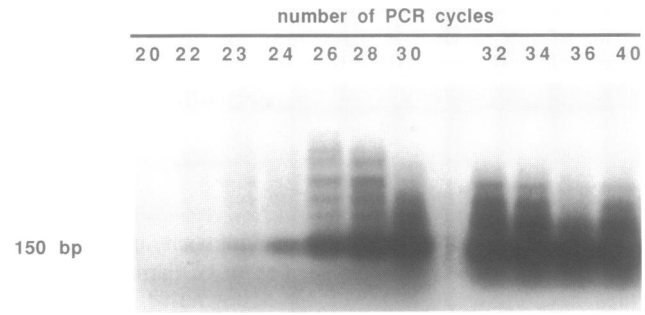

Figure 1. Determination of the conditions to use for quantitative PCR. 500 ng CHO genomic DNA was amplified for the indicated number of cycles using primers for the 150 bp sequence. The PCR products were separated by electrophoresis and stained with ethidium bromide.

To more accurately quantify the amount of amplification, a tracer amount of $\alpha[^{32}P]$-dCTP was included in the reaction mixture. The resulting electrophoresis gels were autoradiographed and the radioactivity in the amplified bands was quantified. The results from amplification of the 2 kbp and 750 bp fragments are shown in Fig 3. One Pt adduct/DNA strand should be adequate to prevent amplification if every lesion blocks the Taq DNA polymerase. As DNA was platinated randomly, the distribution of damage fits a Poisson distribution. Accordingly, at a mean level of platination of one Pt adduct/DNA strand, 37% of the strands remain undamaged and can be amplified. The expected and observed values are presented in Table 2. The correlation between these two values is very close demonstrating that the Taq polymerase is blocked by at least the majority of lesions in DNA. This demonstrates the validity of this method to quantify DNA damage induced by cisplatin.

This method was applied to CHO cells that had been incubated with 0–125 μM cisplatin to determine whether the DNA damage was detectable. The ethidium bromide staining showed a significant reduction in amplification of the 2 kbp fragment at and above 75 μM cisplatin (Fig. 4). A parallel reaction was performed containing $\alpha[^{32}P]$-dCTP (Fig. 5) and the radioactivity was quantified. The results show, for example, that the

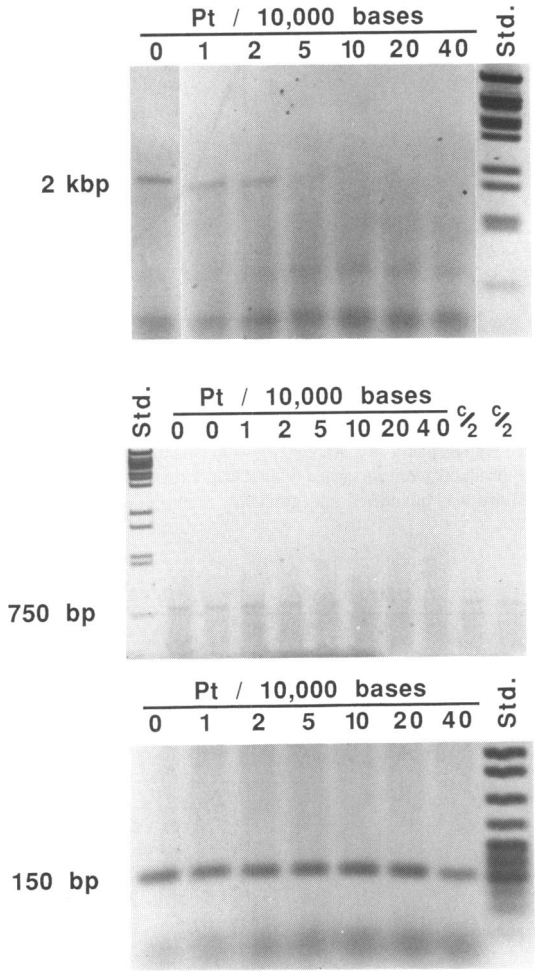

Figure 2. PCR amplification of DNA damaged with cisplatin *in vitro* to defined levels. The PCR products were separated by electrophoresis and stained with ethidium bromide.

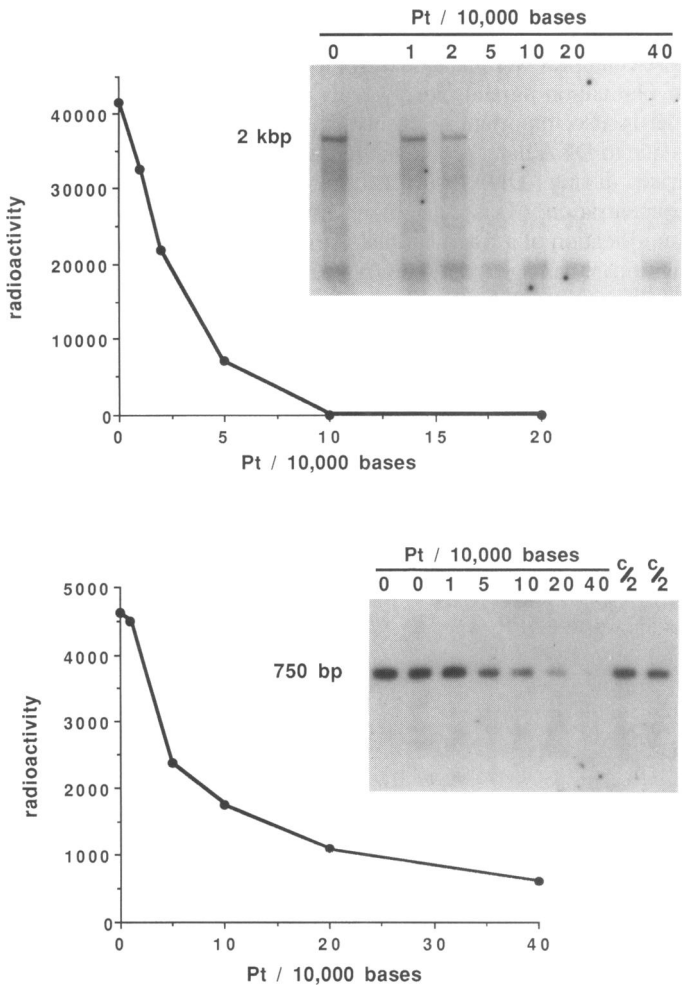

Figure 3. Quantitation of the PCR amplification of DNA damaged with cisplatin in vitro (fig. 2). $\alpha[^{32}P]$-dCTP was included in the PCR step, and the products were separated by electrophoresis and autoradiographed (insets). The radioactivity was quantified and graphed.

Table 2. Calculation of the amount of damage expected in DNA platinated in vitro compared to that observed by inhibition of PCR

Pt/10,000 bases	Pt/2,000 bases	% of 2 kb sequence expected to be undamaged*	% of 2 kb sequence observed to be undamaged
0	0	100	100
1	0.2	82	78
2	0.4	67	53
5	1	37	17
Pt/10,000 bases	**Pt/750 bases**	**% of 750 b sequence expected to be undamaged***	**% of 750 b sequence observed to be undamaged**
0	0	100	100
1	0.075	92	95
5	0.375	68	51
10	0.75	47	38
20	1.5	22	24
40	3	5	13

*expected values calculated according to Poisson distribution.

radioactive signal at 125 μM was reduced to 15% which corresponds to an adduct level of 1.8 Pt/2 kb (Table 3). The same DNA samples were analyzed by atomic absorption and the level of platination agreed closely with both techniques.

It is also important to ensure that inhibition of amplification is due to DNA damage and not to inter-sample variation that may arise during DNA purification or from assessing DNA concentration. This can be examined in every sample by amplification of a fragment that is too small to have accumulated significant damage. A 150 bp fragment was amplified and showed no significant inhibition of amplification up to 125 μM cisplatin (Fig. 4).

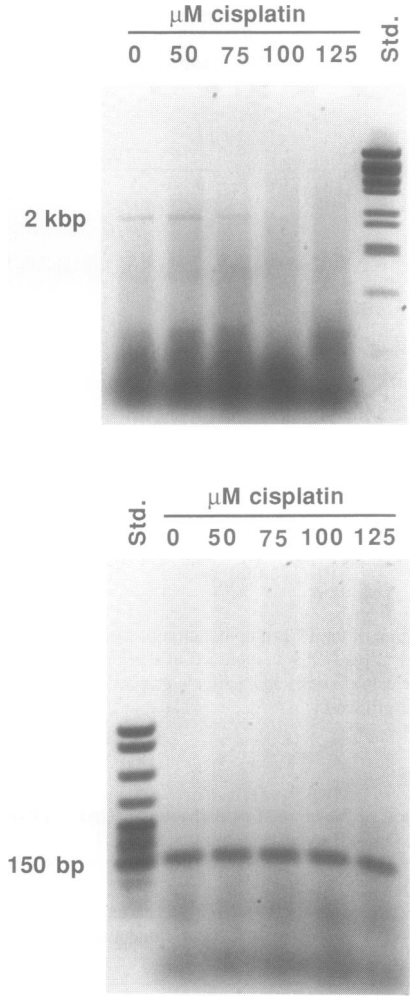

Figure 4. PCR amplification of DNA purified from CHO cells that had been incubated for 2 h with 0–125 μM cisplatin. The PCR products were separated by electrophoresis and stained with ethidium bromide.

Table 3. Comparison of the amount of DNA platination in cells incubated with cisplatin detected by atomic absorption and by inhibition of PCR

concentration of cisplatin (μM)	Pt/2,000 bases determined by atomic absorption	Pt/2,000 bases calculated from PCR
50	0.49	0
75	0.46	0.74
100	1.07	1.37
125	1.66	1.85

One application of the method described here is the confirmation of Pt damage on short DNA fragments. In other studies, we have used the 150 bp fragment in band shift assays to detect proteins that recognize cisplatin-damaged DNA (unpublished). The purified 150 bp fragment obtained by PCR was incubated with various amounts of cisplatin. In these experiments, 60 ng of fragment was incubated with various concentrations of cisplatin in water for 48 h in a total volume of 9 μl. The platinated fragment (0.2 ng) was then subjected to 10 rounds of amplification. Only at the highest level of damage (r_f = 0.25) was inhibition of amplification observed and this inhibition corresponded to 2–4 Pt/150 bp fragment (Fig. 6). This was far less than the 75 Pt/150 bp fragment which was expected

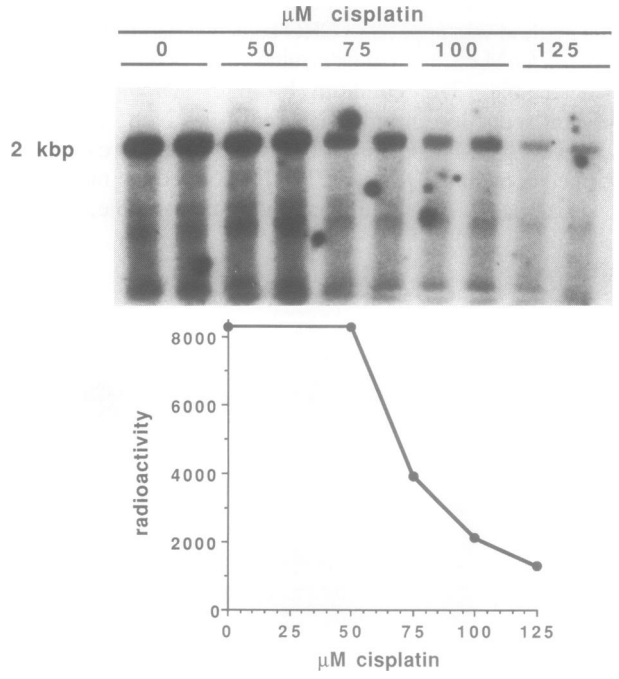

Figure 5. Quantitation of the PCR amplification of DNA purified from CHO cells incubated with cisplatin (fig. 4). α[^{32}P]-dCTP was included in the PCR step, and duplicate products were separated by electrophoresis and autoradiographed. The radioactivity was quantified and graphed.

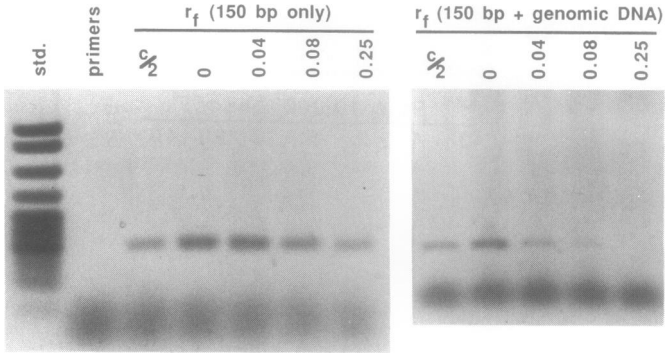

Figure 6. Determination of the damage introduced into a 150 bp fragment of DNA during an *in vitro* incubation with cisplatin. The 150 bp fragment was incubated with cisplatin either alone or diluted with excess of salmon testis DNA. r_f refers to the molar ratio of cisplatin to DNA nucleotide during the platination reaction.

because DNA platination usually approaches 100% of available cisplatin. A subsequent experiment was performed in which 30 ng of 150 bp fragment was mixed with 120 μg salmon testis DNA and incubated with various concentrations of cisplatin in a final volume of 92 μl. In this experiment the same molar ratio of cisplatin to DNA was used, but higher concentrations of both were used compared to the previous experiment. After PCR, it was evident that a much greater inhibition of amplification was obtained indicating much higher levels of platination of the 150 bp fragment (Fig. 5). These results demonstrate that at low concentrations of DNA and cisplatin, other minor components of an incubation can compete with DNA for reaction with cisplatin. Therefore PCR is a useful method to assure damage at low concentrations of DNA and cisplatin.

DISCUSSION

We have demonstrated in this paper that PCR can be used to quantify damage induced in DNA by cisplatin. Using DNA with defined levels of damage, inhibition of PCR was observed to an extent that suggested the Taq DNA polymerase was inhibited at every Pt lesion. About 65% of the lesions in DNA are intrastrand cross-links at the sequence d(GpG), while 25% occurs at d(ApG) and about 6% at dGpNpG) (where N is any nucleotide) (3). Only about 1% of the lesions represent DNA interstrand cross-linksand are therefore too rare to contribute to the observed results. It is feasible that a low percentage of lesions could be by-passed by the polymerase but this would not be detected by this assay and would not contribute significantly to quantitation of the lesions in DNA. In cells that were platinated in culture, inhibition of PCR was also observed and this agreed closely with the amount of DNA damage measured directly by atomic absorption. Therefore, under these conditions, the only detectable blocks to PCR were the Pt lesions.

The sensitivity for detection of DNA damage is dependent upon the size of the target sequence. At any level of platination, there is a greater probability that an adduct lies within a 2 kbp region than within a 150 bp sequence. Therefore, amplification of the longer fragment is expected to be more inhibited than the shorter fragment or, to express it another way, higher amounts of damage must be introduced into a short fragment to produce an equivalent inhibition of amplification. This agrees very well with the experimental observations. It should be possible to increase the sensitivity of the assay by increasing the size of the fragment to be amplified. However, the longest sequence that we are aware of that has been successfully amplified is 10.2 kbp but the efficiency of amplification decreased markedly with increasing size of fragment (15). This suggests that little increase in sensitivity can be obtained in practical terms by increasing the target size much further than the 2 kbp used here.

There is always concern for potential variations in the efficiency of amplification between various DNA preparations. Such variations may arise from contamination in samples, quantitation of DNA, or fluctuations in the amplification reaction. This can be accounted for in the current protocol by amplification of a 150 bp fragment. This acts effectively as a control because it will have insufficient damage to inhibit PCR. In the current experiments, no variation in amplification of the 150 bp fragment was observed in DNA purified from cells. However, should variations be observed, it would be easy to normalize the amplification of the large fragment to that observed for the small fragment. Furthermore, with appropriate selection of primers, it would be feasible to amplify fragments of different sizes and from different genes in a single reaction. This was not possible in the current experiments because the smaller fragments were contained within the larger fragments, and therefore would have been amplified from the product of the other amplifications.

In these experiments, we were able to detect damage in cells incubated with 75 μM cisplatin. The LC_{90} for a 2 h incubation of these cells in cisplatin is 30 μM (10) so the cells had to be incubated with greater than a 99% lethal concentration before lesions were detectable. Using the uvrABC complex to detect cisplatin lesions in specific genes of CHO cells, a 1 h incubation with 100−300 μM cisplatin was used (7). Therefore the sensitivity of the current assay appears to be as good as the previously available method. In the experiments with uvrABC, the target gene was up to 23 kbp, hence a greater sensitivity would be expected. The fact that greater sensitivity was not seen can in part be attributed to the endonuclease complex cleaving at only 30% of the lesions (7), whereas the Taq DNA polymerase appears to stop at every lesion.

The original experiments on gene-specific damage were performed with UV-damaged DNA. Incisions were made at the site of pyrimidine dimers using T4 endonuclease V, then the DNA was denatured and analyzed by Southern hybridization. In those experiments, DNA damage was detected at 10−20 J/m^2 while the LC_{90} value was 15 J/m^2 (16). Hence, the lesions were detectable at somewhat less toxic concentrations than for cisplatin. It can be reasoned that it takes less cisplatin lesions than UV-induced dimers to kill cells.

A recent paper has also demonstrated the use of PCR to detect damage in specific genes, but in this case they studied UV-induced DNA damage (9). A 50% inhibition of PCR was observed in cells irradiated with 6 kJ/m^2. Surprisingly, this inhibition was independent of the size of the sequence amplified; in their case the sequences were 147−440 bp. The radiation required to bring about this inhibition of PCR was 600 fold higher than required to detect gene-specific damage using T4 endonuclease V (16). This is in part due to the smaller size of the target analyzed, 440 bp rather than 9−23 kbp used in the endonuclease studies. It may also suggest that the Taq DNA polymerase does not stop at every pyrimidine dimer. It was previously demonstrated that UV-irradiation of CHO cells with 10 J/m^2 produced one endonuclease-sensitive site/20 kb of the dhfr gene (17). This is in close agreement with the calculated value of 0.8 endonuclease sensitive sites/10^8 daltons/J/m^2 for the overall CHO genome (18). This calculates to 1 pyrimidine dimer/8 bases at 24 kJ/m^2. This is considerably more damage than the 2−3 pyrimidine dimers per 440 bp fragment calculated by Govan *et al* for a radiation dose of 24 kJ/m^2 (9). A likely explanation for these discrepancies is that the previous report started with 100 ng DNA and used 40 cycles of PCR which should be far beyond the exponential phase of amplification.

Most of the studies on damage in specific genes are designed to investigate the potentially different rates of DNA repair in these genes. The toxicity of the agent under investigation is therefore of particular concern. All of the methods used to date require that toxic conditions be used. For UV-unduced DNA damage, it is necessary to use ~90% lethal irradiation, and for cisplatin, it requires ~99% lethal concentration. It is feasible that these conditions could considerably alter the kinetics of repair or even the genomic domains that are repaired. The different toxicities required for each agent may explain why gene-specific or strand-specific DNA repair is not observed for all agents. Perhaps more

significant is the concern that the repair that is observed is occurring in cells that are destined to die. Although the cell membranes may still be intact as assayed by trypan blue exclusion, it is known that genomic DNA is digested by endonucleases in dying cells long before any indication of changes in membrane integrity (19,20). For example, in experiments not shown, the CHO cells incubated with cisplatin above have been further incubated for 24 h in the absence of drug to permit DNA repair. At drug concentrations above 25 μM, the genomic DNA had been cleaved to fragments of 180 bp or oligomers of this length. These fragments are characteristic of the internucleosomal digestion of DNA associated with cell death by apoptosis. Little attention has been given to this concern. Presumably, the degraded DNA was excluded from the analysis. In ongoing studies, we have found that murine leukemia L1210 cells are more resistant to endonuclease digestion associated with cell death, and that gene-specific damage and repair can be assessed with this PCR-based method.

In summary, the PCR-based method for measuring gene-specific damage appears to be as sensitive as other available techniques. Its major advantage is that it requires no specific enzymes to recognize and cleave DNA adducts. The uvrABC excinuclease complex used for such studies is not generally available and the current method will therefore provide a very convenient alternative method.

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