Assessment of DNA damage and repair in specific genomic regions by quantitative immuno-coupled PCR

Mikhail F.Denissenko*, Sundaresan Venkatachalam, Edith F.Yamasaki and Altaf A.Wani
Department of Radiology and Biochemistry Program, The Ohio State University, 103 Wiseman Hall,
400 W. 12th Avenue, Columbus, OH 43210, USA

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ABSTRACT

Fine analysis of DNA damage and repair at the subgenomic level has indicated a microheterogeneity of DNA repair in mammalian cells, including human. In addition to the well established Southern hybridization-based approach to investigate gene-specific DNA damage and repair, alternative methods utilizing the sensitivity of PCR have been evaluated. The latter technique has relied on decreased PCR amplification due to damage in template DNA. We have developed a novel quantitative assay combining the selective recovery of DNA damage containing genomic fragments with the PCR amplification. DNA isolated from 7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*-BPDE) treated human skin fibroblasts was immunoprecipitated with polyclonal antibody BP-1. Recovered target sequences were amplified by PCR using primers encompassing a 149 bp target region around codon 12 of the H-*ras* proto-oncogene. Quantitative DNA damage specific response was observed with nanogram amounts of genomic DNA. This approach allowed analysis of the initial DNA damage at a level less than 1 *anti*-BPDE adduct per 6.4 kbp *ras* gene fragment. Repair proficient GM637 cells exposed to 2 μ M *anti*-BPDE showed a faster removal of the adducts from the H-*ras* gene segment than from the genome overall. Gene-specific repair was not apparent in GM4429 xeroderma pigmentosum (complementation group A) cells. The established technique could be extended to the quantitative measurement of the repair of diverse DNA base lesions in any genomic region of known sequence.

INTRODUCTION

Heterogeneity of DNA repair has been established in different prokaryotic (1) and eukaryotic (2–5) cellular systems. Various structurally altered DNA base lesions are preferentially removed from the transcriptionally active genes as compared to the inactive genomic regions or the overall genome (6,7). In many instances, preferential repair appears to be directed at the transcribed strand (5,8). Some investigators support the notion that the faster repair

of active genes is largely due to selective repair of the transcribed strand, assuming that the repair of non-transcribed strand is comparable to the genome overall (9). Fast repair of DNA lesions located on the transcribed strand may be due to their accessibility to repair proteins and the coupling of DNA repair with other cellular processes e.g., transcription (10). Pyrimidine dimers, induced by UV light, were the first lesions reported to undergo preferential repair (3). Other adducts formed by various genotoxic agents, including alkylators, cisplatin, and the ultimate carcinogen *anti*-BPDE, were found to be also eliminated from DNA in a preferential manner (11–13).

Novel approaches are being designed to explore gene-specific DNA damage and repair and their implication in mutagenesis and carcinogenesis. The initial development of the Southern hybridization-based assay (3,14) provided a powerful tool for measuring preferential repair of various DNA lesions in specific gene segments. However, the applicability of this methodology requires large amounts of DNA and depends on the presence of suitable restriction enzyme recognition sites within the region of interest and a lesion specific endonuclease to incise the DNA adducts (15). An alternative methodology employing the polymerase chain reaction has recently been reported (15–17). This approach has exploited the loss of template activity of a target gene due to DNA damage and the rate of restoration of this activity by the cellular repair machinery. Although very sensitive, this technique necessitates introduction of high levels of lesions in the target DNA segment to obtain a pronounced and quantifiable decrease in amplification. On the other hand, Hochleitner *et al.* specifically separated the damaged DNA template by immunoaffinity purification prior to PCR amplification and quantitation of gene-specific repair of alkylated DNA adducts (18) and recently extended their approach on a set of rat genes of various transcriptional activity (19). Thus, the high specificity and sensitivity of antibody-antigen interaction allowed the detection of very low levels of lesions not amenable to the standard gene specific analysis based on an endonucleolytic cleavage at or near the modification site.

The present study describes a direct quantitative approach for analyzing DNA damage and repair in defined gene sequences with lesion specific antibodies. The assay combines selective immunoprecipitation of lesion bearing genomic fragments and

*To whom correspondence should be addressed

a sensitive PCR of immunorecovered DNA. The reactive diol-epoxide metabolite of benzo[*a*]pyrene, *anti*-BPDE, was used to induce damage in DNA of human fibroblast cells. Cellular *H-ras* protooncogene, a prolific model in experimental carcinogenesis, served as a target for DNA damage induction and repair. Our results demonstrate a faster removal of the *anti*-BPDE adducts from the *H-ras* gene containing fragment compared to the repair within overall genome determined as a loss of the antibody-binding sites from the bulk DNA. The experiments with xeroderma pigmentosum (complementation group A) fibroblasts showed repair deficiency for the overall genome as well as absence of gene-specific repair in these cells.

MATERIALS AND METHODS

DNA modification *in vitro*

Plasmid p220-*ras* DNA was constructed by subcloning a 6.4 kbp fragment of the human *H-ras* protooncogene into the *Bam*HI site of the polylinker region of plasmid p220.2 (20). *Anti*-BPDE (NCI Repository, Midwest Research Institute, MO) was freshly dissolved in 95% ethanol and quantitated by measuring specific absorbance. Plasmid DNA was modified with *anti*-BPDE and purified as recently described (21). N-cyclohexyl-N'- β -(4-methylmorpholinium) ethyl carbodiimide-p-toluene sulphonate (CMC) was obtained from Aldrich (Milwaukee, WI). Modification of plasmid DNA was done at 100 mM CMC in the presence of NaCl as described earlier (22). The conditions for UV irradiation of DNA were as described (20).

Cell culture and treatment

SV40 transformed GM637 (47% diploid – 53% tetraploid) and xeroderma pigmentosum (XP) GM4429 (complementation group A, strain XP12BE) human fibroblast cell lines were purchased from NIGMS Human Genetic Mutant Cell Repository, NJ. Cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with high glucose, 10% fetal calf serum, and penicillin and streptomycin. Cells were maintained at confluence for 3 days before use in the experiments. Culture medium was removed, replaced with Hanks' BSS (Life Technologies), and cells were exposed to freshly prepared ethanolic *anti*-BPDE for 30 min at 37°C. Parallel controls were treated with solvent (0.75% ethanol) during this initial incubation. After treatment, the cells were washed with phosphate-buffered saline (PBS) and either collected immediately or further incubated in serum-free medium for appropriate post-treatment times to allow DNA repair. Pelleted cells were processed immediately for DNA isolation. For uniform prelabeling of DNA, cells were grown to confluence in medium containing 15 nCi/ml [methyl-³H]deoxythymidine ([³H]dThd, 71 Ci/mmol, ICN Biomedicals, Irvine, CA). The resulting specific activity of the DNA isolated from the radiolabeled cultures was approx. 1.7 nCi/ μ g.

Isolation of genomic DNA

Cell pellets were resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 140 mM NaCl, 0.5% v/v Sarkosyl NL-30 (ICN Pharmaceuticals, Plainview, NY), 0.02 U/ml RNase T1 (Life Technologies), 100 μ g/ml RNase A (Sigma, St. Louis, MO), lysed, and incubated for 1 hr at 37°C. Proteinase K (Sigma) was added to a final concentration of 200 μ g/ml and incubation was continued for 15 min at 65°C followed by overnight at 25°C. The lysates were extracted once with

phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), and twice with chloroform:isoamyl alcohol (CI, 24:1, v/v). Genomic DNA in the aqueous phase was ethanol precipitated and solubilized in TE at 4°C. The DNA was digested with restriction endonuclease *Bam*HI (Life Technologies, 5 U/ μ g DNA) at 37°C overnight. Digestions were checked for completion by agarose minigel electrophoresis of an aliquot of the restriction mixture. Since PCR based assays depend critically upon accuracy of DNA concentration, care was taken to ensure precise DNA measurements; i.e. RNA-free DNA preparations were quantitated by absorbance and the concentrations verified by microfluorimetric Hoechst 33258 assay (23) or by a modified microdiphenylamine assay (24).

Damage specific antibody

Polyclonal antibodies against *anti*-BPDE modified DNA were developed in New Zealand white rabbits. The characteristics of BP-1 antibody, used in the present work, have been recently described (21).

Quantitation of *anti*-BPDE adducts in plasmid DNA

Anti-BPDE induced damage in insert and vector DNA sequences of p220-*ras* plasmid was immunoquantitated essentially as described previously (20), except that BP-1 antibody was used. Briefly, the modified plasmid DNA was digested with *Bam*HI endonuclease, denatured, and used in immuno-slot blot (ISB) procedures to quantitate DNA damage. Calf thymus DNA (Sigma) with known levels of modification (0.576 fmoles adduct/ng DNA) was used as a reference standard (25). For the transblot assays, the fragments were electrophoretically separated and Southern transferred onto 0.45 μ m nitrocellulose (NC) filters (Schleicher & Schuell, Keene, NH). The filters containing immobilized DNA fragments were then processed for the immunoquantitation of *anti*-BPDE DNA adducts by a modified ISB assay (26).

Genomic DNA repair analysis

The levels of *anti*-BPDE DNA adducts in total genomic DNA were determined by the ISB assay as described (21,27,28). Increasing amounts of heat denatured DNA samples (10–40 ng) in 0.8 ml of 1 M ammonium acetate (AAc) were applied under low vacuum onto NC filters in a convertible filtration manifold (GIBCO BRL, Gaithersburg, MD). The slots were washed with an additional 0.8 ml of 1 M AAc, the filter removed, rinsed with 1 M AAc, air dried, baked under vacuum at 80°C for 1 hr, and soaked in Blotto (20 mM Tris-HCl, pH 8.0, 120 mM glycine, 5% non fat dry milk powder, 0.5% gelatin, 0.01% thimerosal, 0.01% antifoam-A, and 0.1% Tween 20). Following an overnight incubation at 37°C, the blocked filters were incubated for 1.5 hrs at 37°C with the primary antibody BP-1 (5 μ g/ml in Blotto containing 0.5 M NaCl), washed 4 times with PBS-Tween (PBS containing 0.1% Tween 20), incubated for 1 hr at 37°C with secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG, 1:1,000 dilution in Blotto, Boehringer Mannheim, Indianapolis, IN), and finally washed 3 times with PBS-Tween and twice with AP-9.5 buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM MgCl₂). In the final step, filter bound enzymatic activity was determined by color development with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, both from Sigma) in AP-9.5 buffer. Densitometric evaluation of the color intensity was performed on air dried filters using a LKB laser scanner. The data were

Figure 1. Map of human H-ras protooncogene. The exons are indicated by roman numbered boxes, the primer locations are designated by horizontal arrows. The 149 bp amplified fragment includes the codon 12 of exon 1.

transmitted to an interfaced computer and analyzed with LKBL gelscan XL software as described (28). The adduct level was determined by comparison with *anti*-BPDE modified DNA standards run in parallel within the linear range of the ISB. Data were expressed as a percentage of adducts remaining in DNA at varying times following the exposure to genotoxin. *Anti*-BPDE adducts in DNA from cells treated with varying doses of metabolite were quantitated using the same procedure.

Recovery of adducted DNA by immunoprecipitation

Samples of genomic DNA (25–100 ng) were heat-denatured and brought to a volume of 50 μ l in TN buffer (10 mM Tris–HCl, pH 7.5, 140 mM NaCl). Equal volume of primary antibody BP-1 (0.25 mg/ml in TN containing 5% non fat dry milk powder, 0.5% gelatin, 2% normal rabbit serum (Cappel, Malvern, PA), and 0.8% Tween 20) was added, and the mixture was incubated first for 1 hr at 37°C and then for a minimum of 2 hrs at 4°C. An excess (50 μ l of 0.02 U/ml in TN) of goat antiserum to rabbit γ -globulin (Calbiochem, La Jolla, CA) was added, and the incubation was continued overnight at 4°C. The immunoprecipitate was pelleted by centrifugation in a microcentrifuge (full speed) for 10 min at 4°C and gently washed with TE. To prepare DNA suitable for PCR, the pellet was solubilized in 0.25 M KOH for 30 min at 37°C, then neutralized with 0.15 M HCl and adjusted to 0.075 M Tris–HCl, pH 8.3 and 0.125 M KCl. Deproteinization was performed by repeated CI extraction (4 times), and the aqueous phase was subjected to PCR. The final CI extraction allowed $84 \pm 5\%$ recovery of DNA, as determined with [3 H]-labeled test SV40 DNA added to the neutralized sample prior to first CI extraction. In some experiments, the immunoprecipitated pellets were treated with PreTaq thermophilic protease (Life Technologies) as follows. To the washed pellet, 30 μ l of 10 mM Tris–HCl, pH 8.0 and 20 μ l (6 U) of PreTaq protease were added, and the mixture was incubated for 3 hrs at 75°C. The reaction was stopped by addition of EGTA to give a final concentration of 10 mM, followed by a 30 min incubation at 90°C. Aliquots of the mixture were used for PCR amplification.

PCR amplification of plasmid and genomic DNA

The oligonucleotide primers were synthesized on an automated DNA synthesizer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The primers used in this study were (i) H-ras7 (sense): 5'- AGACCCTGTAGG-

AGGACCCCGGGCC and (ii) H-ras8 (antisense): 5'- ATAGTGGGGTCGTATTCGTCCACAA. This set of primers allows amplification of the 149 bp fragment (1621 to 1770) of exon 1 of the human H-ras gene (29) coding for amino acids 1–34 (Figure 1). Samples of plasmid (0.1–10 pg) and genomic (1–25 ng) DNA or equal aliquots of solubilized immunoprecipitates were amplified (30 cycles) in TempCycler II (COY Corporation, Grass Lake, MI). Each set of samples included DNA-free immunoprecipitation (when necessary) and PCR controls. A typical reaction mixture (30) contained two primers (0.2 μ M each), dATP, dGTP, and dCTP (0.2 mM each), 0.18 mM dTTP, 0.02 mM Bio-11-dUTP (all from Sigma), 50 mM KCl, 25 mM Tris–HCl, pH 8.3, 1 mM MgCl₂, and 2.5 U/ml of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The thermal cycling profile consisted of 30 sec of DNA denaturation at 95°C, 30 sec of primer-template hybridization at 55°C, and 30 sec of primer extension at 72°C. Following amplification, the PCR products were analyzed on a 2.0% agarose gel.

Quantitative analysis of PCR products

For quantitation, the biotinylated PCR product was colorimetrically visualized by streptavidin-alkaline phosphatase binding. The heat denatured aliquots of each sample were immobilized on NC filters, rinsed with 1 M AAc, dried, and baked in a vacuum oven for 1 hr at 80°C. The filters were blocked with Blotto, washed with PBS-Tween and AP-7.5 buffer (0.1 M Tris–HCl buffer, pH 7.5, 0.1 M NaCl, 2 mM MgCl₂, 0.1 % (v/v) Tween-20), and incubated with streptavidin-alkaline phosphatase (1:3,000 dilution in AP 7.5, Life Technologies) for 1 hr at 37°C. After a final washing with PBS-Tween and AP 9.5, the color was developed with NBT and BCIP. The peak intensities were determined by laser densitometry. In initial experiments, enzyme-linked immunosorbant assay (ELISA) was used to quantitate the PCR product. ELISA was performed essentially as described (27), except that incubations with primary and secondary antibodies were omitted. Serially diluted *Bam*HI digests of genomic DNA of known concentrations served as standards and were PCR amplified and ISB or ELISA analyzed in parallel. Immunoprecipitated template DNA (*Bam*HI digested restriction fragments of H-ras gene) was quantitated from PCR standards in the linear response range of the ISB. Background values given by DNA from control untreated cells were subtracted from values obtained upon analysis of DNA from the cells exposed to genotoxin, and the data were expressed as a percentage of damaged DNA template immunorecovered from cells at varying times of repair. Replicate analyses were performed with at least two separate immunoprecipitations followed by two independent PCR amplification for each set of DNA samples. Quantitations were always done with serially diluted PCR standards run in parallel in the linear assay response range.

RESULTS

Immunoquantitation of *anti*-BPDE adducts in plasmid and genomic DNA

To measure repair of specific DNA damage in selected gene sequences, we have established an antibody based assay for quantitative detection of transblotted DNA fragments. Despite identical amounts of DNA in resolved fragments, as revealed by ethidium bromide fluorescence, the antibody binding showed a carcinogen dose dependent increase in the signal intensity (Figure 2 A). Non-specific binding of antibody could not be

Figure 2. Immuno-quantitation of anti-BPDE DNA adduction *in vitro* and *in vivo*. (A) Specific immunodetection of anti-BPDE DNA adducts in insert (6.4 kbp) and vector (8.9 kbp) fragments of p220-ras plasmid. The plasmid DNA samples were treated by anti-BPDE *in vitro* and digested with BamHI to quantitatively release the ras gene containing segment. DNA fragments were separated on 0.8% agarose gel, ethidium bromide stained (upper part), Southern blotted onto NC filter and ISB processed using antibody BP-1 (lower part). Constant amounts of DNA, 10 ng (lanes 1–7) and 100 ng (lanes 8–14), were used for each lane. The concentration of anti-BPDE used for *in vitro* DNA modification was 0 μM (lanes 1,8), 0.03 μM (lanes 2,9), 0.06 μM (lanes 3,10), 0.12 μM (lanes 4,11), 0.25 μM (lanes 5,12), 0.5 μM (lanes 6,13), and 1.0 μM (lanes 7,14). (B) Quantitative antibody binding to the p220-ras DNA as a function of carcinogen dose. 3 ng (filled symbols) and 25 ng (hollow symbols) of *in vitro* anti-BPDE treated plasmid DNA hydrolysates were fractionated and immunodetected by ISB assay with BP-1 antibody. The intensity of each individual band was integrated, peak heights quantitated and plotted against corresponding doses of carcinogen. (C) Quantitative relationship of antibody binding and anti-BPDE DNA adducts. The adduct levels in individual fragments were determined in three separate experiments using appropriately modified DNA standards, and data were expressed as a function of absolute adduct concentration in DNA fragments. (D) Anti-BPDE DNA adduct frequency in ras gene *in vivo*. GM637 cells were treated with indicated doses of genotoxin and the adducts within isolated genomic DNA were quantitated after binding of antibody BP-1 in ISB assay. The adduct level at each dose was determined from analysis of three different DNA concentrations in the linear range of the dose response curves. The data were expressed for 6.4 kbp of genomic DNA assuming a random distribution of specific damage throughout the genome.

detected with an excess amount of control untreated DNA (lanes 1 and 8). At the same time, specific immunodetection of as little as 10 ng of plasmid DNA treated with 0.03 μM anti-BPDE was seen with both insert (6.4 kbp) and vector (8.9 kbp) fragments. The anti-BPDE concentration dependent antibody binding response was plotted for 3 and 25 ng of DNA hydrolysate (Figure 2 B). The fraction of DNA molecules specifically detected by the antibody showed a quantitative relationship of peak height with anti-BPDE concentration. The plateau level of ISB response at anti-BPDE concentrations above 0.5 μM for 25 ng of DNA hydrolysate was overcome by decreasing the amount of DNA. Conversely, a larger amount of DNA could be used for an enhanced sensitivity of detection of very low modification levels in DNA. The antibody binding response normalized for fmoles of anti-BPDE adducts per fragment is shown in Figure 2 C. The overall results indicate that anti-BPDE induced DNA damage can be quantitatively measured in either the ras gene containing insert

or vector sequences of p220-ras by using the linear part of the dose-response curve. Importantly, the equivalent level of DNA adduction corresponded to the equal output ISB signal regardless of the size and sequence context of the fragment studied. Quantitation of damage in plasmid DNA is presented in Table 1. The linearity between the dose of carcinogen and the calculated adduct level per DNA fragment indicated that polyclonal antibody BP-1 can potentially bind a single antigen molecule and detect an adduct within a discrete segment (e.g., a 6.4 kbp ras gene segment). This is important for the quantitative measurement of gene-specific DNA damage and repair (6,31).

The quantitation of anti-BPDE DNA adducts was performed in total genomic DNA isolated from human fibroblasts GM637 after *in vivo* treatment with 2, 4, and 8 μM of genotoxin (Figure 2 D). Immunoassay allowed the quantitative assessment of genomic DNA damage within the indicated range of carcinogen doses. Cellular treatment with 2 μM anti-BPDE resulted in an

Figure 3. Optimization of non-radiolabeled quantitative PCR. (A,B) Reaction was performed in presence of varying concentrations of biotinylated dATP and dUTP in the standard PCR mixture. Plasmid p220-*ras* DNA (10 pg) was amplified for 30 cycles and increasing amounts of PCR product were immobilized and quantitated by ELISA (A) or ISB (B). Incorporated biotin was detected with 1:3,000 diluted streptavidin-alkaline phosphatase. (C,D) Non-radiolabeled quantitative PCR was performed with p220-*ras* plasmid (C) and GM637 genomic (D) DNA templates as described in methods. Varying amounts of DNA were PCR amplified (30 cycles) and the incorporation level of biotinylated dUTP in product analyzed by ISB. Insets show the plots of linear correlation between the amount of template DNA and the PCR product. The amounts of product analyzed were: 0.5% (squares), 1% (triangles), and 2% (circles). Data are representative of experimental standards run alongside each analysis.

adduct/base ratio of 7.65×10^{-5} or 0.979 lesions per 6.4 kbp of genomic DNA. The initial level of *in vivo* DNA adduction in GM4429 cells (XP group A) was found to be 2.44 lesions per 6.4 kbp. It should be noted that DNA immunodetection revealed a good correlation with the data obtained by others using the Southern hybridization method. For example, Chen *et al.* (13) have found that *anti*-BPDE (1.2 μ M) induced 1.31–1.77 lesions per 20 kb of single stranded genomic DNA in *in vivo* treated human fibroblasts.

Non-radiolabeled quantitative PCR amplification of plasmid and genomic DNA

To increase the sensitivity of the antibody-based assay for detection of damage in very small amounts of specific DNA segments, we have established a new method of PCR product analysis by immuno-slot blot or ELISA. In our initial attempts to quantitate the amplified product, we incorporated [32 P]dCTP in the PCR reaction (data not shown). Although this assay is used by others (15–17), quantitation is complicated by the diffused autoradiographic signal and requires the separation of unincorporated label from amplification products. Therefore, biotinylated precursors were substituted in the PCR reaction. In this case, the product analysis is based on the extent of biotin incorporation detected by the binding with streptavidin-alkaline

phosphatase conjugate. Quantitation by ELISA, though easy and quick, indicated decreased binding of PCR product having a high level of biotin (Figure 3 A). Therefore, to circumvent complications with quantitative assessment, a direct ISB procedure was established and used in all subsequent experiments. This assay, while showing comparable sensitivity, elicited the response proportional to product concentration (Figure 3 B). Inclusion of bio-dUTP at 25 μ M concentration in the reaction mixture was determined to be sufficient for quantitation as compared to other labels e.g., bio-dATP (Figure 3) or digoxigenin-dUTP (data not shown).

Specific amplification and quantitative response was seen with very small amounts (0.312–10 pg) of plasmid template DNA (Figure 3 C). A comparable product yield was obtained with genomic template DNA (1.25–25 ng) from repair-proficient GM637 and repair-deficient GM4429 human fibroblast cells (Figure 3 D). PCR was optimized for the cycle number providing proportional response (data not shown). Thirty cycles were found to be adequate for reproducible amplification of small amounts of template DNA. Furthermore, amplification was limited only by the template availability. A linear relationship was shown for the initial amount of template and the resulting amount of PCR product within 1–10 pg for plasmid DNA and 1–25 ng for genomic DNA (Figure 3 C,D, insets). This response ensured

Table 1. Modification of *ras* gene in plasmid by *anti*-BPDE *in vitro*.

<i>Anti</i> -BPDE (μ M)	Adducts/ 10^6 bases	Adducts/p220	Adducts/ <i>ras</i>
0.03	4.55	0.081	0.058
0.06	7.66	0.136	0.098
0.13	14.69	0.261	0.188
0.25	25.71	0.458	0.329
0.5	51.77	0.922	0.663
1.0	93.84	1.670	1.201

Different amounts of p220-*ras* DNA (3.25–25 ng) were treated by *anti*-BPDE, fractionated and immunodetected as in Figure 2 A. The adduct levels were quantitated from analysis of modified standard (calf thymus DNA, adduct level 0.576 fmoles/ng DNA) run in parallel. Data are means of three separate experiments with S.D. < 10%.

Table 2. Effect of target site modification on PCR amplification

DNA template treatment	Target site modifications	PCR product ratio (modified/unmodified template DNA)
Plasmid DNA		
Control	No adducts	—
100 J/m ²	0.21 dimers	1.22
500 J/m ²	1.05 dimers	1.03
CMC + 0.3 M NaCl	0.127 CMC adducts	1.0
CMC + 0.1 M NaCl	0.579 CMC adducts	1.02
Genomic DNA		
Control	no adducts	—
100 J/m ² , <i>in vitro</i>	0.21 dimers	1.06
500 J/m ² , <i>in vitro</i>	1.05 dimers	1.04
8.0 μ M <i>anti</i> -BPDE, <i>in vivo</i>	0.094 adducts	1.01

*Bam*HI digested plasmid (p220-*ras*) or genomic (GM637 cells) DNA at 20 ng/ μ l was modified using a UV source at a fluence rate of 10 J/m²/sec. Modification with CMC (100 mM) was done with form I plasmid DNA in the presence of NaCl followed by *Bam*HI digestion. GM637 cells were treated with *anti*-BPDE as indicated in Materials and Methods. The pyrimidine dimers were calculated using the conversion factor of 7×10^{-3} dimers per J/m² per 1 kb of DNA (57) and CMC adducts were determined by ELISA as described (20,22). Values for *anti*-BPDE modification were taken from Figure 2 D. DNA was subjected to PCR amplification of the target 149 bp *ras* gene fragment, and products were analyzed by ISB assay. The amount of product was normalized to the control unmodified template DNA. Data are mean values from three experiments.

that the values, obtained by the quantitative PCR, reflected the measurement of the original template concentration (15,18).

PCR amplification of damaged template DNA

Of great concern is the possibility that genotoxic modifications of target template DNA may either block the *Taq* polymerase or interfere with effective primer hybridization and thus diminish the yield of the PCR product. Moreover, this inhibitory effect of DNA damage in large size gene fragments has been demonstrated and utilized by others for studying gene-specific DNA repair (15–17). However, our approach is based on a different principle, which assumes proportionality between the amount of immunorecovered damaged template and the output PCR signal. This approach was validated by demonstrating an absence of any influence of a sufficiently high DNA adduct level on the yield of quantitative PCR. Data summarized in Table 2 showed no effect of diverse bulky genotoxic modifications (UV, CMC, and *anti*-BPDE) on amplification of the 149 bp PCR target of the H-*ras* gene in plasmid or genomic DNA. Importantly, DNA isolated from cells treated with a high toxic concentration

Figure 4. Immunorecovery and PCR amplification of *in vivo* *anti*-BPDE modified DNA. GM637 cells were treated with 0–8 μ M *anti*-BPDE for 30 min. Total DNA (prelabeled with [³H]dThd) was isolated from cells immediately after treatment, digested with *Bam*HI, and immunoprecipitated (80 ng) with antibody BP-1. (A) Total DNA recovery by immunoprecipitation was calculated from liquid scintillation counting of aliquots of solubilized pellets. (B) Equal aliquots (30 μ l from total ~40 μ l) of the respective immunoprecipitates were subjected to amplification with primers for a 149 bp target of H-*ras* protooncogene in presence of 25 μ M bio-11-dUTP. PCR product (1%) was quantitated by ISB as in Figure 3.

of *anti*-BPDE (8 μ M) was also found to be a fully suitable template for *Taq* polymerase. For all the genotoxic agents tested, the product yield from damaged template DNA was equivalent to that of the unmodified control template. Effective PCR amplification of modified target sequence has been observed in other studies, particularly with <200 bp amplicons (16,17).

Selective immunoprecipitation and quantitative PCR amplification of *anti*-BPDE adducted genomic DNA

The ability of lesion specific antibodies to selectively separate damaged target DNA molecules from a large excess of unmodified DNA represents a significant advantage of immunoreagent-based techniques (18,32). In our experiments, immunoprecipitation of damage containing DNA restriction fragments with the polyclonal antibody BP-1 allowed recovery of template DNA suitable for PCR amplification. The conditions for incubation, dilution of primary and secondary antibodies, and suppression of non-specific binding were standardized with radiolabeled test DNA templates (data not shown). The results of genotoxin dose dependent immunoprecipitation are shown in Figure 4 A. The amount of control non-adducted DNA precipitated ($7.6 \pm 3.5\%$) did not vary significantly when

Figure 5. Representative non-radiolabeled quantitative PCR amplification of modified human genomic DNA. (A) GM637 cells were exposed to 2 μ M anti-BPDE. DNA was isolated from cells at indicated times and subjected to standard non-radiolabeled PCR as described (see Figure 4 B and Materials and Methods). PCR product was analyzed on a 2% agarose gel (40% of the product, upper part) and by ISB assay (1% of the product, lower part). (B) PCR standard, *Bam*HI digested genomic DNA, was run in parallel and analyzed under identical conditions. C, control unmodified DNA, M, 123 bp DNA marker ladder. The grey background seen in ISB strips is the result of photographic processing of an otherwise white filter.

25–100 ng of DNA was used (data not shown). As expected, DNA samples with higher levels of anti-BPDE adduction were preferentially precipitated by antibody, showing an increase in immunorecovery with increasing dose of genotoxin (0–8 μ M, 0–4 adducts per 6.4 kbp of genomic DNA) (Figure 4 A).

The presence of precipitated IgG protein in the reaction was found to cause an interference with the PCR amplification. Thus, to eliminate extraneous protein, samples were treated with PreTaq thermophilic protease before PCR. However, these deproteinized samples resulted in a low (10%) yield of template DNA (data not shown). On the other hand, repeated (4 times) CI extraction preserved 83.6% of DNA in the aqueous phase with negligible sample-to-sample variation. Template DNA purified by CI extraction of the immunoprecipitates was fully compatible with the PCR reaction and resulted in anti-BPDE dose dependent products upon non-radiolabeled quantitative PCR (Figure 4 B).

Analysis of DNA repair

To study the processing of DNA damage *in vivo*, cells were treated with 2 μ M anti-BPDE and either harvested immediately or allowed time to repair before analysis. Total DNA was isolated from cells, and the elimination rate of the initially induced adducts (0.979 adducts/6.4 kbp of genomic DNA) was determined by (i) direct ISB analysis of the antibody binding to total genomic DNA immobilized on NC filters and (ii) quantitation of the immunorecovered H-*ras* gene template by non-radioisotopic PCR with the primers amplifying a 149 bp fragment (Figure 5). In the case of GM637 cells, ~60% of anti-BPDE adducts were eliminated from a specific *Bam*HI released H-*ras* gene fragment in the first hour followed by slower lesion removal up to 80% in the next 17 hrs (Figure 6). A comparable repair rate, i.e., ~60% and ~90% adduct removal in 7 and 20 hrs, respectively, has been reported in only one other study describing the preferential repair of anti-BPDE adducts in an active gene of human fibroblasts (13). In the genome overall, ~65% of the adducts were present after 3 hrs of repair, and ~42% of the induced damage were not repaired within 18 hrs of incubation.

Figure 6. Repair of anti-BPDE DNA adducts in human cells. Confluent cultures of human fibroblast GM637 (hollow symbols) and GM4429 (filled symbols) cells were treated with 2 μ M anti-BPDE and DNA isolated from cells harvested at various post-treatment times. Repair kinetics was determined for H-*ras* gene target by quantitation of immunorecovered template in standard non-radiolabeled PCR assay (circles). Bulk DNA repair of induced damage was monitored by loss of the antibody binding sites using ISB assay with immobilized genomic DNA (triangles). Results for bulk DNA repair deficiency in GM4429 cells and replication data are omitted for clarity. Plots are mean data for 2 to 4 independent experiments.

This repair rate is identical to the results of Van Houten *et al.* (33) and similar to other studies (34). In contrast, excision-repair deficient GM4429 fibroblasts appeared to show no repair of either the *ras* gene segment (Figure 6) or the whole genome (data not shown) during the 19 hr post treatment time. Interestingly, the same concentration of anti-BPDE (2 μ M) induced higher initial adduct levels in repair-deficient GM4429 cells than in repair-proficient GM637 cells. Such increased levels were also observed by others (33,34) and may be due to enhanced accessibility of genomic sites in XP cells and/or effective initial repair in normal cells during the incubation period with anti-BPDE. Nevertheless, this increased adduct level did not interfere with amplification by *Taq* polymerase (Table 2). The specific radioactivity of DNA from prelabeled GM637 cells did not decrease significantly over the 48 hr post-treatment period after exposure to 1 μ M anti-BPDE (data not shown).

DISCUSSION

An increasing interest in gene-specific repair has stimulated the search for universal and sensitive approaches to study DNA damage processing at subgenomic level (6). Techniques employing DNA damage specific antibodies (18,32) represent a good alternative to the well established assay of Bohr *et al.* (3) which has some site-specific DNA cleavage associated limitations. Antibody-based assays have been successfully used to quantitate the levels of DNA adduction by various genotoxins (22,26,35–38) as well as the repair of specific lesions in cellular DNA (20,31,39,40). Monoclonal and polyclonal antibodies have been shown to clearly separate adducted from non-adducted DNA fragments (32,41). In the present study, we demonstrated the applicability of polyclonal antibody BP-1 to quantitatively detect

anti-BPDE induced DNA damage in discrete fragments of genomic DNA. We have recently shown that this antibody binds to the carcinogenic (+)-*anti*-BPDE-N²-dG lesion with a 40-fold preference over the (-)-*anti*-BPDE-N²-dG adduct (21). Immunoassays of *anti*-BPDE DNA adducts in the plasmid and genomic DNA revealed the high sensitivity and specificity of BP-1 antibody. Less than 0.1 fmoles of *anti*-BPDE adducts per fragment could be detected by immunoslot blot analysis of *in vitro* damaged plasmid DNA. The output ISB signal obtained from analysis of equal amounts of adducts per fragment (insert and vector sequences of p220-*ras*) did not depend on the fragment size and increased linearly with increasing doses of *anti*-BPDE used for treatment. Thus, it appeared that the antibody molecule could specifically recognize as low as one adduct per fragment, the condition necessary for using antibodies in DNA repair studies (6,32).

Based on these findings, we developed a quantitative assay of gene-specific DNA repair, combining the selective immunorecovery of lesion containing genomic fragments and sensitive PCR of the immunorecovered template DNA. Since the first report of Govan *et al.* (16), several approaches employing PCR as a tool to investigate gene-specific DNA damage induction and repair have been described (15,17,18), and a set of transcriptionally active human (16), hamster (17), mouse (15), and rat (18) genes was studied. PCR based assays provided quantitative assessment of DNA damage in very small amounts of single copy genes and were demonstrated to be 3–5 times more sensitive than the traditional Southern transfer based analysis (15). Attempts were made to follow the repair of specific genomic segments by measuring the time dependent restoration of the potential of damaged template DNA to perform PCR amplification. In fact, a variety of bulky DNA lesions can block the *Taq* polymerase (15,16,42) and thus cause a decrease in the amount of PCR product. However, in the study of repair in smaller DNA segments, a necessary requirement of higher DNA damage levels results in an overall lower sensitivity of the assay. As much as 24 kJ/m² of UV irradiation *in vivo* was required to follow the repair in 147–440 bp fragments (16), whereas a dose of 40 J/m² was found to be acceptable for a 2.3 kbp DNA fragment (15). Another study used 75–100 μ M cisplatin (0.46–1.07 adducts per 2,000 bases) to optimize the PCR assay of a 2 kbp genomic segment (17). Moreover, small DNA adducts (e.g., products of N- and/or O-alkylation) located on the template may not interfere with the polymerase (18,43) but still be a crucial substrates for DNA repair. In our study we have overcome these problems; the results show that the amount of PCR product is proportional to the initial amount of damage. Immunorecovered damaged DNA served as a template for amplification of a specific target and its subsequent quantitation. The primers for PCR were designed so that the encompassing DNA region was much smaller than the restricted gene fragment precipitated by the antibody. Data indicate that such target selection and the chosen adduct level (1 or less for the entire double-stranded gene containing segment) caused no interference with the *Taq* polymerase action. This was clearly confirmed by amplification of templates highly modified by diverse UV, CMC, and *anti*-BPDE agents *in vitro* and *in vivo* (Table 2). Thus, for immuno-coupled PCR, the fragment of interest should bear at least 1 antibody binding site (i.e., DNA lesion) and include a small PCR amplifiable 'indicator' sequence. The incorporation of a chemical tag (biotin) in the PCR mixture allowed a quantitative detection of the product. The product yield was strictly dependent on template

recovery by damage specific antibody, and reproducibility of the assay was achieved by replication and analysis of serially diluted template standards in parallel PCR reactions. The efficient and reproducible amplification of 149 bp template as well as the absence of false products and primer dimers, allowed the use of PCR protocol without an internal standardization otherwise necessary for comparative analysis. It might be speculated that with the help of selected sets of primers, the search can be focused on any domain of interest generated by appropriate mixtures of restriction endonucleases.

It should be noted that, if the *anti*-BPDE adducts are distributed randomly, according to Poisson equation, the 'zero-adduct' class molecules (~37%) will not be precipitated by the antibody. Loss of antibody binding site (due to repair) would move the molecules into the 'zero-adduct' class only from the 'one-adduct' class but not from the other groups. In other words, removal of one lesion from the fragment class comprising 2 or more adducts (~26%), would not necessarily lead to changes in the precipitation status of the fragment. As a result, the repair may be somewhat underestimated. Moreover, the interpretation of data might be complicated further, if the efficiency of precipitation is a function of the adduct number or modal size of restriction fragment. This could very well be the reason for lower than expected precipitation of DNA modified *in vivo* with 8 μ M *anti*-BPDE (Figure 4 A). Such saturation of modified DNA immunoprecipitation, at higher doses of inducing agent, has also been observed by others (41). These limitations notwithstanding, and for simplicity, we have assumed that, when the target gene contains ~1 lesion per segment, the post-treatment template immunorecovery provides an indicator of the adduct removal from the specific DNA segment. Within the constraints of this model, the repair in a gene sequence, determined by immuno-coupled PCR, offers a comparison to the bulk DNA repair derived from the direct antibody binding to specific adducts in immobilized genomic DNA. Nevertheless, further refinements and fine mapping should allow the evaluation of microheterogeneity of DNA damage and repair by this approach and provide new insights into the target preferences of mammalian repair systems as well as the role of repair deficiency in mutation predisposition and carcinogenesis.

The phenomenon of gene-specific repair has been demonstrated in a variety of organisms (6,10). Preferential repair of UV-damage has been well documented in several active genes of mammalian cells (3–5,9,44,45). Less is known about many other types of critical DNA lesions. Cisplatin induced intrastrand adducts (12) and alkylation damage introduced by nitrogen mustard (46,47) and methylnitrosurea (11) were shown to be repaired preferentially while no gene-specific repair was seen for cisplatin interstrand crosslinks (12) and dimethylsulfate alkylation lesions (46,47). Repair analysis of bulky adducts of polycyclic aromatic hydrocarbon *anti*-BPDE has shown equivocal results. Recently, Chen *et al.* (13) have explained the strand bias for *anti*-BPDE induced premutagenic lesions (48) to be a result of preferential repair in the hypoxanthine(guanine) phosphoribosyltransferase (*HPRT*) gene of human cells. However, others found no preferential repair of the DNA damage induced by *anti*-BPDE (49) or N-acetoxy-acetylaminofluorene (50) in the hamster dihydrofolate reductase gene. Adducts of carcinogenic 4-nitroquinoline-1-oxide were also shown to be removed non-preferentially from the hamster genome (51). Our finding that the *anti*-BPDE induced bulky adducts are eliminated faster from the transcriptionally active *H-ras* protooncogene than

from the overall genome of human GM637 fibroblasts, is consistent with the data of Chen *et al.* (13). Importantly, DNA replication in GM637 cells was effectively blocked at 1 μ M of *anti*-BPDE (data not shown). Therefore, the reduction in *anti*-BPDE induced DNA damage was not due to DNA synthesis resulting in lesion dilution. It should be noted that in our experiments exposure to 2 μ M *anti*-BPDE, introducing 1 adduct per 6.4 kbp *ras* fragment, did not show significant cell death during the 24 hr post-treatment. Thus, our repair results support the suggestion of Chen *et al.* (13) attributing the aforementioned discrepancy between the studies (13,49) to the differences in cells and genotoxin doses.

Gene-specific repair of UV induced DNA damage has been recently shown in the actively transcribing genes in human fibroblast strains from XP group C patients (52–54). Data presented here indicate absence of gene-specific as well as overall genomic DNA repair of *anti*-BPDE adducts in XP group A GM4429 fibroblasts. This data is consistent with (i) inability of XP group A cells to perform effective nucleotide excision repair (34,55), (ii) reported lack of strand bias for mutations induced by *anti*-BPDE in the *HPRT* gene of XP12BE cells (complementation group A) (56) and (iii) a non-preferential UV repair phenotype for cells from individuals in XP complementation groups A and D (54).

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