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PROPERTIES OF AN XERODERMA PIGMENTOSUM REVERTANT CELL LINE EXPRESSING ENDONUCLEASE V

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Summary. We have developed a set of cell lines to help distinguish the sequelae of specific lesions in DNA after UV irradiation. Irradiation results in two primary lesions: cyclobutane dimers and pyrimidine-pyrimidone (6-4) photoproducts. The contributions of each to mutation are considered utilizing a spectrum of cell lines with increasing abilities to repair these lesions. In particular, we focus on a revertant of the XP12Ro(M1) cell line from a patient with Xeroderma pigmentosum, XP129, which is capable of repairing (6-4) photoproducts but not cyclobutane dimers. We have successfully introduced the *denV* gene into these cells which confers the ability to repair cyclobutane dimers. By comparing the results of a shuttle vector mutation experiment with the vector pZ189, we can correlate specific mutations to specific lesions.

The autosomal recessive disease Xeroderma pigmentosum (XP) is a paradigm of the lesion → mutation → cancer cascade. Patients with this disease present with multiple cutaneous skin neoplasms (1). Cells from XP patients have an inability to repair the two predominating lesions in DNA after exposure to UV irradiation (254 nm): the 5,6 cyclobutane dimer and the pyrimidine-pyrimidone (6-4) photoproduct (2). Considerable effort has been made to determine the relative contribution of each lesion towards mutation and cell survival. It has been suggested that the cyclobutane dimer plays a minimal role in survival while maintaining a crucial role in mutagenesis (3-5).

We have analyzed a series of cell lines of varying degrees of repair competence for mutational frequency and spectrum using the shuttle vector pZ189 with the *supF* gene as the target for mutagenesis (6). These cell lines include XP12Ro(M1) (XP, complementation group A) which is deficient in the ability to remove both cyclobutane dimers and (6-4) photoproducts (7) and the XP revertant, XP129, which can repair (6-4) photoproducts but not cyclobutane dimers (8). We have also used two cell lines developed in our laboratory: XP12Ro(M1) cells transformed with the *denV* gene (9) and XP129 cells transformed with the

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denV gene developed for this study. We now have cell lines that can repair cyclobutane dimers only (*denV*-transformed XP12Ro(M1)) and cell lines that can repair both lesions (*denV*-transformed XP129). Previous work has shown a significant increase in the frequency of mutation in XP cells when compared to repair competent cell lines with a significant bias towards transitions in the resulting mutations (10). In contrast, treating the target with photoreacting enzyme such as *E. coli* photolyase to remove cyclobutane dimer shifts mutation towards transversions (11). We test the hypothesis that the spectrum of mutations seen in XP129 should be biased towards transitions whereas the mutations seen in *denV*-transformed XP129 should shift towards transversions.

Materials and Methods

Cell lines. The XP129 revertant of XP12Ro(M1) (complementation group A) was produced after exposure to MNU and was obtained from J. Cleaver. The other cell lines used in this study [GM637A, XP12Ro(M1), RSVdenV-SVgpt] have all been described previously (8)). All cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. Cells under selection were supplemented with G-418 (Genectin, GIBCO, Grand Island, NY) at 500 μg/ml.

Plasmids. The plasmid pNEOTK-denV has been previously described (12). For transfection, the plasmid was purified on CsCl gradients. Transfection of plasmid DNA (~15 μg) into host cell lines was done by a modification of Graham and van der Eb (13). Plasmid pdenV-52 contains the 457 bp ClaI fragment used for the detection of *denV* specific sequences (14). For RNA analysis, the plasmid pGEM-denV was made with the ClaI fragment of pdenV-52 cloned into pGEM7Z(f) (Promega, Madison, WI). For RNase protection analysis, this plasmid was linearized with XhoI and anti-sense RNA probes made using SP6 RNA polymerase (Promega). Plasmid pRSVcat (15) used in host cell reactivation studies was a gift of K. Kraemer.

Isolation and amplification of DNA, total RNA and cDNA. Isolation of DNA was done according to Painter and Schaeffer (16). Isolation of total RNA was done by the guanidinium isothiocyanate method according to (17). Amplification of DNA and RNA was performed according to (18, 19) using *denV*-specific primers (Figure 1A).

Analysis for *denV* specific sequences and transcripts. Analysis for *denV* specific sequences and transcripts was done by PCR amplification/Southern blot analysis of DNA, RNA (cDNA) or by RNase protection (18-20). Eight μl of PCR product was separated on 1.4% agarose gels, transferred to nitrocellulose (BAS85, S&S, Keene, NH), and bonded to the support by UV-crosslinking (Stratalinker, Stratagene, La Jolla, CA). Hybridization was done according to (20) using ^{32}P-labeled *denV* specific probe. For RNase protection, 5 μg of total RNA was hybridized to a full-length *denV* specific ^{32}P-labeled antisense probe from pGEMdenV at 55°C.

Host cell reactivation. *In vivo* repair capabilities were tested using the reporter gene *cat* according to (15). The target gene *cat* was part of the plasmid pRSVcat. The plasmid was irradiated at 800 J/m^2 prior to transfection. For transient expression cell lines were co-transfected with pRSVdenV (9).

Mutation frequency analysis. The mutation frequency of varying cell lines was determined using the shuttle vector pZ189 and done according to Seidman et al. (6) with pZ189 shuttle vector sequencing primers based on Protić-Sabjić et al. (21).

Results

Establishment of *denV* transformants of XP129 cells. Two cell lines (1A1 and 3D5) were established from single clones of pneotk-denV transfected XP129 cells. Presence of the *denV* gene and *denV* transcripts was determined by PCR amplification of *denV*-specific sequences followed by Southern blotting. PCR amplification of DNA and RNA isolated from 3D5 demonstrates the expected bands on ethidium bromide stained gels [Figure 1B, lanes 2 (DNA), 3 (RNA)] but no band in the XP129 parent (lane 1). Southern blots of this gel demonstrates the amplified band is *denV* specific (Figure 1C). In Figure 1D, specific bands representing full-length transcripts as determined by RNase protection can be seen in both *denV* transformants 1A1 and 3D5, while no band is seen in the parent XP129 cell line.

Host cell reactivation of a reporter gene. We tested the *in vivo* repair capabilities of cell lines to repair a UV-irradiated reporter gene, *cat*, in both transformed and untransformed cells lines. In Fig. 2, the parent XP129 cell line has increased endogenous repair capabilities with 27% reactivation of CAT activity after irradiation when compared to its XP12Ro(M1) parent (2.1%). The addition of exogenous *denV* by co-transfection increases this reactivation substantially (48% vs 27%). Additional exogenous *denV* (3:1 ratio) does not further increase reactivation. The abilities of stable *denV* transformants to reactivate CAT are increased as

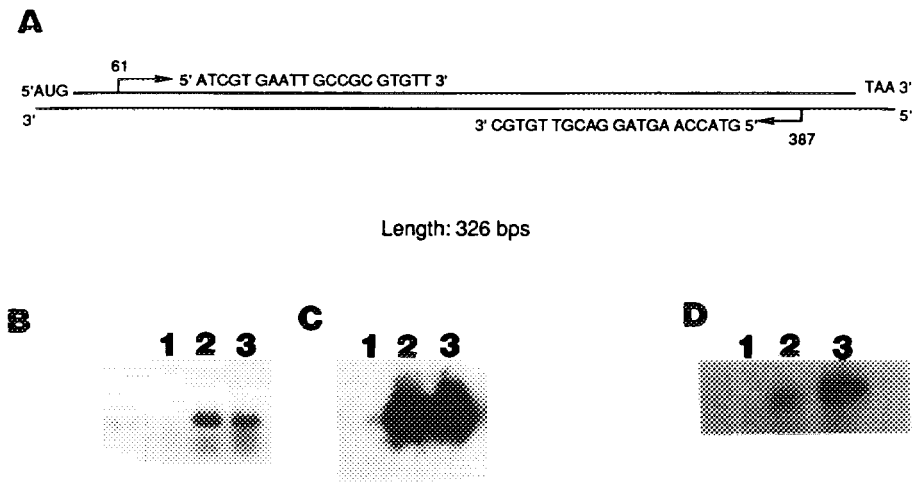

Figure 1. A-D. PCR amplification/Southern blot of DNA, RNA (cDNA) and RNase protection of RNA from XP129 and transformants. Eight μl of the resulting sample was analysed on 1.4% agarose gels. **A.** Sequence of oligonucleotides and location on the *denV* gene of amplimers used. **B.** Ethidium bromide stain and **C.** Southern Blot of PCR products: Lane 1, XP129 ; Lane 2, XP129 transformant 3D5 DNA; Lane 3, 3D5 RNA; **D.** RNase protection of *denV*-specific sequences in XP129 transformants.

Cell Line	pRSV-CAT UV	pRSV-denV	%/μg	%REACT
GM637A	+	-	3.5	58
GM637A	-	-	6	
XP12RoM1	+	+ (3:1)	0.27	57
XP12RoM1	+	-	0.01	2.1
XP12RoM1	-	-	0.47	
RSV #9	+		0.14	30
RSV #9	-		0.48	
3D5	-		0.2	
3D5	+		0.17	85
XP129	+	+ (3:1)	0.6	50
XP129	+	+ (1:1)	0.57	48
XP129	+	-	0.32	27
XP129	-	-	1.2	

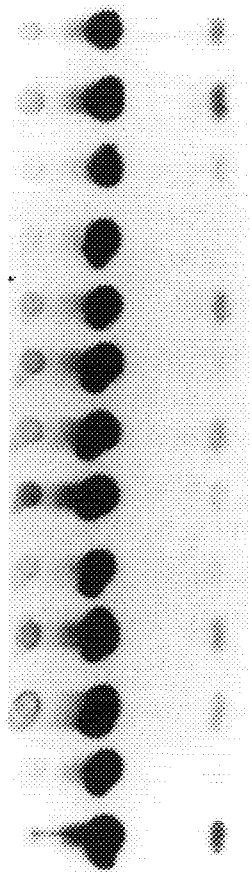

Figure 2. Host cell reactivation assay in normal, XP, XP revertant and transformed cell lines. % reactivation calculated according to (14).

compared to the XP129 parent (85% vs 27%) reflecting the combinatorial repair properties of the parent and the addition of *denV* to repair both types of UV-induced lesions.

Mutation frequencies and spectrum in XP129 and XP129 transformants (Table 1). Using the shuttle vector pZ189, the mutation frequency of the XP129 parent is high in irradiated cells when compared to repair-competent GM637A cells (97.5×10^{-4} vs 17.5×10^{-4}) but about half the frequency seen in the XP12Ro(M1) parent and agrees with previously published results (8). This frequency is roughly equal to the frequency seen in *denV* transformed XP12Ro(M1) cells (e.g. RSVdenV #9). Unlike assays that measure host cell reactivation, the expression of the *denV* gene in the XP129 transformants does **not** improve the mutation frequency. One explanation for this apparent discrepancy is that the *cat* gene can sustain some mutations which do not necessarily inhibit, or inhibit to a limited degree, the

Table 1. Mutations in UV-irradiated shuttle vector pZ189*

	Cell Lines					
	GM637A	XP12Ro(M1)	#9†	XP129	1A1	3D5
1. Mutation Analysis:						
%Survival	36	21	27	27	21	17
Mutation frequency‡	17	97	24	46	55	49
2. Mutation Spectrum [No. changes (%)]						
Transitions:		9 (90)	14 (70)	17 (85)	8 (80)	13 (65)
G:C to A:T		9 (90)	14 (70)	15 (75)	7 (70)	12 (60)
A:T to G:C		0	0	2 (10)	1 (10)	1 (5)
Transversions:		1 (10)	6 (30)	3 (15)	2 (20)	7 (35)
G:C to T:A		0	1 (5)	0	0	1 (5)
G:C to C:G		1 (10)	3 (15)	2 (10)	0	3 (15)
A:T to T:A		0	2 (10)	1 (5)	2 (20)	3 (15)
A:T to C:G		0	0	0	0	0
Total:		20 (100)	20 (100)	20 (100)	10 (100)	20 (100)

*Irradiated at 300 J/m^2; †RSV-denV-SVgpt/XP12Ro(M1), ref. 8; ‡ x 10^{-4}.

enzymatic activity of the protein. In contrast, the cumulative data on pZ189 mutagenesis suggests that **any** mutation within the gene can effect function. Table 1 also shows mutational spectrum in pZ189 derived from each of the cell lines. There is a significant shift in the spectrum of mutations when compared to the parent line XP12Ro(M1) in which 90% of the mutations are transitions, confirming previous data on this cell line (10). When this cell line is transformed with the *denV* gene, the spectrum shifts towards transversions (30% vs 10%). A similar shift was seen after exposing the UV-irradiated vector to photolyase (6% to 36% after treatment, ref. 11). By contrast, the spectrum of mutations seen in the revertant line (XP129) is shifted significantly towards transitions (85%). The addition of the *denV* gene shifts the spectrum back towards transversions (20% in 1A1 cells and 35% in 3D5 cells) and is consistent with the notion that these cells can repair **both** lesions and thus behave more like repair competent cells [25% transversion for repair-competent GM637A cells (11)].

Discussion

We have developed a library of cell lines with which to test the relative contribution of the two main UV-induced photoproducts to mutation. Host cell reactivation studies confirm

the endogenous ability of the XP129 cell line to repair UV-induced lesions; the percent reactivation in untransformed cell lines was 27% as compared to only 2% in the XP12Ro(M1) parent. Reactivation in stable *denV*-transformants increases to 85%, reflecting the contributions of each repair pathway to reactivation. Despite this, the XP129 cell line has a high mutation frequency (3,8). However, in contrast to the data from Cleaver, we show a relative improvement in the mutation frequency between XP12RO(M1) and XP129 cells (97% vs 46%; Table 1), but still significantly higher than the repair-competent cell line GM637A (17%). The addition of the *denV* gene to XP12Ro(M1) cells decreases the mutation frequency, but has no effect on *denV*-transformed XP129 cells. This suggests that when a cell has limited endogenous repair capabilities, any contribution helps. However, in those cell lines with moderate repair capabilities, this additional contribution (in the form of *denV*) gets lost. This seems to confirm that the (6-4) photoproduct may in fact be the more important lesion with regard to mutation and survival (4,8).

Differences in the spectrum of mutations are seen in the varying cell lines tested. XP129 cells, with its inherent capabilities to repair (6-4) photoproducts has a high percentage of transitions (similar to XP12Ro(M1)); the addition of the *denV* gene shifts that spectrum towards transversions. This is consistent with the effect of *denV* in XP12Ro(M1) cells (e.g. RSV #9) and the work of Kraemer, et al. (11) using photoreactivating enzyme. The ability of cells to repair cyclobutane dimers decreases the proportion of transitions, whereas the ability to repair (6-4) photoproducts decreases the proportion of transversions.

Acknowledgments

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