

Transient and Stable Complementation of Ultraviolet Repair in Xeroderma Pigmentosum Cells by the *denV* Gene of Bacteriophage T4¹

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ABSTRACT

In this paper we report both transient and stable complementation of pyrimidine dimer repair in xeroderma pigmentosum cells by the *denV* gene of bacteriophage T4, coding for endonuclease V, a dimer-specific DNA glycosylase. Cotransfection with pRSVdenV in SV40-transformed XP12RO(M1) cells (complementation group A) restored transient expression of an indicator plasmid (pRSVcat) bearing a UV-inactivated chloramphenicol acetyltransferase (*cat*) gene. In addition, XP12RO(M1) clones stably transformed by pRSVdenV-SVgpt expressed transient chloramphenicol acetyltransferase activity when transfected with UV-inactivated pRSVcat plasmid. These clones also showed partial restoration of colony forming ability and excision repair synthesis after UV irradiation. Immunofluorescence, using an endonuclease V polyclonal antibody, showed the presence of the phage glycosylase in stably transformed xeroderma pigmentosum cells. The cotransfection assay affords a rapid, sensitive procedure to screen for functional cloned DNA repair genes and to test mutant cells for the deficiency of specific steps in DNA repair, such as incision.

INTRODUCTION

XP⁴ is an autosomally inherited disease characterized by extreme sensitivity to the UV component of sunlight and by multiple cutaneous neoplasms (for review, see Ref. 1). Cultured cells derived from XP patients are defective in initiation of DNA repair at UV-damaged sites such as cyclobutane pyrimidine dimers, resulting in increased cell death (2), elevated frequencies of mutation (3), and malignant transformation (4) following exposure to UV. The human DNA repair complex responsible for repair at UV damage sites, like the Uvr ABC excinuclease complex of *Escherichia coli*, has broad specificity for distorted helix structures, including several forms of UV damage (5). The complexity of the initial incision step may be inferred from the identification of at least nine distinct XP complementation groups which are defective in incision (1, 5). In contrast, T4 endonuclease V, a pyrimidine dimer-DNA glycosylase with an associated apyrimidinic endonuclease activity, has the capacity to initiate DNA repair as a single enzyme by incising specifically at pyrimidine dimers (6, 7). *In vitro* as well as *in vivo* studies have shown that XP cells are able to carry out excision repair of dimers initiated by endonuclease V (8, 9, 10).

To create a pyrimidine dimer-specific repair system in XP cells, we placed the *denV* gene (11) linked to the RSV LTR into the selection vector pSV2gpt (12), containing the *E. coli*

xanthine-guanine phosphoribosyl transferase (*gpt*) gene under transcriptional control of the SV40 early gene promoter. Selection for the *gpt* gene allows for the advantageous coselection of stable *denV* transformants in the absence of UV. We chose as our target cells the well characterized XP12RO(M1) cell line, an SV40-transformed XP fibroblast cell line (complementation group A) which permits mycophenolic acid (MAX) selection for the *gpt* gene (12, 13), and as control cells the SV40-transformed human fibroblast line GM637A.

MATERIALS AND METHODS

Plasmids and Cell Lines. Plasmid pRSVdenV contains the RSV LTR of pRSVcat (14) on a 578-base pair *NdeI-HindIII* fragment followed by 6 base pairs (AGCTTG) from the *HindIII-SphI* linker of pEMBL19 (15), the 457-base pair Klenow filled-in *Clat* fragment of pdenV-52 (16), and 11 base pairs (CGACTCTAGAG) from the *AccI-BamHI* linker of pSP65 (Promega Biotec, Madison, WI), cloned into the ~4.5-kilobase *BglII-NdeI* fragment from pSV2dhfr (17), containing the SV40 large T splice and polyadenylic acid signals, as well as portions of pBR322. The *denV* gene and junctions of pRSVdenV were sequenced and confirmed to be intact by Maxam and Gilbert chemical cleavage (18). The plasmid pRSVdenV-SVgpt (see Fig. 1) was constructed by ligating the ~5.1-kilobase *NdeI-PvuII* fragment of pSV2gpt (12) to the ~1.9-kilobase *NdeI-BamHI* (Klenow filled-in) fragment of pRSVdenV. pRSVcat plasmid was obtained from B. Howard and K. Kraemer. The XP12RO(M1) cell line was obtained from B. Royer-Pokora and GM637A from the National Institute of General Medical Sciences Cell Repository, Camden, NJ. The cells were maintained in Dulbecco's modified essential medium plus 10% fetal calf serum and antibiotics. For MAX selection, the medium was supplemented with hypoxanthine (15 µg/ml), thymidine (10 µg/ml), mycophenolic acid (25 µg/ml), aminopterin (42 µg/ml), and xanthine (250 µg/ml). All cells used in this study were tested and found to be negative for *Mycoplasma*.

Enzymes and Chemicals. Restriction endonucleases, T4 DNA ligase, and DNA polymerases were purchased from New England Biolabs, Bethesda Research Laboratories or Boehringer-Mannheim Biochemicals and used as directed by the manufacturer. All chemicals were from Sigma (St. Louis, MO) or CalBiochem (La Jolla, CA) unless otherwise noted.

Host Cell Reactivation. Transfection was performed using calcium phosphate/DNA precipitates as described (19, 20) with ~10 µg of control or UV-irradiated (800 J/m²) carrier-free plasmid DNA per 60-mm plate and incubation for ~40 h. CAT activity in crude cell lysates was determined by the procedure of Gorman *et al.* (21) with 4 mM acetyl-coenzyme A and 2-5 µCi of [¹⁴C]chloramphenicol (New England Nuclear)/ml. The reactions were incubated at 37°C for 1 h before extraction with ethyl acetate. Acetylated metabolites of [¹⁴C]chloramphenicol were separated from the parent compound by ascending thin-layer chromatography (chloroform:methanol, 95:5), located by autoradiography, and quantitated by scintillation counting. Specific activity was expressed as a percentage of total cpm per µg protein. Protein concentration was determined by the Bio-Rad assay. Percentage of reactivation was calculated as the CAT activity obtained with UV-irradiated plasmid divided by the activity obtained with control plasmid ×100. As positive control for the CAT assay we used a crude protein extract of *E. coli* MM294 carrying the plasmid pBR328 (Cm^r, Amp^r, Tet^r) containing Tn9.

Detection of *denV* DNA in XP Cells. DNA (~10 µg) isolated from fibroblasts were fractionated on a 1.0% agarose gel. The DNA was

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⁴ The abbreviations used are: XP, xeroderma pigmentosum; LTR, long terminal repeat; MAX, mycophenolic acid-aminopterin-xanthine; RSV, Rous sarcoma virus; CAT, chloramphenicol-3-O-acetyl transferase.

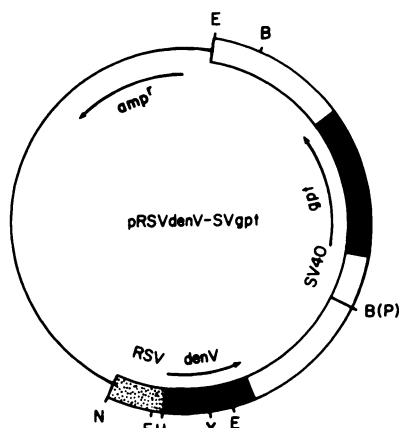


Fig. 1. Map of pRSVdenV-SVgpt (~7.0 kilobases). Restriction enzymes: N, *NdeI*; E, *EcoRI*; H, *HindIII*; X, *XhoI*; B, *BamHI*; P, *PvuII*; *amp^r*, ampicillin resistance gene.

blotted onto nitrocellulose and hybridized to a ³²P-labeled DNA probe derived from the *Clal* fragment of pdenV-52 (16), specific for the *denV* gene. *HindIII*-digested λ -phage and *Bst*NI-digested pBR322 were used as size markers.

Immunofluorescence of Endonuclease V. Cells were fixed on glass cover slips in ice-cold acetone and treated with an affinity purified rabbit polyclonal antibody directed against endonuclease V⁵, followed by 1:100 dilution in phosphate-buffered saline of fluorescein-conjugated goat anti-rabbit Ig antibody (Cappel, Cochranville, PA).

Postirradiation Colony Forming Ability. Approximately 2.5×10^5 cells from GM637A, XP12RO(M1), or selected MAX-resistant clones in a 0.5-ml droplet of phosphate-buffered saline were irradiated in a Petri dish with a calibrated 15-W germicidal UV source. Aliquots of 50 μ l ($\sim 3 \times 10^4$ cells) were withdrawn after various doses, seeded in microtiter plates for 2-fold serial dilution, and then allowed to form colonies for 7–10 days. The cells were then fixed with 10% formaldehyde, stained with crystal violet, and the colonies were counted. The D_0 value refers to the dose increment that results in a decrement in survival to 0.37 on the exponential portion of the survival curve.

Measurement of Excision Repair. Replicated and unreplicated DNA was separated by isopycnic sedimentation in neutral cesium chloride gradients essentially as described (22). Cells were preincubated in 5-bromodeoxyuridine (10 μ g/ml) for 30 min, then UV-irradiated with 40 J/m². Immediately, [*methyl*-³H]thymidine (52 Ci/mmol) (ICN, Irvine, CA) was added to the medium (10 μ Ci/ml), and the cells were incubated for an additional 3 h, harvested, and resuspended in 0.15 M NaCl/0.015 M sodium citrate. The suspension was sequentially treated with 0.2% sodium dodecyl sulfate, ribonuclease A, and proteinase K, and then phenol extracted and dialyzed. Solid cesium chloride was added to give a final density of 1.7 g/ml, followed by hydrodynamic shearing by five successive passages through a 22-gauge syringe needle. The samples were centrifuged for 60 h in a Beckman SW50.1 rotor at 30,000 rpm and 25°C. Gradients were collected from the bottom, and the fractions were analyzed for absorbance at 260 nm. Five fractions with the highest absorbance were rebanded by pooling the fractions, dialyzing, and processing as above. After rebanding, the gradients were processed for absorbance at 260 nm and for trichloroacetic acid-precipitable radioactivity. Specific activities were calculated from the five fractions with peak absorbance.

Measurement of Incision Activity. Incising activity was measured as described (23). Briefly, cells were labeled for 24 h (0.10 μ Ci/ml; 52 Ci/mmol), irradiated with 10 J/m², incubated for 1 h in fresh medium containing 1- β -D-arabinofuranosylcytosine (10 μ M), and lysed directly on a 5–30% sucrose gradient (pH 12.1) prior to sedimentation (45 min; 25,000 rpm) in an SW50.1 rotor. Fractions were collected from the bottom and analyzed for trichloroacetic acid-precipitable radioactivity. Uniformly labeled T4 DNA was used as a standard size marker.

⁵ K. Valerie *et al.* Production and detection of T4 endonuclease V polyclonal and monoclonal antibodies by staphylococcal protein A hybrid proteins, submitted for publication.

RESULTS

Transient Complementation of UV Repair. Initially we tested the effectiveness of the *denV* gene product in repairing UV damage *in vivo* by measuring the ability of normal GM637A or XP12RO(M1) cells to express transient CAT activity after transfection with UV-irradiated pRSVcat (19) alone or together with unirradiated pRSVdenV. XP12RO(M1) cells were less efficient at expressing CAT activity from unirradiated pRSVcat than were the normal control cells. As expected, UV irradiation (800 J/m²) of pRSVcat prior to transfection led to a relatively modest decrease in CAT expression in GM637A (~38%) but resulted in a dramatic decrease in CAT activity in XP12RO(M1) cells (~93%) in line with what has been reported earlier for XP (A) cells (19). Cotransfection with a 2:1 or 3:1 molecular ratio of unirradiated pRSVdenV to UV-irradiated pRSVcat resulted in restoration of 26 and 93%, respectively, of the CAT activity obtained from the unirradiated pRSVcat transfection (Fig. 2).

Stable Complementation of UV Repair. Stable pRSVdenV-SVgpt transformants of XP12RO(M1) were obtained by transfecting 10^6 cells with ~10 μ g of pRSVdenV-SVgpt (see Fig. 1) by the calcium phosphate precipitation method of Graham and van der Eb (20) and after 1-day replating in MAX selective medium (12). MAX-resistant colonies were expanded and tested for ability to reactivate UV-irradiated pRSVcat (Fig. 2). A majority of the seven tested MAX-resistant clones showed significantly higher CAT activity than the parent XP12RO(M1) cells (Fig. 2). Three clones (clones 5, 10, and I-A5) restored CAT activity close to the level observed after transfection of parental XP12RO(M1) cells with unirradiated pRSVcat.

Detection of *denV* DNA and Protein. A Southern blot of cellular DNA from clone 5 confirmed the presence of pRSVdenV-SVgpt (Fig. 3A). An *EcoRI* digest of DNA from clone 5 resulted in the appearance of a ~400-base pair fragment corresponding to the 383-base pair RSV-*denV* sequence (see Fig. 1) and a fragment larger than 4 kilobases, indicating the association of the *denV* sequences with higher-molecular-

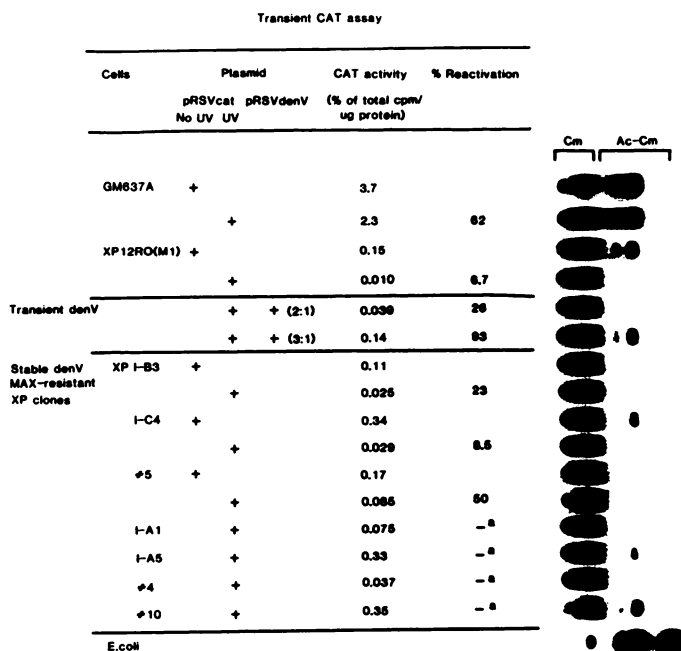


Fig. 2. CAT assay of normal, XP, and transformed cell lines. Cm, chloramphenicol; Ac-Cm, acetylated chloramphenicol; *, not determined. The percentage of reactivation was calculated as described in "Materials and Methods."

B

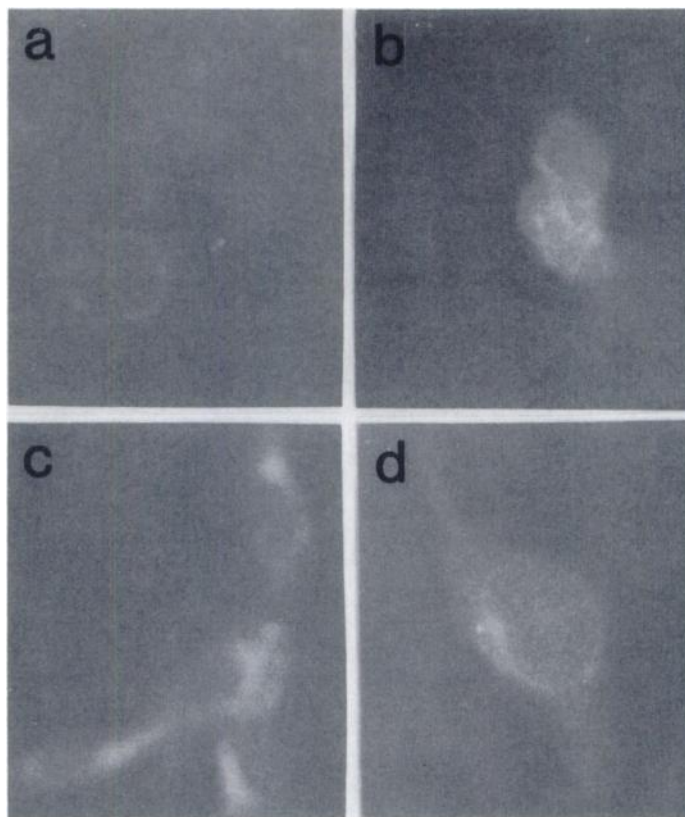
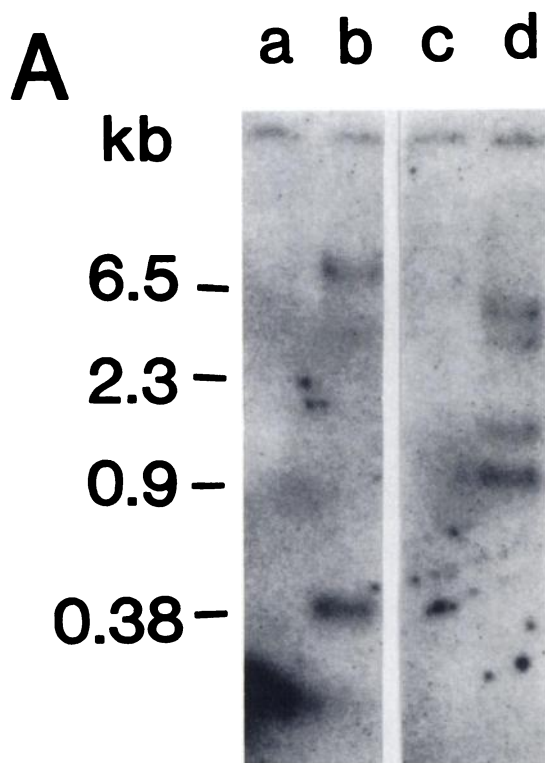


Fig. 3. *A*, Southern blot of parent and pRSVdenV-SVgpt-transformed XP fibroblasts. *Lanes a and c* contain XP12RO(M1) DNA; *lanes b and d* contain DNA of pRSVdenV-SVgpt clone 5/XP12RO(M1). *Lanes a and b*, *EcoRI*; *lanes c and d*, *BamHI-XhoI*. *kb*, kilobase. *B*, immunofluorescent detection of endonuclease V in stably transformed clones. The fluorescent cells were visualized under a Zeiss Universal microscope. *a*, XP12RO(M1); *b*, pRSVdenV-SVgpt clone 5/XP12RO(M1); *c*, pRSVdenV-SVgpt clone 9/XP12RO(M1); *d*, pRSVdenV-SVgpt clone 10/XP12RO(M1). $\times 500$.

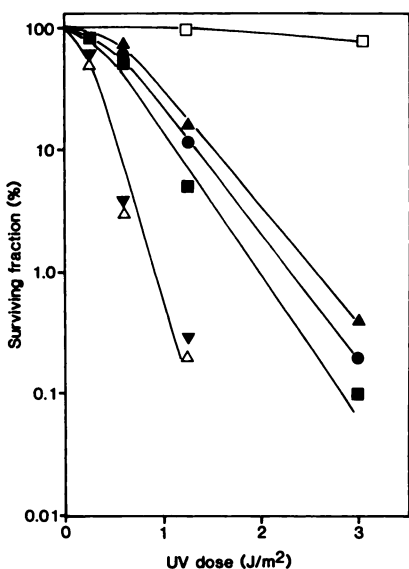


Fig. 4. Survival curves of normal, XP, and pRSVdenV-SVgpt-transformed XP fibroblasts. \square , GM637A; Δ , XP12RO(M1); \bullet , pRSVdenV-SVgpt clone 5/XP12RO(M1); \blacktriangle , pRSVdenV-SVgpt clone 9/XP12RO(M1); \blacksquare , pRSVdenV-SVgpt clone 10/XP12RO(M1); \blacktriangledown , pRSVdenV-SVgpt I-B2/XP12RO(M1).

weight DNA. Clone 5 seems to contain at least two copies of the plasmid, indicated by the two doublets at ~ 1 and ~ 4 kilobases in the *BamHI-XhoI* digest (Fig. 3*A*, lane *d*). Similarly, clone 10 also showed the presence of *denV*-specific sequences

(data not shown). Indirect immunofluorescence of clones 5, 9, and 10 revealed the presence of endonuclease V protein in pRSVdenV-SVgpt containing XP cells (Fig. 3*B*).

Detection of T4 Endonuclease V Activity in XP Cells. To determine whether transformants which showed an ability to repair exogenous DNA were also able to incise and repair cellular DNA after UV irradiation, colony-forming ability, UV-specific DNA incision, and nucleotide excision repair were measured. Two clones (5 and 10) which had rescued CAT activity from UV-irradiated pRSVcat and one clone which was not tested for CAT activation (clone 9) had a significant increase in cell survival following UV irradiation, although not to wild-type levels (Fig. 4). D_0 values were $0.37 J/m^2$ for XP12RO(M1) and 0.99 , 1.07 , and $0.85 J/m^2$ for clones 5, 9, and 10, respectively. A similar UV response was observed in a Chinese hamster ovary cell line expressing the *denV* gene (23). Incomplete restoration of UV resistance is presumed to be the result of the inability of endonuclease V to recognize other kinds of UV-induced DNA damage which are removed by the endogenous repair system in normal cells. On the other hand, clone IB-2, another MAX-resistant clone obtained after transfection with pRSVdenV-SVgpt, showed no increase in UV resistance compared to the XP12RO(M1) parent line. XP12RO(M1) and clone 10 showed equal sensitivity to 4-nitro-quinoline-oxide (data not shown), indicating that the endogenous mechanism for repair of bulky DNA adducts had not been reactivated in clone 10. Nucleotide excision repair replication was quantitated by measuring the specific activities of precursor nucleotide

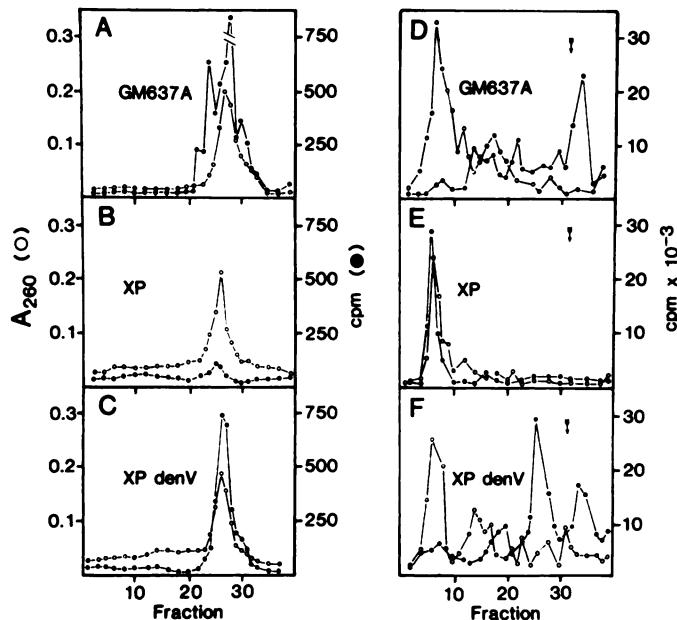


Fig. 5. A-C, measurement of excision repair synthesis. A, GM637A; B, XP12RO(M1); C, pRSVdenV-SVgpt clone 5/XP12RO(M1). D-F, measurement of incision activity. Alkaline sucrose gradients of [³H]thymidine-labeled UV-irradiated DNA. D, GM637A; E, XP12RO(M1); F, pRSVdenV-SVgpt clone 5/XP12RO(M1); ○, unirradiated; ●, irradiated. Direction of sedimentation is from right to left. Arrow, position to which ¹⁴C-labeled T4 DNA sedimented.

incorporation into unreplicated DNA (24). From the shape of the curves in Fig. 5, A-C, it can be concluded that the normal cell line (GM637A) had extensive repair following UV irradiation, whereas XP12RO(M1) had greatly reduced levels of repair, consistent with complementation group A fibroblasts (22). In contrast, the XP12RO(M1)-derived UV-resistant pRSVdenV-SVgpt transformants had an extensive level of repair synthesis restored. Specific activities of UV-induced DNA repair were 26 cpm/ μ g for GM637A, 4 cpm/ μ g for XP12RO(M1), and 29, 14, and 15 cpm/ μ g for clones 5, 9, and 10, respectively (only the curve for clone 5 is shown in Fig. 5C). The ability to carry out the incision step was measured by assessing the accumulation of DNA strand breaks by velocity sedimentation (23). The reduction of the mean molecular weight of DNA from GM637A and clone 5 following UV-irradiation but not from XP12RO(M1) cells demonstrated that incision occurred after UV-damage in *denV*⁺ XP cells (Fig. 5, D-F).

DISCUSSION

The results presented here strongly suggest that the *denV* gene under control of the RSV LTR reactivates exogenously introduced DNA, extensively restores excision repair activity on endogenous DNA, and partially restores viability after UV irradiation. Therefore XP cells, like UV-sensitive mutant Chinese hamster ovary cells (23), can be genetically restored to repair competence by an exogenous DNA repair gene. The rescue of CAT activity from a UV-irradiated template in *denV*-transformed XP cells suggests that many of the UV-induced lesions which block cellular transcription are removed from transfected DNA. Previous results have shown that a single thymine cyclobutane dimer is sufficient to block *cat* gene transcription in XP cells (19, 25). From these observations it might be concluded that endonuclease V recognizes and initiates repair of UV-induced pyrimidine dimers which are blocks to

transcription, while leaving behind some lethal UV-induced lesions. These conclusions are in agreement with what is known about the biological effects of pyrimidine dimers and (6-4) pyrimidine-pyrimidone photoproducts (26), neither of which are efficiently removed by XP cells (27). Alternatively, restoration of UV survival may be hampered by inefficiency in a subsequent step in the repair of endonuclease V-induced single strand breaks. It might be possible to test this notion with an endonuclease V which retains its pyrimidine dimer-DNA glycosylase activity but has lost the AP endonuclease activity through site-specific mutagenesis, allowing endogenous AP endonucleases (28) to carry out strand breakage.

Although T4 endonuclease V can not remove all UV-induced lethal lesions, it has an advantage over other repair systems for the study of cyclobutane dimer repair in mammalian cells due to its apparent ability to gain access to DNA within chromatin (9, 10, 23).

The cotransfection assay used in this study affords a rapid sensitive screen for the presence and function of a cloned DNA repair gene, which correlates well with repair capability measured by standard criteria and can be performed on bulk transfected cell populations. This minimizes the risk of selection artifacts that accompanies the study of stable transformants. Using the cotransfection assay in conjunction with a cloned DNA repair gene of known function, it should also be possible to assay mutant cells for deficiency in a specific step in DNA repair.

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