Ribozyme-mediated REV1 inhibition reduces the frequency of UV-induced mutations in the human *HPRT* gene

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Received June 3, 2003; Revised and Accepted July 13, 2003

ABSTRACT

In yeast, mutations induced by UV radiation are dependent on the function of the *Rev1* gene product, a Y-family DNA polymerase that assists in translesion replication with potentially mutagenic consequences. Human *REV1* has been cloned, but its role in mutagenesis and carcinogenesis remains obscure. To examine the role of REV1 in UV mutagenesis in human cells and to evaluate its potential as a therapeutic target to prevent such mutations, we developed a ribozyme that cleaves human REV1 mRNA *in vitro*. Stable expression of the ribozyme in human cells reduced the target REV1 mRNA up to 90%. We examined the cytotoxic and mutagenic response to UV of seven independent clones that had reduced levels of endogenous REV1 mRNA. In each case, the clonogenic survival after UV was not different from that of the parental cell strains. In contrast, the UV-induced mutant frequencies at the endogenous *HPRT* locus were reduced up to 75% in cells with reduced levels of REV1 mRNA. The data support the idea that targeting the mutagenic translesion DNA replication pathway can greatly reduce the frequency of induced mutations.

INTRODUCTION

Data indicate that most mutations induced by UV occur when DNA that contains residual (unrepaired) damage is replicated during S phase of the cell cycle. Such lesions perturb the structure of DNA (1) and are likely to block replicative DNA polymerase complexes, which have stringent base-pairing requirements. Until recently, there has been little understanding of how cells complete the replication of damaged genomes, or of the factors that determine if this process will be error free or error prone. Despite recent advances in this area (2,3), the fundamental mechanisms that generate mutations are poorly understood, but such knowledge 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*. In this organism, mutagenic bypass of DNA damage is equivalent to error-prone translesion replication (4). Virtually all mutations induced by UV are dependent on the activity of DNA polymerase ζ (pol ζ) acting in concert with another DNA polymerase encoded by the *Rev1* gene. REV1 is required for UV mutagenesis *in vivo*, and interacts with pol ζ *in vitro* to stimulate translesion replication activity (5). 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 (6).

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 *hREV1* (7,8), *hREV3* (9–11) and *hREV7* (12) have been cloned. *In vitro* primer extension studies using purified hREV1p (13) indicate that the protein is highly distributive and catalyzes the insertion of dCMP when it encounters guanine, guanine adducted with a large chemical adduct, or an abasic site, but is unable to bypass UV photoproducts. In addition to REV1p, higher eukaryotic cells have at least three other DNA polymerases in the Y-family, including pol η , ι and κ . These enzymes are best characterized by their low fidelity when copying undamaged templates and their ability to bypass lesions that block DNA polymerases in other families (2). The relative contributions of each of these enzymes to UV-induced mutagenesis *in vivo*, if any, are unknown. However, it is likely that REV1p contributes to UV mutagenesis in human cells, since cells that express antisense to REV1 were found to have lower UV-induced mutant frequencies than a non-transfected parental cell line (7).

To examine the role of REV1p in UV mutagenesis in human cells, we developed ribozymes that are catalytically active against hREV1 mRNA. Ribozymes are short RNA molecules that hybridize to a complementary target sequence of RNA and cleave this sequence catalytically and site-specifically in

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the absence of proteins (14). The 'hammerhead' ribozyme element is usually only 30–40 bases long, and is composed of two target recognition arms and a correctly folded catalytic domain (15). The advantages of employing such structurally simple RNA enzymes for *in vivo* settings are the ease of preparation, their specificity and their negligible toxicity and immunogenicity. On the other hand, concerns that need to be considered prior to *in vivo* applications of ribozymes include their intracellular stability, sufficient expression levels, the accessibility of the cleavage site in the native folded mRNA target and the compartmentalization of the ribozyme versus target RNAs in the cell. Although not all parameters critical for efficient intracellular and *in vivo* ribozyme activity have been explored systematically, it is apparent that the ribozyme approach has high potential for inhibition of gene expression in both diagnostic and therapeutic strategies (16).

We found that intracellular expression of the ribozymes in several clones derived from two independent normal human fibroblast cell lines resulted in a significant reduction in REV1 mRNA. Moreover, the UV-induced mutant frequency was greatly reduced in each clone at every dose examined. These data indicate that REV1 function is required for most mutations induced by UV in human cells, and support the development of ribozymes that target REV1 to reduce sunlight-associated mutagenesis and carcinogenesis.

MATERIALS AND METHODS

Cells and cell culture

The primary human fibroblast cell strain GM0024 (Coriell Institute) was originally derived from a dermal biopsy of an adult male. Dr Jerry Shay (University of Texas Southwestern Medical Center, Dallas) immortalized the cells by expression of the catalytic subunit of human telomerase (hTERT) under a license from Geron Corporation. The immortalized cells (tGM24) were obtained from Dr Christopher States (University of Louisville) under the terms of the Material Transfer Agreement (MTA) 3025 between W. G. McGregor and Geron Corporation. The primary fibroblast cell strain GM1604 (Coriell Institute) was originally derived from human fetal lung tissue, and was immortalized as above (17). The immortalized cells (NF1604) were provided by Dr Lisa McDaniels (University of Texas Southwestern Medical Center, Dallas) under the terms of MTA 3025. Cells were maintained in exponential growth in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Hyclone), 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml). For selection of thioguanine-resistant (TG^r) cells, cells were plated in DMEM with 5% FCS, 5% supplemented calf serum, penicillin and streptomycin as above, and 40 µM 6-thioguanine. The cloning efficiency at the time of selection was determined by plating cells at cloning density in the same medium, but omitting 6-thioguanine.

Plasmids were transfected into cells using standard lipid-mediated transfection protocols. Briefly, cells in exponential growth were plated onto p100 dishes at a density of 1.8×10^3 cells/cm². For each plate, 4 µg of plasmid DNA was mixed with 20 µg of DOTAP/Dope (Avanti Polar Lipids, Huntsville, AL) and incubated for 30 min at room temperature. The

DNA–lipid complex was then mixed with 3 ml of DMEM without serum or antibiotics. The cells were incubated with the DNA–lipid–medium mix for 4 h. The transfection mix was removed and replaced with DMEM containing 10% FCS but no antibiotics. After 48 h, the medium was replaced and selection begun with G418. After 3 weeks, macroscopic drug-resistant colonies were identified and expanded.

Determination of the mutagenic and cytotoxic effects of UV radiation

Cell synchronization. Cells were driven into the G₀ state by density inhibition and mitogen deprivation, as described previously (18), and stimulated to enter the cell cycle by replating at a density of 10⁴ cells per cm². The time of onset of S phase was ~16 h after replating, determined by flow cytometry.

Exposure to UV light and determination of mutant frequency.

A series of independent populations (1.5×10^6 cells each, plated in 150 mm diameter dishes) was irradiated 17 h after release (~1 h after the onset of S phase). For irradiation, the culture medium was aspirated, and the cells were washed with sterile phosphate-buffered saline (PBS; pH 7.4). The cells were irradiated as described previously (18) with 0–10 J/m² UV_{254 nm}. For each dose of UV, sufficient dishes were used to insure at least 1×10^6 surviving cells. Cells plated at cloning density were similarly irradiated and used to determine survival of colony-forming ability. Cells exposed at cloning density were allowed 14 days to form colonies, with fresh culture medium added ~24 h and 1 week after UV, and then stained. Cells exposed at higher density were allowed to grow for 4–5 days, then were detached from the plates, pooled and 2×10^6 were replated to continue growing for an additional 4 days. After the 8–9 day expression period, at least 1×10^6 cells from each population were selected for resistance to TG as described (18). The colony-forming ability of the cells at the time of selection was also determined by plating the cells at cloning density in non-selective 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 and the mutant frequency was determined. Mutant frequency is defined as the observed number of TG^r clones per million cells, corrected for cloning efficiency on the day of selection.

Design of ribozymes

Potential ribozyme target sites within REV1 mRNA were identified using the MFOLD RNA structural modeling program to identify possible unpaired or loop regions (19). A sequence surrounding the GUC triplet with the predicted cleavage at position 618 was chosen. The ribozyme sequence was determined based on the appropriate complementary sequence recognition arms and the conserved hammerhead catalytic core. Oligonucleotides that contained this sequence and its complement, together with a 5' HindIII and a 3' XhoI restriction site for cloning purposes, were commercially synthesized (Integrated DNA Technologies). The sequence of the non-transcribed strand was 5'-AGC TTC ACA CTG CTG ATG AGT CCG TGA GGA CGA AAC TGC TTG GTG TC. The sequence of the transcribed strand was: 5'- TCG AGA CAC CAA GCA GTT TCG TCC TCA CGG ACT CAT CAG

CAG TGT GA. DNA that was predicted to code for a catalytically inactive ribozyme that differed by a single base (G to A in the non-transcribed sequence) was also synthesized. Annealed double-stranded DNA oligonucleotides coding for active or inactive ribozyme were cloned between the HindIII and XhoI sites of pCDNA3 (Clontech); sequence analysis indicated that no mutations were introduced during the cloning process.

Cloning of REV1 5' fragment into a T7 expression system

The plasmid pEGLh6-REV1, which contains the full-length REV1 cDNA, was kindly provided by Dr Zhigang Wang (University of Kentucky). A 2.2 kb XbaI-HindIII fragment encoding the 5' portion of REV1 was excised and cloned into pBluescript II KS (Stratagene) (pBS-KS-REV1).

In vitro RNA transcription

RNA was transcribed *in vitro* from plasmids that encoded the active or inactive ribozyme sequences, or from pBS-KS-REV1 that encoded a portion of the target (REV1) mRNA using the Ampliscribe T7 transcription kit (Epicentre). Briefly, 1 µg of the plasmid to be transcribed was linearized by digesting with either EcoRI (*REV1*) or XhoI (ribozymes). The final transcription reaction mix contained DNA template, 7.5 mM of each NTP, 40 mM Tris-HCl pH 7.5, 10 mM NaCl, 6 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 1 µl of RNasin and 2 µl of T7 Ampliscribe Transcriptase (Epicentre) in 20 µl. The reaction proceeded for 2.5 h at 37°C, followed by DNase I digestion of the template. After the addition of 5 mM EDTA, the RNA was extracted with phenol:chloroform and ethanol precipitated. The integrity of the RNA was determined by running aliquots on a denaturing 5% polyacrylamide gel.

Ribozyme catalytic activity in vitro

Active or inactive ribozyme was mixed with REV1 target RNA and heated to 80°C for 4 min. The RNAs were then slowly cooled to allow annealing before 20 mM MgCl₂ was added to start cleavage reactions. Aliquots were taken at 0, 15, 45, 90 min and 4 h, and run on a denaturing 5% polyacrylamide-7 M urea gel to check for the expected size (400 and 200 bases) cleavage fragments.

RT-PCR analysis of ribozyme expression

Total RNA was isolated from drug-resistant clones using the Purescript Total RNA Isolation kit (Gentra Systems). RNA was quantified, and reverse transcription (RT) (Promega) was performed using oligo(dT) primers and 200 ng of RNA for each reaction. PCRs were performed using the products of the RT reactions and using RNA that had not been reverse transcribed as template(s). The PCR primers were Sp6 (IDT) and a ribozyme gene-specific primer, 5'-ATT GAT GTT GCA TGG AGG TC. Briefly, 0.2 mM dNTP mix, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM MgCl₂, 0.6 pmol gene-specific primer, 0.6 pmol Sp6 primer (IDT), 4 µl of RT product, 5 U of *Taq* polymerase (Promega) and water to 50 µl were mixed. The following cycles were then run: 94°C, 4 min; 35 cycles of 94°C, 1 min, 42°C, 1 min, 72°C 2 min; and final extension of 72°C for 7 min. The products were examined on a 1.5% agarose gel.

Figure 1. Structures of human REV1 ribozymes. The two sequence recognition arms are shown base paired with the REV1 target mRNA, surrounding the cleavage site at base 618, marked as C[^]. The central catalytic core is invariant. G* = G to A change in the inactive ribozyme.

Northern blot analysis of REV1L mRNA expression

mRNA was isolated from the expanded clones and parental cells using the Gen Elute Direct mRNA Isolation Kit (Sigma). The samples were run on a 1.2% agarose/formaldehyde gel for 3.5 h at 100 V. Downward transfer was performed overnight onto Immobilon Ny+ (Millipore). After transfer, the membrane was washed and cross-linked using a DNA Transfer Lamp (Fotodyne). The membrane was hybridized with a ³²P randomly labeled 800 bp PCR fragment derived from the 5' portion of REV1 cDNA, washed and placed on a PhosphorImager cassette. The imaging screens of the cassettes were scanned with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant analysis software. Loading was normalized using a probe for GAPDH.

RESULTS

Ribozyme sequences and expected cleavage sites

The secondary structures of REV1 mRNA were studied using the MFOLD program. A suitable GUC sequence in an unpaired region of REV1 mRNA near the 5' end of the RNA was identified around base 618 (Fig. 1). The sequence arms were modified accordingly and are shown matched with the mRNA sequence from REV1L. The G to A base change in the catalytic core inactivates the ribozyme cleavage function without perturbing the folded structure.

Ribozyme-mediated cleavage activity in vitro

Active and inactive ribozyme RNAs at different ratios were mixed with the REV1 RNA, and different time points were taken from 0 to 4 h. The active ribozyme cut the target into the expected 200 and 400 base sizes (Fig. 2A). As the ratio of inactive to active ribozyme was increased, the amount of REV1 cleavage decreased (Fig. 2B and C), indicating that the inactive ribozyme inhibited the cleavage activity of the active ribozyme by binding to the target without cutting. The inactive ribozyme alone did not cleave the mRNA even after 4 h (Fig. 2D).

Screening and characterization of tGM24-derived clones

Ribozyme expression in clones derived from tGM24 fibroblasts was examined using RT-PCR of total RNA extracted from the cells (Fig. 3). Clones 5 and 6 express the ribozyme.

Figure 2. *In vitro* cleavage tests for REV1 ribozyme 618 using different ratios of active to inactive ribozyme. Target RNA (0.02 fM final concentration) was mixed with inactive ribozyme (0.126 fM final concentration), active ribozyme (0.100 fM) or a mixture of the two. Aliquots were taken at 0.2 min (lane 4), 15 min (lane 5), 45 min (lane 6), 90 min (lane 7) and 4 h (lane 8). The expected 400 and 200 base fragments are seen between the top target RNA and the bottom ribozyme bands. The ratios (w/w) of active/inactive ribozyme were: (A) 1/0, (B) 2/3, (C) 1/3 and (D) 0/1. The gel was visualized with ethidium bromide. The amount of uncut REV1 increased as the amount of inactive ribozyme increased, and no cleavage was seen with the inactive ribozyme alone. Marker lanes: active ribozyme (lane 1), inactive ribozyme (lane 2), target RNA (lane 3).

Figure 3. RT-PCR analysis of ribozyme expression in tGM24 cells and transfectants. RNA samples without (–) or with (+) reverse transcriptase were used as templates for PCR. Lanes: 1, Clone 3; 2, Clone 5; 3, Clone 6; 4, Clone 7; 5, parental cells tGM24; 6, positive control (pcDNA3-Rz618); 7, negative control (no template). The gel was visualized with ethidium bromide. Clones 3 and 7 show DNA contamination in their RNA; clones 5 and 6 show ribozyme expression. The DNA in lanes 1 and 6 is larger because a primer that anneals to the T7 promoter was used instead of the internal gene-specific primer.

Clones 3 and 7 could not be evaluated because a PCR product was obtained in the negative RT samples, indicating that the RNA was contaminated with DNA. Northern analysis revealed that clones 6 and 7 had greatly reduced REV1 mRNA (Fig. 4). In preliminary experiments, cells from clone 6 were shown to grow well in culture, and were chosen for further analysis.

UV-induced clonogenic survival and mutation frequency in tGM24-derived cells

A series of independent populations of tGM24 cells and Clone 6 cells were synchronized and irradiated with 6 J/m² of UV_{254 nm} 17 h after release, as the cells were entering S phase. After an 8 day expression period, *HPRT* mutagenesis assays

Figure 4. Northern blot analysis of REV1 RNA levels in transfected tGM24 clones. (A) Probing for REV1 RNA levels (upper panel). Lanes: 1, Clone 5; 2, Clone 6; 3, Clone 7; 4, parental cell strain tGM24; and probing for 18S rRNA as reference (lower panel). (B) Quantitation and normalization of the above blots, showing the reduction of REV1 RNA in clones relative to the parental strain tGM24 (set to 100%).

were performed. The results of assays performed on tGM24 and Clone 6 are shown in Table 1. There was no difference in the clonogenic survival of the two cell lines. However, at equivalent clonogenic survival, Clone 6 had a 2.5-fold reduction in the mutant frequency when compared with the parental tGM24 strain.

Screening and characterization of NF1604-derived cells

Plasmids expressing either active or inactive ribozymes (pcDNA3-Rz618 and pcDNA3-MtRz618) were transfected into NF1604 cells, and several drug-resistant NF1604 colonies were screened by RT-PCR (Fig. 5). Each of the clones expressed the ribozymes, but the levels of expression were qualitatively different. Northern blot analysis was done to examine the levels of endogenous REV1 transcript in each of the clones (Fig. 6A). ImageQuant analysis showed that REV1 was significantly reduced in each of the transfected clones (Fig. 6B).

UV-induced cytotoxicity and mutagenicity in NF1604-derived cells

Populations of NF1604 cells and the ribozyme-expressing clones were plated at cloning density and exposed to 0, 4, 6, 8 or 10 J/m² UV_{254 nm}. There was no detectable difference in the clonogenic survival of any of the ribozyme-expressing clones compared with the parental NF1604 cells (Fig. 7A). Initial mutagenesis experiments were performed in asynchronous NF1604 cells and clones. At each dose examined, there was a reduction in the mutant frequency of the cells expressing the

Table 1. Effect of REV1 ribozyme on the UV-induced cytotoxicity and mutagenicity in synchronous populations of tGM24 cells irradiated at the beginning of S phase

Cell strain	Ribozyme expression level	Endogenous REV1 RNA level	Dose of UV ₂₅₄ (J/m ²)	Percent survival	No. of cells selected (10 ⁻⁶)	Thioguanine-resistant colonies	Mutants/10 ⁶ clonable cells ^a
tGM24	None	100%	0	100	1	2	6
			6	29	2	56	78
Clone 6	High	10%	0	100	1	3	9
			6	29	2	20	19

^aCorrected for cloning (15–22%) at the time of selection.

Figure 5. RT-PCR analysis of ribozyme expression in NF1604 cells and transfectants. RNA samples without (–) or with (+) reverse transcriptase were used as templates for PCRs. Lanes: 1, active Rz1; 2, active Rz15; 3, active Rz20; 4, inactive Rz5; 5, inactive Rz10; 6, inactive Rz15; 7, parental cell line NF1604; 8, negative control (no template); 9, positive control (pcDNA3-Rz618). The gel was visualized with ethidium bromide. All clones showed ribozyme expression without DNA contamination. Qualitatively, clones active Rz20 and inactive Rz5 showed the highest ribozyme expression levels.

Figure 6. Northern blot analysis of REV1 mRNA levels in transfected NF1604 clones. (A) REV1 mRNA levels (upper panel) for clones compared with their GAPDH levels (lower panel). Lanes: 1, NF1604 parent strain, performed in triplicate; 2, inactive Rz5; 3, inactive Rz15; 4, active Rz1; 5, active Rz13; 6, active Rz20. (B) Quantitation and normalization of the above blots, showing the reduction of REV1 RNA in clones relative to the parental strain NF1604 (set to 100%, shown as the average of the three replicate lanes).

Figure 7. Effects of ribozyme expression on colony-forming ability and mutant frequency at the *HPRT* locus of cells exposed to UV radiation. (A) Clonogenic survival after UV: open diamond, NF1604 synchronous; filled diamond, NF1604 asynchronous; open square, inactive Rz5; filled square, active Rz20 asynchronous; open circle, active Rz1; cross, inactive Rz15; star, active Rz20 synchronous; $R^2 = 0.91$. (B) Comparison of UV-induced mutant frequencies of synchronous NF1604 cells (solid line, filled diamonds, $R^2 = 0.9398$) and synchronous active Rz20 cells (dashed line, open squares, $R^2 = 0.96$). Cells were irradiated at the beginning of S phase.

Table 2. Effect of REV1 ribozyme on the UV-induced cytotoxicity and mutagenicity in asynchronous populations of NF1604 cells

Cell strain	Ribozyme expression level	Endogenous REV1 mRNA level	Dose of UV ₂₅₄ (J/m ²)	Percent survival	No. of cells selected (10 ⁻⁶)	Thioguanine-resistant colonies	Mutants/10 ⁶ clonable cells ^a
NF1604	None	100%	0	100	0.5	1	11
			8	26	1	8	44
Active Rz 1	Low	30%	0	100	0.5	1	11
			8	28	1	4	15
Active Rz 20	High	23%	0	100	0.5	1	10
			8	18	1	2	4
Inactive Rz 5	High	24%	0	100	0.5	3	14
			6	23	1	6	18
Inactive Rz 15	Low	20%	0	100	0.5	3	12
			6	29	1	10	25

^aCorrected for cloning (22–36%) at the time of selection.**Table 3.** Effect of REV1 ribozyme on the UV-induced cytotoxicity and mutagenicity in synchronous populations of NF1604 cells irradiated at the beginning of S phase

Cell strain	Ribozyme expression level	Endogeneous REV1 mRNA level	Dose of UV ₂₅₄ (J/m ²)	Percent survival	No. of cells selected (10 ⁻⁶)	Thioguanine-resistant colonies	Mutants/10 ⁶ clonable cells ^a
NF1604	None	100%	0	100	2	7	14
			4	37	1	13	28
			6	23	1	10	52
			8	18	1	19	64
Active Rz 20	High	23%	0	100	2	2	4
			4	36	1	2	10
			6	29	2	10	18
			8	18	1	5	21

^aCorrected for cloning (32–39%) at the time of selection.

active ribozyme, and also a reduction in cells expressing the inactive ribozyme (Table 2). The clone with the lowest mutant frequency, Act Rz 20, was chosen for further study. In order to minimize the effect of nucleotide excision repair on the frequency of mutations, we synchronized populations of cells and irradiated them at the beginning of S phase, as described (18). There was no difference in the clonogenic survival of the two cell lines at doses up to 10 J/m² (Fig. 7A). However, the mutant frequency of Act Rz 20 was reduced by at least 60% of the original value at each UV dose examined (Table 3; Fig. 7B).

DISCUSSION

The mechanisms that generate mutations in eukaryotes have been studied most intensively in the budding yeast *S.cerevisiae*. In this organism, mutations induced by DNA-damaging agents such as UV occur during translesion DNA replication, a process in which primers are extended past sites of helical distortions that would otherwise block elongation of the nascent DNA. Proteins encoded by *Rev3* and *Rev7* make up pol ζ, which is required for translesion replication together with the dCMP transferase encoded by *Rev1*. Deletion of any of these genes results in a similar phenotype in which the cells do not generate mutations after exposure to a variety of mutagenic agents, including UV. It is likely that the DNA damage tolerance pathways that have been extensively analyzed in yeast have been conserved in higher eukaryotic cells, including human. However, the presence of several

non-homologous accessory DNA polymerases in such cells suggests that there are additional layers of complexity.

The human REV1 protein, like its yeast homolog, belongs to the Y family of DNA polymerases (20). The deoxycytidyl transferase activity of this protein has been conserved throughout evolution (21), implying a role for this activity in cellular homeostasis. However, it is likely that the function of the protein in translesion replication is not dependent on this activity, since yeast mutants that are deficient in mutagenesis retain dCMP transferase activity (22). *In vitro* studies of the purified human protein indicate that it is able to insert dCMP opposite a variety of damaged bases and apurinic/apyrimidinic (AP) sites (13), but is unresponsive to thymine–thymine cyclobutane dimers or 6–4 photoproducts in the template (13). Nevertheless, it is likely that REV1 plays a role in translesion replication past UV photoproducts *in vivo*, since expression of antisense RNA to REV1 was shown to reduce the frequency of UV-induced mutations in a human cell line (7). This activity is presumably mediated through its interactions with pol ζ, possibly by associating with REV7 (23,24). Recently, REV1-deficient chicken DT40 lymphocytes were shown to be sensitive to a variety of mutagens, including UV. These cells have normal resting and damage-induced sister chromatid exchange, but have an increased frequency of chromosome and chromatid breaks after UV (25). These studies suggest that REV1 is involved in mutagenic translesion replication, but it is unclear if this function can be separated from other roles the protein may have in the maintenance of genomic stability.

To address these questions, and to evaluate potential therapeutic agents, we chose to modulate the activity of REV1 *in vivo* using hammerhead ribozymes. This choice was governed by the potential advantages such molecules have over conventional antisense approaches in gene therapy applications. First, the cleavage action of ribozymes permanently inactivates the target mRNA, and, secondly, the catalytic nature of ribozyme activity implies that one ribozyme can act on many target molecules. The pre-formed secondary structure of the short ribozyme molecules imparts a target accessibility that is not enjoyed by long antisense molecules. Finally, ribozymes may have fewer non-specific effects than antisense molecules (26).

We examined the cytotoxic and mutagenic effects of UV on two independent normal human fibroblast cell strains, and in clones derived from them that express ribozymes and have greatly reduced levels of REV1 mRNA. There was no apparent difference in survival between the two parental strains and their ribozyme-expressing derivatives after UV irradiation (Fig. 7). These data are consistent with Gibbs *et al.* (7), who found that a human fibroblast line and two derivative clones that express antisense to REV1 exhibit no change in survival in response to UV. In contrast, yeast *rev1* null mutants have moderately enhanced UV cytotoxicity compared with wild-type strains. One explanation for the ability of the human cells with reduced REV1 levels to survive after UV is that higher eukaryotes have a greater variety of Y-family DNA polymerases to accomplish the replication of damaged templates, thereby avoiding double-strand breaks and cell death (2).

In contrast, reduction in REV1 mRNA mediated by catalytically active ribozymes resulted in a greatly reduced UV-induced mutation frequency at each dose examined. These data support a central role for REV1 in UV mutagenesis, and indicate that reducing the level of this protein reduces the frequency of UV-induced mutants without adversely affecting cell survival after UV. In addition, we found that catalytically inactive ribozymes also had measurable inhibitory activity on REV1 activity, and also reduced the UV-induced mutant frequency. This behavior is not surprising since the mutated ribozyme can still bind to its target mRNA, and thus may elicit antisense-like inhibitory effects on protein production. In addition, it is conceivable that the short double-stranded regions in the mutant ribozyme–target RNA complex may attract intracellular double-strand-specific RNase activity, resulting in decreased target mRNAs as observed here for some clones (27). Also, small interfering RNA (siRNA)-like effects may potentially contribute to such decreases, although the strict sequence and length requirements for siRNAs make this less likely (28,29).

Our results indicate that targeting the mutagenic translesion DNA replication pathway can greatly reduce the frequency of induced mutations. We hypothesize that delivery of plasmids that express REV1 ribozymes to sun-exposed skin will reduce the frequency of UV-induced mutations and sunlight-associated skin cancer. We are currently examining various expression cassettes and delivery systems to test this hypothesis in a UV-induced skin cancer model in hairless mice. Delivery methods for DNA-based expression vectors which produce such optimized and stabilized REV1 ribozymes intracellularly in treated skin will be developed and applied

prior to UV exposure to achieve preventive protection from UV-induced DNA damage.

ACKNOWLEDGEMENTS

We thank Dr Zhigang Wang, University of Kentucky, for the human REV1 expression plasmid pEGLh6-REV1L, Dr Lisa McDaniels, University of Texas Southwestern Medical Center, for NF1604 cells, Dr Chris States, University of Louisville, for tGM24 cells, and Geron Corporation for the use of cells that were immortalized with hTERT. This work was supported by grants to W.G.M. from the University of Louisville School of Medicine, Philip Morris USA, Inc., Kentucky Lung Cancer Research Board and NIH CA73986, and by NIH DE13150 to W.Z.

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