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hRev7, putative subunit of hPol ζ , plays a critical role in survival, induction of mutations, and progression through S-phase, of UV_(254nm)-irradiated human fibroblasts

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Abstract

Translesion synthesis (TLS) refers to mechanisms by which specialized DNA polymerases incorporate nucleotides opposite fork-blocking lesions and extend replication until standard replicative polymerases take over. The first eukaryotic TLS polymerase discovered, *S. cerevisiae* Pol ζ , consists of catalytic subunit Rev3 and non-catalytic subunit Rev7. Human homologs of these two proteins have been identified. Studies by Lawrence, Maher, and colleagues comparing UV_(254nm)-irradiated human fibroblast cell strains expressing high levels of *hRev3* antisense to their normal parental strains, demonstrated that there was no difference in cell survival, but that the frequency of UV-induced mutations in the derivative strains was 10-fold lower than that of the parental strains, indicating that hRev3 plays a critical role in such mutagenesis. To examine the role of hRev7 in TLS, we generated human fibroblasts expressing *hRev7* siRNA, identified two derivative cell strains with significantly reduced levels of hRev7, and compared them to their parental strain and a vector control for cell survival, induction of mutations, and ability to traverse the cell cycle following exposure to UV radiation. Cells with reduced hRev7 were ~2-times more sensitive to UV-induced cytotoxicity than the controls, indicating that unlike hRev3, hRev7 plays a protective role for cells exposed to UV radiation. When these cell strains were assayed for the frequency of mutations induced by UV in their *HPRT* gene, cell strains with reduced hRev7 were 5-times less sensitive to UV-induced mutagenesis than control strains. In addition, when these four strains were synchronized at the G1/S border, released from the block, UV-irradiated, and allowed to traverse the cell cycle, the rate of progression through S-phase of the cell strains with reduced hRev7 was significantly slower than that of the control strains. These data strongly support the hypothesis that hRev7 is required for TLS past UV-photoproducts, and together with hRev3, comprise hPol ζ .

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1. Introduction

Human cells are continually exposed to endogenous and exogenous DNA damaging agents, many of which create fork-blocking lesions. If DNA replication past such lesions cannot take place, this can lead to cell death, nevertheless replication past such lesions can result in mutations. Because mutations play a crucial causal role in the development of cancer, it is important to examine processes that produce them.

Human cells have efficient, error-free repair pathways for excising DNA fork-blocking lesions from either strand of their DNA. They also possess cell cycle checkpoints [1], some of which, when activated, provide additional time for excision repair to occur before the replicative polymerases encounter fork-blocking lesions, such as UV-induced pyrimidine dimers. In spite of these protective processes, replication forks still encounter lesions. Cells have evolved damage tolerance mechanisms to cope with such lesions, viz., translesion synthesis and damage avoidance pathways. Such methods of dealing with fork-blocking damage have been, and continue to be actively examined. Overviews summarizing in detail such areas of research can be found in reference [2].

Translesion synthesis in both prokaryotes and eukaryotes involves specialized DNA polymerases capable of incorporating nucleotides directly across from fork-blocking DNA lesions. This insertion step can be error-free or error-prone, depending upon 1) the type of DNA lesion encountered, 2) the specialized polymerases involved, and 3) the sequence context surrounding the site of the damage. Insertion of a nucleotide or nucleotides by one or other such polymerases is followed by extension, i.e., the addition of nucleotides beyond the site of the blocking lesion. This latter step also involves TLS DNA polymerases. Such extension beyond the damage is necessary if the high fidelity replicative DNA polymerases are to resume their function. Thus, TLS is a two-step process whereby specialized DNA polymerases, with relaxed fidelity, incorporate and/or extend nucleotides at sites of fork-blocking DNA damage, allowing DNA replication to continue, but often introducing mutations.

Reports and summaries of the discovery of many translesion synthesis polymerases, first in *S. cerevisiae*, and later in mammalian cells, can be found in the cited references (see for ex., [3-6]). However, many aspects still remain to be clarified. Pol ζ was found using *S. cerevisiae* cells whose specific mutated phenotypes could not be reverted to wild type by exposure to mutagenic agents. Genes that complemented the deficiencies in such strains of *S. cerevisiae*, i.e., allowed them to revert, were identified and subsequently shown to code for proteins that allow replication past fork-blocking DNA damage [7,8]. For example, the yeast Rev3 protein was found to exhibit polymerase activity in primer extension assays *in vitro*. The addition of yeast Rev7 to such assays enhanced the polymerase activity of Rev3 over 20-fold. Together, Rev3 and Rev7 were recognized as constituting yeast Pol ζ [9].

Genes coding for the human homologs of yeast Rev3 [10-13] and Rev7 [14] were subsequently identified. By using antisense directed against *hRev3* mRNA, Lawrence, Maher, and their colleagues [10,15] demonstrated that hRev3, the putative catalytic subunit of hPol ζ , is critically involved in generating UV-induced mutations in diploid human

fibroblasts. These results indicate that hRev3 is essential for a mutagenic process involving DNA lesions that interfere with replication, just as yeast Rev3 is. The hRev3 protein of human cells, a predicted 353 kDa molecule [10], has not yet been isolated, but the non-catalytic subunit, hRev7, a much smaller molecule, has been isolated [14].

The present study was carried out to test the hypothesis that hRev7, the putative noncatalytic subunit of hPol ζ , is also involved in human cell mutagenesis. For such a study, an approach similar to that used for investigating the role of hRev3 was employed, but instead of using antisense RNA to block expression of the target protein, siRNA against hRev7 was used to reduce the level of this protein in human fibroblasts. The fact that antibodies capable of detecting very low levels of hRev7 protein were available allowed us to identify independent cell strains in which the level of hRev7 protein had been greatly reduced by siRNA. Comparing the results obtained using these cell strains with those obtained using their parental human fibroblasts and a vector control strain allowed us to demonstrate that hRev7, the noncatalytic subunit of hPol ζ , plays a role in the survival of UV-irradiated human cells, and has a significant role in UV-induced mutagenesis. Using these human cell strains, we also demonstrated that reduction in the expression of hRev7 impedes the cells' ability to progress through S-phase.

2. Materials and methods

2.1. Cell culture

Cells were grown in Eagle's minimum essential medium, supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1 mM sodium pyruvate, 10% supplemented calf serum (HyClone), 100 units/ml penicillin, 100 μ g/ml streptomycin, 1 μ g/ml hydrocortisone and 1 μ g/ml tetracycline.

2.2. Cell strains

The parental human cell strain used for these studies, designated MSU-1.2.9N.58, was derived from the cell strain MSU-1.2, a spontaneous derivative of the infinite life span cell strain MSU-1.1, whose origin from the foreskin-derived from a normal neonate and subsequent acquisition of an unlimited life span in culture has been described [16]. MSU-1.2 cells are near-diploid, chromosomally-stable, and grow vigorously as a result of expressing their endogenous gene for platelet-derived growth factor.

2.3. Derivation of cell strains with reduced hRev7

Oligonucleotides designed to target *hRev7* mRNA were annealed to a complementary oligonucleotide according to the manufacturer's protocol (Ambion). Using T4 DNA ligase (New England Biolabs), annealed-oligonucleotides were ligated into the pSilencer3.1 vector (Ambion), which includes the gene coding for puromycin resistance, and purified. The parental MSU-1.2.9N.58 cells were transfected with such siRNA vectors, using Lipofectamine (Invitrogen) according to the manufacturer's protocol, and stable transfectants were selected and maintained in medium containing 1 μ g/ml puromycin.

2.4. Preparation of nuclear protein extracts and Western blot analysis

Subconfluent monolayers of cells were washed with ice-cold phosphate buffered saline (PBS), scraped from the 150-mm-diameter dishes in 1 ml of lysis buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), and incubated on ice for 15 min. 10% NP-40 (62 μ l) was added to each sample of lysed cells, and the samples were vortexed for 10 sec, and centrifuged for 30 sec at 10,000 RPM, 4°C. The supernatant containing cytoplasmic proteins was removed, and the nuclear pellet was washed once in 1 ml of buffer A containing 10% NP-40. Nuclear proteins were extracted by disruption of the nuclei in 40 μ l of lysis buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF) and incubated on ice for 15 min with vortexing every 5 min. Nuclear extracts were centrifuged at for 5 min at 16,000 RPM, 4°C. The supernatants, which contained the nuclear proteins, were saved. Protein was quantified using the Bradford method (Pierce). Protein lysates were subjected to gel electrophoresis using 14% SDS-polyacrylamide, transferred to a PDVF Immobilon membrane (Millipore), and probed with a 1:600 dilution of a custom-made (Bethyl) rabbit polyclonal antibody raised against the C-terminal 19 amino acids of the human Rev7 protein. The membrane was probed with a 1:7500 dilution of goat anti-rabbit secondary antibody (Sigma) and visualized using SuperSignal chemiluminescent detection reagent (Pierce). Equal protein loading was confirmed by probing with a 1:10,000 dilution of a rabbit Ku80 antibody (Santa Cruz) and a 1:10,000 dilution of anti-rabbit secondary antibody (Santa Cruz).

2.5. Assay of UV cytotoxicity

The cytotoxic effect of UV_(254nm) radiation was determined by assaying the colony-forming ability of the cells as described [17]. Briefly, cells in exponential growth were detached from the dishes with trypsin, plated at cloning densities (100-600 cells per 100-mm-diameter dish), and allowed 12 h for attachment. The cells were rinsed twice with PBS, the excess PBS was removed, and the cells were irradiated with the designated doses of UV_(254nm) as described [17]. Immediately after irradiation, the cells were given fresh culture medium. The culture medium was renewed 24 h after irradiation and again after 7 days. After 14 days, the resulting clones were stained with crystal violet. Cell survival was determined by comparing the cloning efficiency of the irradiated cells with that of the sham-irradiated control cells. Cell survival at each dose was expressed as a percent of the cloning efficiency of the unirradiated control cells for each cell strain.

2.6. Assay for frequency of UV-induced mutations

The mutagenic effect of UV radiation in each strain was determined from the frequency of cells that lost expression of the *HPRT* gene and, therefore, were resistant to 6-thioguanine. As described [17], sufficient sets of cells plated at densities of 0.5-1.5 x 10⁶ cells per 150-mm-diameter dish, were used, to ensure at least 1 x 10⁶ surviving target cells per dose. Cells were allowed 12 h for attachment, then rinsed twice with PBS, UV-irradiated at the designated doses, and immediately covered with fresh culture medium. The culture medium was renewed 24 h after irradiation, and the cells were allowed to replicate for four days. Cells were then detached using trypsin, pooled, plated at densities of 0.5-1.0 x 10⁶ cells per

150-mm-diameter dish, and allowed to grow exponentially for four additional days in order to deplete the pre-existing wild-type HPRT protein. Cells were then detached using trypsin, and plated at a density of 500 cells/cm² in medium containing 40 μ M 6-thioguanine (TG) to select for cells lacking functional HPRT protein. At the same time, a portion of cells from each population was plated in non-selective medium at a density of 100 cells per 100-mm-diameter dish to assay the colony-forming ability of the cells at the time of selection. The medium on these cells was renewed after seven days. After 14 days, the colonies that had formed were stained with crystal violet, and the frequency of 6-TG-resistant colonies was calculated using the cloning efficiency of the cells at the time of selection. The induced frequencies for each cell strain were calculated by subtracting the background frequencies in the sham-irradiated control populations that accompanied each experiment.

2.7. HPRT (hypoxanthine phosphoribosyl transferase) mutation spectrum analysis

HPRT-defective colonies were obtained essentially as described above for the UV-induced mutation frequency protocol, except that populations were kept independent to avoid sibling mutations. The TG-resistant mutant clones were isolated by treating with trypsin, and each independent mutant was subjected to reverse transcription, followed by two rounds of PCR to amplify the *HPRT* coding region. PCR products were purified (Qiagen) and sequenced at the MSU macromolecular structure facility to determine the specific mutation in the coding region of *HPRT*. Only base substitutions at adjacent pyrimidines that resulted in an amino acid change were considered UV-induced mutations.

2.8. Cell synchronization and flow cytometry analysis

Each cell strain was plated at a density of 0.2×10^6 cells per 100-mm-diameter dish and allowed 12 h for attachment. Cells were then re-fed with complete culture medium containing lovastatin at a final concentration of 60 μ M to synchronize the cells in early G1 phase. After 12 additional hours of incubation, the medium containing lovastatin was removed, the cells were washed twice with PBS, and culture medium containing aphidicolin at a final concentration of 2 μ g/ml and mevalonic acid at a final concentration of 6 mM was added to the dishes for 12 h to synchronize the cells at the G1/S border. The cells were released from the aphidicolin/mevalonic acid block by rinsing twice with PBS, and immediately irradiated with the designated doses of UV as described [17]. At the designated times post-irradiation, cells were detached using trypsin, fixed in 80% ethanol, and stained with a propidium iodide solution (PBS, 1 mg/ml propidium iodide, 10% Triton X-100, 0.5 mM EDTA, 10 mg/ml RNase A) for cell cycle analysis by flow cytometry. Asynchronously-growing cells were assayed in parallel experiments. Statistical methods. To compare the slopes of the curves in Fig. 2A and 2B, a regression model was used. The slopes indicate the effect of UV on the survival (A) of the cells' colony-forming ability and on their frequency of UV-induced mutants (B). The data were taken from a series of experiments that were treated as blocks in the regression analysis.

For comparing the types of mutations induced (see Table 1), the sparse categories, i.e., C→G and T→G, were collapsed for the chi-square analysis. They showed no statistically significant difference between the clones and the comparison group (P-value = 0.60). Other

categories were collapsed to reduce the number of table cells analyzed with small expected frequencies.

3. Results

3.1. Efficient reduction of hRev7 protein using siRNA

The parental cell strain, designated MSU-1.2.9N.58, was transfected with a vector expressing an siRNA targeted against *hRev7* and also carrying the gene for puromycin resistance to allow selection of transfectants. As a control, we similarly transfected the parental cells with a vector containing this selectable marker and siRNA that has no significant homology to any human gene sequence. Puromycin-resistant clones were isolated and expanded. Nuclear protein extracts from the parental cells, vector control transfectants, and from candidate transfectants that received the siRNA against *hRev7* were prepared and analyzed by Western blotting for their level of expression of hRev7. Figure 1 shows a representative Western blot. Lane 1 shows hRev7 protein from the parental cell strain (P), migrating as expected for a 24 kDa protein. Lane 2 shows hRev7 protein from the vector control cell strain (VC). Lanes 3 and 4 were loaded with protein from two clones, designated 2-2 and 2-6, that had been transfected with a vector expressing *hRev7* siRNA. No hRev7 protein could be observed in the latter two clones (Fig. 1). However, when the Western blots were allowed 24 h of exposure, a very low level of hRev7 protein could be detected on the blots (data not shown). The morphology of clones 2-2 and 2-6 did not differ from that of the parental or the vector control cells. Neither did the rate of growth in culture of these two cell strains differ from that of their parent or the vector control cells (data not shown).

3.2. Effect of reduction of the level of hRev7 protein on the sensitivity of the cell strains to the cytotoxic effect of UV_(254nm) radiation

To examine the sensitivity of these cell strains to the cytotoxic effect of UV, we irradiated the parental cells, the vector control cells, and the two cell clones virtually devoid of hRev7 protein and assayed them for survival of colony-forming ability. As shown in Fig. 2A, the parental cell strain and the vector control strain exhibited identical sensitivity to the cytotoxic effect of UV_(254nm) radiation. The dose required to reduce their survival to 37% that of unirradiated cells was 12 J/m². The survival of clones 2-2 and 2-6 were identical to each other, but both were 1.7 times more sensitive to UV-induced cell killing than the control cells. The dose required to reduce their survival to 37% that of unirradiated cells was only 8.5 J/m². These differences were shown to be statistically significant. The data indicate that hRev7 plays a protective role in survival of cells exposed to UV irradiation.

3.3. Effect of reduced hRev7 protein on the frequency of UV-induced mutations

The frequency of mutations induced by UV in the *HPRT* gene of these four cell strains was determined using resistance to 6-thioguanine as an indicator of cells with a mutation in their *HPRT* gene. As shown in Fig. 2B, the frequency of mutations induced by UV in the parental cells and vector control cells expressing hRev7 were identical. The dose that reduced their survival to 37%, i.e., 12 J/m², induced an *HPRT* mutation frequency of $\sim 135 \times 10^{-6}$. In contrast, the two clones with greatly reduced expression of hRev7 protein showed a statistically significant decrease in the frequency of UV_(254nm)-induced mutations (P-value

<0.001 for comparison of slopes). The frequency of mutants induced by 12 J/m² in clones 2-2 and 2-6 was only 32 x 10⁻⁶ clonable cells. At a dose that reduced their survival to 37%, i.e., 8.5 J/m², the frequency of induced mutants was ~20 x 10⁻⁶, significantly lower than the 135 x 10⁻⁶ seen in the two control cell strains. This significant reduction in the frequency of UV-induced mutations in cells with reduced hRev7 indicates that hRev7 plays an important role in UV-induced mutagenesis in human fibroblasts.

3.4. Effect of decreased hRev7 protein expression on kinds of UV-induced mutations

The kinds of base substitutions induced by UV in the coding region of the *HPRT* gene of cells expressing or not expressing hRev7, as determined by nucleotide sequencing, are shown in Table 1. The data report kinds of mutations induced in the two clones extremely deficient in expression of hRev7 (clones 2-2 and 2-6) and those induced in the comparison group (parent and vector control). [A table detailing the kinds and showing the context of 95 independent UV_(254nm)-induced mutations in *HPRT* of these two sets of human skin fibroblasts can be found in the Supplement]. There was no statistically significant difference in the kinds of mutations induced in the parental and vector control cells compared to those induced in the two derivative cell strains, virtually devoid of hRev7 protein. These data indicate that reducing the level of hRev7 results in a decrease in the frequency of UV-induced mutations without altering the types of base substitutions generated.

3.5. Evidence that reduction of expression of hRev7 protein in human fibroblasts results in a UV-induced delay in traversing S-phase

To assess potential effects of decreased hRev7 on cell cycle progression after UV irradiation, the parental and vector control cell strains and the two clones with greatly reduced hRev7 were synchronized at the G1/S border as described, released from the block, and immediately UV-irradiated with 12 J/m², the dose determined to reduce the survival of normal cells to ~37% and that of the hRev7-deficient cells to ~20% (see Fig. 2A). Independent synchronized populations of these UV-irradiated cells were assayed by flow cytometry immediately following release from the G1/S block (0 h), or after 10 h or 16 h. As shown in Fig. 3A, at the time of release from the replication block (0 h), all four strains were synchronized at the G1/S border. Ten h later, the parental (P) and vector control cells (VC) were predominantly in S-phase and G2 phase. In contrast, after 10 h, cell strains 2-2 and 2-6 were still predominantly in G1 and S-phase, i.e., their cell cycle progression was greatly delayed, compared to that of the two control strains. The data from cells assayed 16 h following UV irradiation show that the cell strains with reduced hRev7 were still delayed in S-phase, compared to their parental strain and the vector control cell strain.

These four cell strains were similarly irradiated with 12 J/m² while growing asynchronously and assayed by flow cytometry. As shown in Fig. 3B, UV-irradiation of asynchronously-growing cells with decreased expression of hRev7 also delayed their progression through S-phase compared to control cells. As a control, we synchronized the parental strain and the derivative strains with reduced hRev7, released them from the G1/S block, as described above, but did not expose them to UV radiation. The subsequent flow cytometric analysis (Fig. 3C) demonstrated that all three unirradiated cell strains proceeded through the cell

cycle at an equal rate. Taken together, the data in Fig. 3 indicate that cells with reduced hRev7 replicate their DNA more slowly in the presence of UV-induced DNA damage.

4. Discussion

A role for hRev7 as the non-catalytic subunit of Pol ζ was suggested by its homology to the yeast Rev7 protein, as well as its physical interaction with human Rev3 in a yeast-two-hybrid assay [14]. The results of our study establish a functional role for hRev7 in UV-induced mutagenesis, and what is more, a role in the survival of colony-forming ability. The fact that at every dose of UV radiation, the cells with greatly decreased levels of hRev7 protein exhibited ~5-fold lower frequency of induced mutations than the control cell strains is strong evidence that in human cells the hRev7 protein plays a role in error-prone TLS past UV-induced DNA photoproducts, as does yeast Rev7.

The mutagenesis data from the present study, using human fibroblasts that have greatly reduced levels of hRev7 protein, confirm and greatly strengthen the results obtained previously in this laboratory using similar cell lines and antisense against *hRev3* [10,15]. The ~5-fold decrease in UV-induced mutation frequency observed in cells virtually devoid of hRev7 is similar to the decrease in frequency observed previously in this laboratory using human fibroblasts expressing antisense *hRev3* [10,15]. These data strongly support the hypothesis that hRev3 and hRev7 function in the same pathway, most likely as catalytic and non-catalytic subunits, respectively, of human polymerase ζ .

Our results demonstrating that human fibroblasts with reduced hRev7 are more sensitive to the cytotoxic effects of UV-radiation than the control strains (Fig. 2A) support the results obtained by Cheung et al., which demonstrated that nasopharyngeal carcinoma cell lines with reduced hRev7 are also more sensitive to the cytotoxic effects of specific DNA damaging anticancer drugs than the control cells [18]. The fact that reduction of hRev7 results in increased sensitivity to the cytotoxic effects of various DNA damaging agents in both normal and cancer cells, underscores the importance of hRev7 for protecting human cells from DNA damage.

In addition to sharing a high degree of amino acid sequence similarity to the yeast Rev7 protein, hRev7 also has a high degree of similarity to the mitotic checkpoint protein hMAD2, and thus is also referred to as hMAD2B [14,19]. In fact, hRev7 has been shown to interact with hMAD2 and furthermore, to inhibit the anaphase-promoting complex by binding to activators Cdh1 and Cdc20 in *Xenopus* extracts, suggesting a role for hRev7 in regulating the mitotic checkpoint [14,20,21]. One might hypothesize that the sensitivity to the cytotoxic effects of UV we observe in our human fibroblasts reflects interference with the mitotic checkpoint response of UV-irradiated cells. However, Cheung et al., [18] found that reducing hRev7 in nasopharyngeal carcinoma cells had no effect on their mitotic checkpoint response. Therefore, we consider it unlikely that the increased sensitivity to the cytotoxic effects of UV-radiation that we observe in human fibroblasts with reduced hRev7 results from an aberrant mitotic checkpoint response. Nevertheless, further experimentation could be conducted to specifically determine whether or not hRev7 affects the mitotic checkpoint of human fibroblast cells in the presence of DNA damage.

In addition to decreasing the survival of UV-irradiated human fibroblast strains, reduced levels of hRev7 also resulted in a marked decrease in their rate of progression through the DNA synthesis phase of the cell cycle (Fig. 3). This suggests that for human cells with reduced expression of hRev7, and therefore with reduced hPol ζ , the presence of DNA damage during S-phase presents a major problem for DNA replication. If cells with decreased expression of hRev7 were simply unable to resume DNA replication as a result of impaired TLS, this situation would be expected to result in replication fork breakdown and ultimately lead to cell death. It is always possible that replication arrest due to impaired TLS accounts for the increase in sensitivity to the cytotoxic effects of UV that we observe when cell strains with reduced hRev7 are irradiated. However, previous results in our laboratory using human cells expressing antisense against *hRev3*, the catalytic subunit of hPol ζ , demonstrated that such cells did not differ from the parental cells in sensitivity to the cytotoxic effects of UV [15]. Taken together, these results suggest that in our clones with reduced hRev7, factors other than impaired TLS contribute to the observed increase in sensitivity to the cytotoxic effects of UV.

In 2005, a study by Bi et al. [22], demonstrated mouse embryonic fibroblasts lacking Polk were unable to recover from a benzo[a]pyrene diol epoxide (BPDE)-induced S-phase checkpoint and, in addition, were more sensitive to the cytotoxic effects of BPDE than were the wild-type cells. The fact that we found a clear indication of a UV-induced delay in S-phase, and an increase in sensitivity to the cytotoxic effects of UV in cell strains with reduced hRev7, suggests that there is a similar requirement for hRev7 in recovery from a UV-induced S-phase checkpoint as there is for Polk in a BPDE-induced S-phase checkpoint.

In summary, our mutagenesis data demonstrate that hRev7, like hRev3, is required for TLS past UV photoproducts and is causally involved in producing the mutations that result from such TLS. These data strongly support the hypothesis that hRev7, together with hRev3, comprise hPol ζ . In addition, our data demonstrate a requirement for hRev7 in protecting human fibroblasts from the cytotoxic effects of UV-induced DNA damage that was not found in human fibroblast cells expressing antisense against *hRev3*. This suggests that the protective role of hRev7 for cells exposed to UV radiation is independent of the requirement for hRev7 in hPol ζ -dependent TLS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Western blot analysis of hRev7 protein levels in the cell strains. A polyclonal antibody against hRev7 was used to analyze the level of hRev7 protein in nuclear lysates extracted from the parental (P) cells, the vector control (VC) cells, and two derivative clones expressing a transfected siRNA targeted against *hRev7*, viz., clones 2-2 and 2-6. The arrowhead on the right indicates the location of a 22 kDa marker. Ku80 was used as the loading control.

Fig. 2. Effect of reduced expression of hRev7 on the survival of cell strains exposed to UV_(245nm) radiation and on the frequency of UV-induced mutations. (A) Clones 2-2 and 2-6 (closed symbols), which have greatly reduced levels of expression of hRev7 protein, along with their parental strain and a vector control strain (open symbols) were UV-irradiated and assayed for cell survival as determined by colony forming ability. Some data points have been offset slightly to make them visible. The lines represent least squares lines. (B) The frequency of UV-induced mutations in the *HPRT* gene of these four cell strains was

determined by resistance to 6-thioguanine. The frequency of 6-thioguanine-resistant cells was calculated using the cloning efficiency of cells at the time of selection, which averaged ~44% for the parent and the vector control cells, and ~25% for clones 2-2 and 2-6. Induced frequencies were calculated by subtracting the background frequencies observed in sham-irradiated populations. For the vector and parental control cells, these values were always $<11 \times 10^{-6}$. For clone 2-2, they ranged from 0 to 18×10^{-6} , with the majority being 0 to 4×10^{-6} . For clone 2-6, they ranged from 8 to 25×10^{-6} , with the majority being 8 to 11×10^{-6} . Some data points have been offset slightly to make them visible. The lines represent least squares lines.

Fig.3

Fig. 3.

Flow cytometry analysis of UV-irradiated cells. (A) Cell strains with reduced levels of hRev7 [2-2 and 2-6], their parental strain [P], and a vector control transfectant [VC] were synchronized at the G1/S border as described, and UV-irradiated immediately after release from synchrony. The cell strains were assayed by flow cytometry for DNA content at 0 h, 10 h, and 16 h post-irradiation. (B) These four cell strains were UV-irradiated while growing asynchronously and were assayed by flow cytometry for DNA content at 0 h and 10 h post-irradiation. (C) As a control, clones 2-2, 2-6, and their parental strain were synchronized at the G1/S border, as above, released from synchrony, but not exposed to UV, and analyzed by flow cytometry at 0 h, 4 h, and 6 h after release from the G1/S block.

TABLE 1

Kinds of UV-induced base substitutions in the *HPRT* gene of cells with normal or greatly decreased levels of hRev7

Base changes	Parent and vector control	Clone 2-2 and clone 2-6
C→T	25 (47.2%)	19 (45.2%)
T→C	6 (11.3%)	2 (4.8%)
T→A	8 (15.1%)	11 (26.2%)
C→A	10 (18.9%)	7 (16.7%)
C→G	4 (7.5%)	1 (2.4%)
T→G	0	2 (4.8%)
Total	53 (100%)	42 (100%)