REV1 accumulates in DNA damage-induced nuclear foci in human cells and is implicated in mutagenesis by benzo[a]pyrenediolepoxide

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ABSTRACT

The REV1 gene encodes a Y-family DNA polymerase that has been postulated to have both catalytic and structural functions in translesion replication past UV photoproducts in mammalian cells. To examine if REV1 is implicated in DNA damage tolerance mechanisms after exposure of human cells to a chemical carcinogen, we generated a plasmid expressing REV1 protein fused at its C-terminus with green fluorescent protein (GFP). In transient transfection experiments, virtually all of the transfected cells had a diffuse nuclear pattern in the absence of carcinogen exposure. In contrast, in cells exposed to benzo[a]pyrene-diolepoxide, the fusion protein accumulated in a focal pattern in the nucleus in 25% of the cells, and co-localized with PCNA. These data support the idea that REV1 is present at stalled replication forks. We also examined the mutagenic response at the HPRT locus of human cells that had greatly reduced levels of REV1 mRNA due to the stable expression of genespecific ribozymes, and compared them to wild-type cells. The mutant frequency was greatly reduced in the ribozyme-expressing cells. These data indicate that REV1 is implicated in the mutagenic DNA damage tolerance response to BPDE and support the development of strategies to target this protein to prevent such mutations.

INTRODUCTION

Most mutations induced by carcinogens occur when DNA containing residual (unrepaired) damage is replicated during S-phase of the cell cycle. Such lesions perturb the structure of DNA and are likely to block replicative DNA polymerase complexes, which have stringent base-pairing requirements. Knowledge of the fundamental mechanisms used by cells to complete the replication of damaged genomes, and of the factors that determine if this process will be error-free or error-prone, is likely to be useful in elucidating the origins of cancer and other human diseases. At present, these mechanisms have been studied most intensively in the budding yeast, Saccharomyces cerevisiae (1). In this organism, virtually all mutations induced by bulky DNA adducts are dependent on the activity of DNA polymerase ζ, which is encoded by the REV3 and REV7 genes, acting in concert with another DNA polymerase encoded by the REV1 gene (2). In vitro, pol ζ inserts bases across from DNA damage very inefficiently but extends mispaired primer termini with high efficiency. These data led to a two-step, two polymerase model for translesion replication (3–5). This model proposes that a translesion DNA polymerase in the Y-family incorporates 1–2 nt directly opposite a lesion, and pol ζ extends the primer beyond the lesion downstream of the damage. The high fidelity replicative machinery then extends the primer, but the 3′–5′ proofreading exonuclease activity cannot remove the nucleotides incorporated during translesion replication (6). In yeast, the activity of pol ζ is stimulated by REV1. The molecular mechanism whereby REV1 stimulates pol ζ activity has not been fully elucidated, but it has been proposed to play a structural role that is independent of its polymerase activity (7).

The strategies used by yeast cells to complete the replication of damaged genomes appear to have been conserved in higher eukaryotes, but with additional layers of complexity. The human homologs of REV1 (8,9), REV3 (10–12) and REV7 (13,14) have been cloned, and the REV1 protein has been purified and studied in vitro (15). Primer-extension studies using purified REV1 indicate that the protein is highly distributive and catalyzes the insertion of dCMP when it encounters a template guanine, a guanine with a large adduct at the C8 or N2 position, or an abasic site. However, it is completely unresponsive to thymine–thymine cyclobutane pyrimidine dimers or to 6-4 photoproducts, the principal lesions induced by ultraviolet light (UV) (16). Despite this, REV1 plays a central role in mutagenic translesion replication past UV photoproducts in vivo in human cells. Reducing the level of REV1 using a variety of genetic approaches, including...

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expression of antisense to REV1 mRNA (8) or intracellular expression of ribozymes targeting endogenous REV1 mRNA (17), greatly reduces the frequency of mutations induced by UV. This activity is presumably mediated through its interactions with DNA polymerase ζ, possibly by associating with its REV7 subunit (14,18,19).

The ubiquitous environmental carcinogen benzo[a]pyrene is metabolized to the ultimate carcinogen (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, (BPDE), which principally reacts with the N² position of deoxyguanosine residues in DNA. These adducts block DNA replication and are highly mutagenic. In vitro, human REV1 is capable of accurate nucleotide insertion across from these adducts, although error-prone insertion of dG or dT was also observed (16). Extension of the nascent chain can be accomplished by sequential action of purified human REV1 and Pol κ, where REV1 inserts dCMP and Pol κ catalyzes the extension of DNA synthesis from opposite this lesion (18,20). However, the most common mutations induced by BPDE in human cells are G to T transversions (21–23). This implies that the translesion polymerase inserts dA across from the lesion, which was not observed in the primer extension studies using purified human REV1.

To further define the function of REV1 in human cells, the subcellular localization of REV1–GFP fusion protein was examined before and after exposure to BPDE. In undamaged cells, REV1–GFP expression was confined to the nucleus in a diffuse pattern in virtually all the transfected cells. After exposure to BPDE, the fusion protein localized in a distinct focal pattern in the nucleus in 25% of the cells. These foci were only observed in cells that stained for PCNA in a pattern characteristic of cells in S-phase, and the foci co-localized with PCNA. These data support the idea that REV1 is present at presumed stalled replication forks. The frequency of mutations induced by BPDE was determined in normal human fibroblasts and in cells derived from them that had greatly reduced levels of REV1 mRNA. In the latter cells, the BPDE-induced mutant frequency at the endogenous HPRT locus was reduced by at least two-thirds compared with parental cells that had wild-type levels of REV1. The cytotoxicity was unchanged. These data implicate REV1 in DNA damage-induced mutagenesis, and support the development of countermeasures for cancer prevention using this protein as a potential therapeutic target.

**MATERIALS AND METHODS**

**Localization studies**

**Construction of the REV1–GFP expression plasmid.** Full-length REV1 cDNA was amplified by PCR using the DNA from plasmid pEGLh6-REV1 (kindly provided by Dr Zhigang Wang, University of Kentucky) as a template and REV1F (5’-atagaggcaggttgatggagga-3’) and REV1R (5’-tgtaaccttttaa-ttgatttcatga-tttggt-3’) oligonucleotides as primers under standard PCR conditions. The PCR product was analyzed on a 0.8% agarose gel in TBE and the REV1 band was gel extracted and purified using the Strata Prep Gel Extraction Kit (Stratagene, La Jolla, CA). The REV1 fragment was then cloned into pcDNA3.1/CT-GFP-TOPO vector according to manufacturer’s instructions (Invitrogen, Carlsbad, CA). The positive transformant in correct orientation was identified using restriction analysis with the enzyme XbaI. The positive transformant produced two restriction fragments of sizes 8.8 kbp and 1.1 kbp. The sequence and orientation of the gene encoding the fusion protein were confirmed by sequencing with T7 and GFP reverse primers. A plasmid expressing only the GFP protein (Clontech, Palo Alto, CA) was used as a control.

**Cell lines and DNA transfection.** Plasmids expressing GFP or REV1–GFP fusion protein were electroporated into 293T cells. The 293 cell line was originally derived from human renal epithelial cells that had been transformed by the adenovirus E1A gene product; 293T is a derivative that also expresses SV40 large T antigen, allowing episomal replication of plasmids containing the SV40 origin and early promoter region. The cells were maintained in exponential growth in complete medium, DMEM (Mediatech Inc, Herndon, VA) supplemented with 10% calf serum (SCS, Hyclone), 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Plasmids expressing GFP or REV1–GFP fusion proteins were electroporated into 293T cells using a Nucleofector 1 device (Amaza Inc, Gaithersburg, MD). Following the manufacturer’s instructions, 1 × 10⁶ cells were suspended in Cell line Nucleofector™ solution V (Amaza Inc, Gaithersburg, MD), with 15 μg of plasmid expressing REV1–GFP fusion protein or 5 μg of plasmid expressing GFP. Electroporation was accomplished using program Q-01 specified for 293T cells. Subsequently, the cell suspension was plated in 2 ml complete medium without antibiotics in a 6-well plate containing Lysine-coated coverslips (Biocoat, Becton Dickinson, Bedford, MA). The expression of the fluorescent proteins was observed 24 h after electroporation.

**Exposure to BPDE.** The culture medium was aspirated and the cells were washed with sterile phosphate buffered saline (PBS, pH 7.4) and replaced with serum-free medium (0.5 ml). Powdered BPDE (NCI Chemical Carcinogen Repository, Midwest Research Institute) was dissolved in anhydrous tetrahydrofuran (THF) at a concentration of 150 μM. The stock was added to the medium to a final concentration of 0.15 μM. After 1 h incubation at 37°C, the medium was replaced with complete medium. The control cells were exposed to solvent only.

**Antibody labeling.** After a post-treatment incubation of 0 or 6 h, cells were fixed in 100% methanol at −20°C for 15 min. Methanol was aspirated and the coverslips were air-dried for 5–10 min. The coverslips were incubated in PBS for 5 min at room temperature followed by incubation in PBST (1× PBS containing 0.2% Triton X-100) for 5 min at room temperature. Cells were blocked in GPBST (PBST containing 10% goat serum) for 1 h at room temperature. One microgram of primary mouse antibody against human PCNA (PC 10, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was incubated with 7.5 μl Zenon mouse IgG labeling reagent (4.5:1 molar ratio of Fab to antibody target for PCNA) for 5 min in the dark followed by another 5 min incubation in the dark with 7.5 μl Zenon blocking reagent (Zenon mouse IgG labeling kit, Alexa fluor 594, Molecular Probes, Eugene, OR). The cells were then incubated with the antibody (1:80 dilution in GPBST) at room temperature in the dark for 1 h, followed by fixation in 4% formaldehyde in PBS for 15 min at room temperature and by three washes in PBS. Cells were incubated in equilibration...
buffer [Slow Fade Light Antifade kit with 4',6-diamidino-2-phenylindole (DAPI), Molecular Probes, Eugene, OR] for 10 min and mounted onto slides using antifade reagent in glycerol buffer.

**Confocal microscopy.** Fluorescent cell images were acquired using an Olympus LSM Fluoview 500 Confocal laser scanning microscope (Olympus Optical Co Ltd, Tokyo, Japan) and V Plan/Apo 100× oil objective (NA 1.35; α/0.17). The images were analyzed using Olympus LSM Fluoview 500 software (Version 4.3). GFP and Alexa 594 were imaged sequentially by exciting GFP with 488 nm Argon Laser and Alexa 594 with 543 nm He/Ne Laser and then the fluorescence was merged to study co-localization. A 405 nm LED laser was used to image the DAPI-stained nuclei.

**Mutagenesis studies**

**Cells and cell culture.** The primary fibroblast cell strain GM1604 (Coriell Institute) was originally derived from human fetal lung tissue, and was immortalized by expression of the catalytic subunit of human telomerase (hTERT), under a license from Geron Corporation. The cells were immortalized by Dr Lisa McDaniels (University of Texas Southwestern Medical Center, Dallas) and named NF1604. They were provided to W.G.M. by Dr McDaniels under the terms of Material Transfer Agreement 3025 between W.G.M. and Geron Corporation. Cell strain WR20 was derived from these cells by stable transfection of a plasmid that expresses a ribozyme, (Coriell Institute). WR20 line was cultured in the same medium but supplemented with neomycin (100 μg/ml). For selection of thioguanine-resistant (TG) cells, cells were plated in selective medium, which is DMEM medium with 5% fetal calf serum (FCS), 5% supplemented calf serum, penicillin and streptomycin as above, and 40 μM 6-thioguanine (6-TG, Sigma, St Louis, MO).

**Determination of the cytotoxic and mutagenic effects of BPDE.** Populations of exponentially growing cells were plated at a density of 10^6 cells/cm^2^, and were allowed ~12 h to attach. Immediately before exposure to BPDE, the culture medium was aspirated and replaced with serum-free medium. The carcinogen, dissolved in anhydrous THF, was delivered into each culture dish by micropipette, the final concentration ranging from 0 to 0.15 μM. After 1 h, the medium was removed and replaced with complete medium. The cells in one dish for each dose of BPDE and for the solvent-only control were immediately replated at cloning density to evaluate the survival of colony-forming ability (cytotoxicity). The rest were maintained in exponential growth for 8 days to allow expression of resistance to 6-TG. After 8 days, the cells from each population were plated in selective medium. The colony-forming ability of the cells at the time of selection was also determined by plating the cells at cloning density in nonselective medium. This value was used to correct the observed frequency of mutants. When macroscopic drug-resistant clones were identified after 14 days in selective medium, the plates were stained with crystal violet and the mutant frequency was determined. Mutant frequency is defined as the observed number of TG clones per million cells, corrected for cloning efficiency on the day of selection.

**RESULTS**

**Subcellular localization of fluorescent proteins**

Preliminary experiments showed that the expression of the REV1–GFP fusion protein was very low to absent in a variety of human cell lines, including the NF1604 cell line used in the mutagenesis assays. This is presumably due to the very large size of the fusion protein, predicted to be ~165 kDa, since the GFP with a mass of ~40 kDa was expressed in most cells after gene transfer. We found that 293T cells have a higher transfection efficiency, allowing us to examine the localization of the fusion protein.

The GFP protein expressed by the control plasmid was homogeneously distributed in both the cytoplasm and the nucleoplasm of all transfected cells (Figure 1A). This pattern did not change after BPDE exposure (Figure 1D). In contrast, expression of the REV1–GFP fusion protein was confined to the nucleus. We examined 200 control (undamaged) cells derived from each of three independent experiments. In virtually all of the transfected cells, the fusion protein was expressed in a diffuse pattern throughout the nucleus (Figure 1B and C). Within 6 h of exposure to BPDE, REV1–GFP accumulated in a distinct focal pattern in the nucleus (Figure 1E and F). In the 200 cells examined, this pattern was observed in 50 cells (25%).

We noted that the PCNA fluorescence in asynchronous populations of 293T cells showed two distinct patterns, which can be seen in the images in Figure 1E and F. In about half of the cells, PCNA was present in the cytoplasm and in the nucleus with a diffuse pattern. In the rest, there was intense nuclear staining that had a fine punctuate pattern, and little cytoplasmic staining. There are four such cells in Figure 1E and F, indicated by arrows. We only observed the REV1–GFP focal pattern in cells that had the nuclear PCNA fluorescence, and the foci demonstrated fluorescence due to the presence of both the fusion protein and PCNA in most cases (merged images in Figure 1E and F). Asynchronous populations of 293T cells have 42% of the cells in S-phase, as determined by flow cytometry (data not shown), and we hypothesized that the focal pattern was observed in damaged cells that were in S-phase. To examine if the focal pattern of the fusion protein only occurred in S-phase, we attempted to synchronize the 293T cells with serum starvation, mimosine or lovastatin but were unsuccessful. However, we examined the pattern of PCNA staining in NF1604 fibroblasts, which are easily synchronized by serum starvation (17). As shown in Figure 2A, cells in G1 demonstrate cytoplasmic, perinuclear and diffuse nuclear PCNA fluorescence. In populations enriched for S-phase, the great majority of the cells have PCNA in a distinctive punctuate pattern in the nucleus.

**BPDE-induced cytotoxicity**

Populations of NF1604 cells or the ribozyme-expressing WR20 cells derived from them were examined for cytotoxicity induced by BPDE. The cells were exposed to the carcinogen (0–0.15 μM) at same density used in the mutagenesis
experiments, but immediately after carcinogen exposure, the cells were replated at cloning density. Three independent experiments were done at each dose, and the average was determined. The clonogenic survival of the cell populations is shown in Figure 3A (top panel) and Table 1(A). There was a BPDE dose-dependent exponential decline in survival in all populations we examined, and there was no detectable difference in the clonogenic survival of the ribozyme-expressing cells compared with the parental NF1604 cells. At the highest dose examined (0.15 \text{mM}), the survival ranged from 11 to 15% compared with the untreated control populations.

BPDE-induced mutagenesis

The mutant frequency in populations of control cells increased as a function of BPDE dose. Table 1(B) presents representative data from experiments with cells derived from control NF1604 cells, indicating that the mutant frequency was 44 TG\textsuperscript{+} cells per 10\textsuperscript{6} clonable cells after 0.10 \text{mM} BPDE (survival, 50%), and progressively increased to 113 mutants per 10\textsuperscript{6} clonable cells at 0.15 \text{mM} (17% survival). The background frequency in control cells (exposed to solvent only) was 11 per 10\textsuperscript{6} clonable cells. The cloning efficiency of the cells at the time of selection ranged from 16 to 18%. The average mutant frequency induced at each dose is presented in Figure 3B, with the solid line drawn through the data for the control cells.

We compared the mutant frequencies induced in control cells with those induced in WR20 cells, which we previously reported to have 15% of the level of endogenous REV1 mRNA compared to the control NF1604 cells (17). At every dose examined, the mutant frequencies induced in the REV1-depleted cells were reduced by at least two-thirds compared with those induced in the control cells. Table 1(B) presents representative data from experiments done on WR20 cells, and the data are plotted in Figure 3B. In these cells, exposure to the highest dose of 0.15 \text{mM} BPDE resulted

Figure 1. Subcellular localization of GFP and REV1–GFP fusion proteins. In each case, PCNA is red, nuclei fluoresce blue due to DAPI staining, and GFP is green. Untreated controls: (A) Diffuse nuclear and cytoplasmic distribution of GFP. (B and C) Distribution of REV1–GFP fusion protein within the nucleus in a diffuse pattern. Six hours after BPDE treatment: (D) Distribution of GFP, which remains diffuse throughout the nuclear and cytoplasmic compartments and is unchanged by BPDE treatment. (E) Nuclear localization of REV1–GFP fusion protein in a focal pattern in the nucleus after BPDE treatment. REV1–GFP fusion protein co-localizes with the PCNA foci, seen as yellow foci. (F) Co-localization of REV1–GFP fusion protein and PCNA (enlarged view). The arrows indicate cells in a punctuate pattern of PCNA in the nucleus. REV1–GFP foci were only observed in cells that showed this nuclear PCNA pattern.
in 12% survival and 45 TG<sup>+</sup> cells per 10<sup>6</sup> clonable cells, which is a 66% reduction in the frequency of induced mutants. The background mutant frequency and the cloning efficiency of the ribozyme-expressing cells were not different from that of the control cells.

**DISCUSSION**

The focal nuclear pattern of REV1–GFP accumulation after BPDE exposure was only observed in cells that were in S-phase, and the REV1–GFP foci co-localized with PCNA. These observations strongly support the hypothesis that REV1 is present at replication forks that have been stalled by BPDE-induced adducts in the template DNA. Recently, REV1 was observed in a similar focal pattern after UV irradiation, which also induces replication blocking lesions (24). The co-localization of PCNA with several Y-family polymerases, including pol η, τ, κ (25–27) and REV1 [(24), present study] after DNA damage suggests at least two possibilities: either the stalled replication fork recruits these proteins, or the polymerases are present at the replication fork and the
The presence of DNA damage facilitates their visualization. The latter view is supported by the observation that exposure to hydroxyurea, which inhibits DNA replication by depleting nucleotide pools but does not induce DNA damage, also induces nuclear foci containing pol k (27).

The functional role of REV1 in carcinogen-induced cytotoxicity was examined by reducing the level of endogenous REV1 transcripts with intracellular expression of gene-specific ribozymes. In such cells, BPDE did not have a measurable effect on the clonogenic survival compared with genetically identical cells that do not express the ribozyme. These data are consistent with the finding that reducing the level of REV1 transcript in human cells does not affect UV-induced cytotoxicity (8,17). These cytotoxicity results contrast with those in chicken lymphoblastoid DT40 cells that have a complete deletion of REV1 (28). In those cells, there is moderately enhanced cytotoxicity to several agents that induce structurally diverse kinds of DNA damage, including UV, H2O2, ionizing radiation, nitroquinoline oxide and cis-platinum. This discrepancy may reflect the high proportion of DT40 cells in S-phase (20–30%) compared with the human fibroblasts used in this study (20–30%). Cells in late G1 or S at the time of carcinogen exposure have a higher probability that the replication fork will be blocked by DNA adducts, and may have a greater reliance on REV1-associated translesion replication mechanisms. Alternatively, the reduced but not absent level of REV1 in the human cells may be sufficient to rescue the cells. This question requires further investigation.

Reduction of the level of REV1 mRNA within the cell greatly reduces the frequency of mutations induced by BPDE in the endogenous HPRT gene. These results are consistent with earlier observations that UV-induced mutagenesis is also greatly reduced in such cells (8,17), and leave little doubt that both REV1 and REV3 (11,29) are centrally involved in mutagenic translesion replication in human cells. The molecular mechanisms are far less clear. In the case of replication past adducts induced by BPDE, in vitro studies suggest it is possible that REV1 catalyzes the error-free insertion of dCMP across from the adducted dG with extension by another polymerase. However, in vitro studies indicate that REV1 cannot catalyze the insertion of bases across from UV-induced photoproducts. It is likely that the role REV1 plays in translesion replication is independent of its dCMP transferase activity. Friedberg and colleagues recently showed that mouse REV1, which is 86% identical to the human protein,

### Table 1. BPDE-induced cytotoxicity and mutagenicity in asynchronous populations of fibroblasts

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<th>Cell strain</th>
<th>Ribozyme expression level</th>
<th>Endogenous REV1 mRNA level</th>
<th>Dose (μM)</th>
<th>Percent survival</th>
<th>No. of cells selected (10⁶)</th>
<th>Thioguanine-resistant colonies</th>
<th>Mutants/10⁶ clonable cells</th>
<th>Induced mutant/10⁶ clonable cells</th>
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<td>2</td>
<td>11</td>
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<td>18</td>
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<tr>
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<td>100</td>
<td>1</td>
<td>2</td>
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*These values have been corrected for cloning efficiency on the day of selection.

Figure 3. Effects of ribozyme expression on colony-forming ability and mutant frequency at the HPRT locus of cells exposed to BPDE. (A) Clonogenic survival after BPDE exposure: filled diamonds, NF1604 cells; filled squares, WR20 cells. (B) Comparison of BPDE-induced mutant frequencies of NF1604 cells (solid line, filled diamonds) and WR20 cells (dashed line, open squares).
interacts with a variety of translesion DNA polymerases when
the respective mouse proteins are over-expressed in
human cells (30). A C-terminal 100 amino acid fragment of
mREV1 was shown to be required for its interaction with
murine Pol κ, Pol λ, Pol η and the noncatalytic mREV7 subunit
of Pol ζ (30). These data support an important role for REV1
in coordinating the activity of translesion polymerases,
possibly by providing a scaffold to allow polymerase switch-
ing at the lesion site.

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diploid human fibroblasts on the kinds and locations of mutations induced by (+/-)
beta,8 alpha-dihydroxy-9 alpha,10 alpha-epoxy-7,8,9,10-
tetrahydrobenzo[a]pyrene in the coding region of the HPRT gene.
Kinds and locations of mutations induced by (+/-)
beta,8 alpha-dihydroxy-9 alpha,10 alpha-epoxy-7,8,9,10-
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