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Genetics

One pyrimidine dimer inactivates expression of a transfected gene in xeroderma pigmentosum cells

(DNA repair/ultraviolet radiation/host cell reactivation/skin cancer)

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ABSTRACT **We have developed a host cell reactivation assay of DNA repair utilizing UV-treated plasmid vectors. The assay primarily reflects cellular repair of transcriptional activity of damaged DNA measured indirectly as enzyme activity of the transfected genes. We studied three plasmids (pSV2cat, 5020 base pairs; pSV2catSVgpt, 7268 base pairs; and pRSVcat, 5027 base pairs) with different sizes and promoters carrying the bacterial *cat* gene (CAT, chloramphenicol acetyltransferase) in a construction that permits *cat* expression in human cells. All human simian virus 40-transformed cells studied expressed high levels of the transfected *cat* gene. UV treatment of the plasmids prior to transfection resulted in differential decrease in CAT activity in different cell lines. With pSV2catSVgpt, UV inactivation of CAT expression was greater in the xeroderma pigmentosum group A and D lines (D_0 = 56 J·m^{-2}) than in the other human cell lines tested (normal, ataxia-telangiectasia, Lesch–Nyhan, retinoblastoma) (D_0 = 680 J·m^{-2}) (D_0 is the dose that reduces the percentage of CAT activity by 63% along the exponential portion of the dose–response curve). The D_0 of the CAT inactivation curve was 50 J·m^{-2} for pSV2cat and for pRSVcat in the xeroderma pigmentosum group A cells. The similarity of the D_0 data in the xeroderma pigmentosum group A cells for three plasmids of different size and promoters implies they all have similar UV-inactivation target size. UV-induced pyrimidine dimer formation in the plasmids was quantified by assay of the number of UV-induced T4 endonuclease V-sensitive sites. In the most sensitive xeroderma pigmentosum cells, with all three plasmids, one UV-induced pyrimidine dimer inactivates a target of about 2 kilobases, close to the size of the putative CAT mRNA.**

Cells from patients with the autosomal recessive, cancer-prone disease xeroderma pigmentosum are hypersensitive to killing by UV radiation and have defective repair of UV-damaged DNA (1). This DNA repair defect has been measured by demonstrating reduced proliferation in xeroderma pigmentosum cells of UV-treated infecting viral particles (2), or viral DNA (3), a process known as "host cell reactivation."

We have developed a host cell reactivation assay utilizing UV-treated recombinant DNA expression vectors. By using nonreplicating plasmids we are able to examine the influence of precisely quantified DNA damage on plasmid transcription and to study excision repair independently of postreplication repair. We chose three plasmids of known sequence with differing size and with different promoters. They all carry the same bacterial gene, *cat* [coding for chloramphenicol acetyltransferase (CAT)], inserted between viral promoters [simian virus 40 (SV40) or Rous sarcoma virus (RSV) long terminal repeat (LTR)] and SV40 polyadenylylation sequences that permit gene expression in mammalian cells. In addition, the constructs carry pBR322 sequences that permit their replication in bacteria.

We found that expression of CAT activity was much more sensitive to UV inactivation in xeroderma pigmentosum cells than in repair-proficient cells. In the xeroderma pigmentosum cells the UV-inactivation target size for *cat* gene expression was independent of plasmid size or promoter. Measurement of T4 endonuclease V-sensitive sites in irradiated plasmids indicates that one pyrimidine dimer inactivates a target close to the size of the putative *cat* transcriptional unit.

MATERIALS AND METHODS

Cells. The human SV40-transformed and primary skin fibroblast cell lines studied are indicated in Table 1. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 20 mM L-glutamine and 10% fetal calf serum. The ataxia-telangiectasia and retinoblastoma cells were grown in minimal essential medium (Eagle) with the same supplements. Cells were grown at 37°C in a CO_2 concentration sufficient to keep the pH of the growth medium constant at 7.35.

Plasmids. pSV2catSVgpt [7268 base pairs (bp)], pSV2cat (5020 bp), and pRSVcat (5027 bp) [a generous gift from B. Howard, constructed as described (4–7)] contain the bacterial *cat* gene coding for CAT (Fig. 1). The plasmid pSV2catSVgpt also contains the bacterial *gpt* gene coding for xanthine phosphoribosyltransferase. The *cat* gene was inserted between either the SV40 early promoter or the RSV LTR and SV40 polyadenylylation sequences to make a gene transcription unit. Plasmid DNA was prepared by lysosyme/Triton X-100 lysis and double banding in cesium chloride/ethidium bromide equilibrium gradients (8). More than 90% of the DNA was in the supercoiled, form I configuration. DNA was stored as a concentrated solution at 4°C in 10 mM Tris·HCl, pH 7.5/1 mM EDTA.

UV Treatment. For UV treatment, the DNA was diluted with sterile water to 0.03–0.05 mg/ml immediately before irradiation and pipeted into a sterile plastic 60-mm tissue culture dish placed on ice. Irradiation was performed with an unfiltered germicidal lamp at a dose rate of 1.39 J·m^{-2}·sec^{-1}, as measured with a calibrated International Light Company radiometer, model IL770A, with PT171C detector.

Enzyme Digestions. T4 endonuclease V (a gift of K. Dixon) was prepared by the procedure of Seawell *et al.* (9) and was highly specific for pyrimidine dimers, producing <3% nicking of unirradiated DNA. UV-treated plasmids were digested

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Abbreviations: CAT, chloramphenicol acetyltransferase; bp, base pair(s); SV40, simian virus 40; RSV, Rous sarcoma virus; LTR, long terminal repeat; D_0, dose that reduces the percentage of CAT activity or form I molecules by 63% along the exponential portion of the dose–response curve.

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Table 1. SV40-transformed and primary skin fibroblast cell lines studied

Phenotype	Cell line
Normal	GM0637*
	GM1652
Xeroderma pigmentosum	
Group A	XP20s (GM4312)*
Group A	XP12Be (GM4429)*
Group A	XP12Ro*†
Group D	XP6Be*‡
	XP6Be (CRL1157)
Ataxia-telangiectasia	GM5849*
Retinoblastoma	GM3022*
Lesch–Nyhan	GM0487*

Lines with identification numbers beginning with GM were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ, and those beginning with CRL were from the American Type Culture Collection, Rockville, MD.
*Transformed with SV40.
†Transformed in Rotterdam; obtained from R. Day.
‡Primary culture obtained from the American Type Culture Collection was transformed with SV40 (a gift from M. Seidman) and used after 10 passages following the crisis period. More than 50% of the cells expressed large T antigen, as measured by immunofluorescence staining (testing by Human Genetic Mutant Cell Repository).

with T4 endonuclease V in 50 mM Hepes buffer, pH 7.6/50 mM NaCl/1 mM Na_2EDTA at 37°C for 30–60 min. After incubation, DNA samples were mixed with sample loading buffer (1% $NaDodSO_4$/20 mM Na_2EDTA/4% Ficoll/0.05% bromphenol blue) and separated by overnight electrophoresis on 0.8% neutral agarose gels. Ethidium bromide (1 μg/ml) was added to the gel and the buffer and the gels were photographed with Polaroid 55 film. The photographic negatives were scanned at 700 nm on a Beckman DU-8 gel scanner that directly integrates the absorption peaks due to supercoiled (form I) and open circular (form II) plasmid molecules. The percentage of form I molecules remaining after UV irradiation and endonuclease V digestion was determined from the relative absorbance of this band in comparison to the proportion of form I molecules in unirradiated plasmids. The number of T4 endonuclease V-sensitive sites per molecule was calculated as described in ref. 10 by assuming a Poisson distribution of nicks among the molecules. The D_0 (dose that reduces the percentage of form I molecules by 63% along the exponential portion of the dose–response curve) thus represents the dose that yields an average of one site per molecule (10).

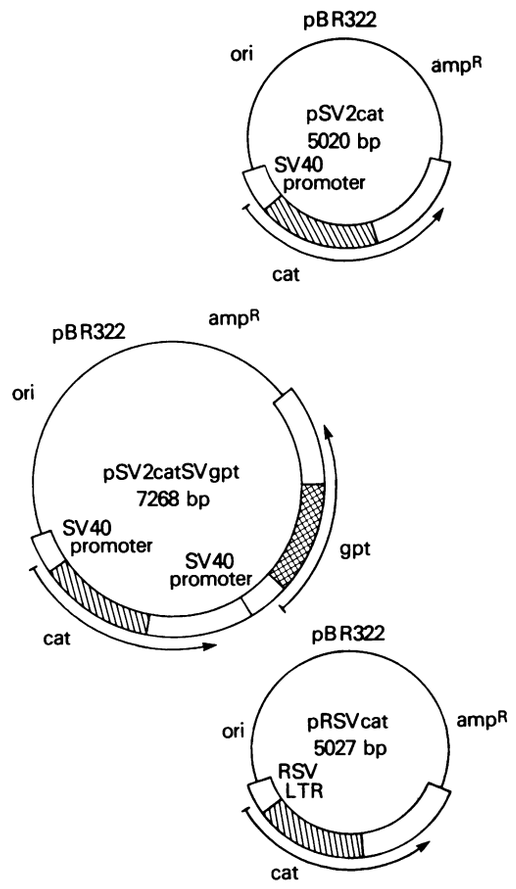

FIG. 1. Recombinant DNA expression vectors used for DNA repair studies. amp^R, Ampicillin resistant; ori, origin.

pSV2catSVgpt and pRSVcat were digested with DNase I (Worthington) in 50 mM Tris·HCl, pH 7.5/3 mM $MgCl_2$ for 40 min at 22°C. The reaction was stopped by addition of Na_2EDTA to a final concentration of 10 mM. DNase I was used at 0.02 μg/50 μg of DNA in a total volume of 500 μl. With these conditions, DNase I produced >99% nicking of form I plasmids, as judged by gel electrophoresis.

Transfection. Transfection was performed by using calcium phosphate/DNA precipitates as described (5, 11) on 100-mm tissue culture dishes containing 1.5–2.0 × 10^6 cells plated 24 hr previously. Five to 10 μg of carrier-free plasmid DNA was used per plate. Cells were incubated with DNA precipitate for 2 hr at 37°C and then were washed once in serum-free medium and shocked with 15% glycerol for 30 sec. Cells were then fed with complete medium and incubated at 37°C for 48 hr. This procedure was found to be the least toxic for human cells and produced the highest transient expression of transfected genes (11).

CAT Assay. CAT activity in crude cell lysates was determined by the procedure of Gorman *et al.* (5) modified by use of 4 mM acetyl-coenzyme A (P-L Biochemicals) and 2.5 μCi (1 Ci = 37 GBq) of [^{14}C]chloramphenicol (New England Nuclear) per ml. Acetylated metabolites of [^{14}C]chloramphenicol were separated from the parent compound by thin-layer chromatography and were quantitated by scintillation spectroscopy. Linearity of the reaction was controlled by using different amounts of protein or incubation times in repeated assays. To accurately determine low CAT activities in samples when DNA-damaged plasmids were used as substrates, reaction mixtures were incubated for up to 6 hr. Cell extracts prepared from mock-transfected cells served as negative controls. Protein concentration of cell extracts was determined by Bio-Rad microassay with bovine serum albumin as standard (12). CAT activity was expressed as nmol of metabolites formed per milligram of cell extract protein per minute after subtraction of background activity. Zero UV-dose CAT activity with 10 μg of plasmid DNA per plate ranged from 0.02 to 0.86 nmol·mg^{-1}·min^{-1} in different SV40-transformed cell lines and from 0.02 to 0.06 nmol·mg^{-1}·min^{-1} in primary cells.

Extraction of Plasmid DNA from Transfected Cells. Forty-eight hours after cell transfection, plasmid DNA was extracted by the method of Hirt (13). DNA was purified by RNase and proteinase K digestions and by phenol extraction. One half of each sample was digested with *Dpn* I (Bethesda Research Laboratories) to remove all nonreplicating DNA. Samples were fractionated on 1% agarose gels, transferred to nitrocellulose paper (14), and hybridized with ^{32}P-labeled pBR322 (1 × 10^7 cpm/μg of DNA).

RESULTS AND DISCUSSION

SV40-transformed xeroderma pigmentosum and normal human cells (Table 1) efficiently express enzyme activity

encoded by bacterial genes that are introduced by transfection of recombinant plasmids (4, 11, 15, 16). Transient CAT expression in xeroderma pigmentosum cells was 5–100% of that in normal cells, with the highest expression obtained with the plasmid pRSVcat (11). There was a linear increase in CAT expression with input plasmid concentrations from 1 to 8 μg per 10^6 cells (11). Peak CAT expression was seen 48 hr after transfection of untreated or UV-irradiated (2, 20, or 200 J·m^{-2}) pSV2catSVgpt in normal or xeroderma pigmentosum cells. By 96 hr CAT activity was <25% of the peak activity (data not shown).

Expression of CAT activity was inhibited by UV treatment of pSV2catSVgpt in all cell lines studied (Fig. 2). The normal cell line and cell lines established from patients with ataxia-telangiectasia, retinoblastoma, or Lesch–Nyhan syndrome all showed similar UV inactivation curves with $D_0 \approx 680$ J·m^{-2} (n = 25; correlation coefficient = −0.87). These diseases are not associated with defective repair of UV damage to DNA (1). In marked contrast, the xeroderma pigmentosum cell lines all showed much steeper inactivation curves for CAT activity with D_0 = 56 J·m^{-2} (n = 22; correlation coefficient = −0.98). The initial slope of the inactivation curve for the group D line was similar to that of the three group A lines. Earlier studies of host cell reactivation of UV damage in SV40 DNA (3) and in adenovirus (17) showed a similar marked sensitivity of the group A and D lines to inhibition of viral replication despite a higher residual rate of UV-induced unscheduled DNA synthesis in the group D cells (18). Our results indicate that cells in both of these complementation groups are equally sensitive to inhibition of expression of transfecting plasmids by UV photoproducts.

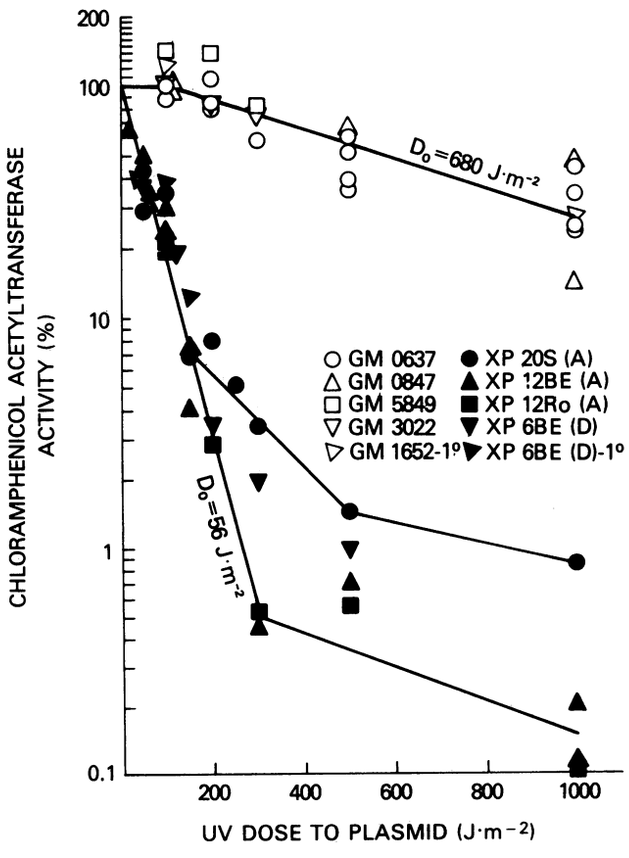

FIG. 2. Transient expression of *cat* gene in SV40-transformed xeroderma pigmentosum, ataxia-telangiectasia, retinoblastoma, Lesch–Nyhan, and normal human cells and primary human skin fibroblasts transfected with UV-treated pSV2catSVgpt DNA. The data for primary line XP6Be were obtained with pRSVcat.

The nature of the target inactivated by UV radiation in pSV2catSVgpt was examined by transfecting the xeroderma pigmentosum and normal cells with smaller plasmids containing the *cat* gene under control of two different promoters and having different levels of CAT expression: SV40 early region (pSV2cat) and RSV LTR (pRSVcat). These plasmids lack the gpt transcription unit found in pSV2catSVgpt but the pBR322 and other sequences are identical. The initial slope of the CAT inactivation curves for all three plasmids is presented in Fig. 3. Over the dose range studied the normal cells showed only a slight fall in CAT activity. The D_0 of the CAT inactivation curves for the xeroderma pigmentosum group A cells was similar for all three plasmids (Table 2). This result implies that all three plasmids have similar-sized UV inactivation targets and, consequently, that the target is independent of the plasmid size and type of promoter.

Induction of damage in plasmid DNA by 254-nm UV radiation was measured by the formation of lesions that make the form I DNA susceptible to single-strand nicking by T4 endonuclease V with resulting loss of superhelicity (10) (Fig. 4). T4 endonuclease V has specificity for cyclobutane-type pyrimidine dimers induced in DNA (9, 10, 19) and does not cut at other types of UV photoproducts, such as pyrimidine-pyrimidone(6-4) adducts (20). The D_0 for the larger plasmid pSV2catSVgpt (7268 bp) was 7 J·m^{-2} and for the smaller plasmid pSV2cat (5020 bp) it was 10 J·m^{-2} (Fig. 4 *Lower* and Table 2). These values reflect the same rate of UV induction of T4 endonuclease V-sensitive sites per base pair in both

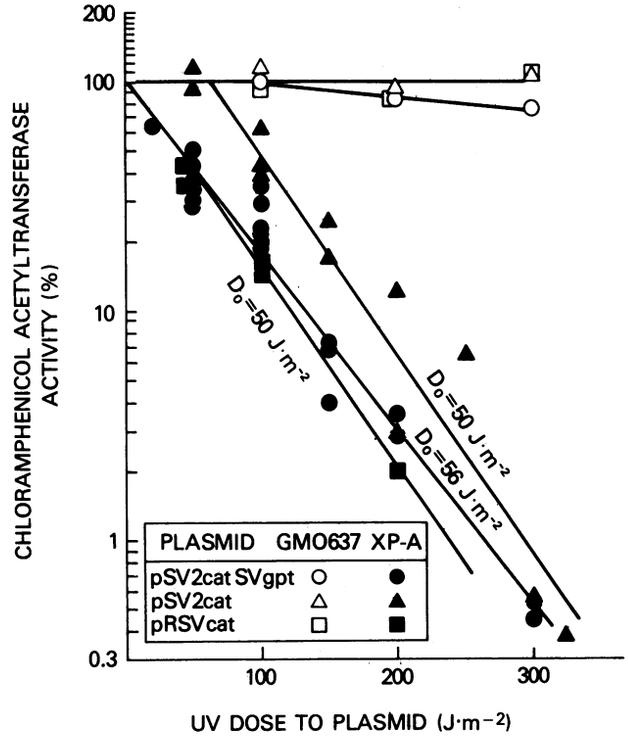

FIG. 3. Transient expression of *cat* gene in SV40-transformed xeroderma pigmentosum group A and normal human cells transfected with UV-treated plasmids. Normal (GM0637) (open symbols) and xeroderma pigmentosum group A (XP12Be, XP20s, and XP12Ro) (closed symbols) SV40-transformed cells were transfected with UV-treated plasmids containing the *cat* gene: pSV2catSVgpt (circles), pSV2cat (triangles), pRSVcat (squares). Zero UV-dose CAT activities for the normal cells were 0.45, 0.62, and 1.2 nmol·mg^{-1}·min^{-1} and for the xeroderma pigmentosum cells were 0.02–0.27, 0.14–0.45, and 1.8 nmol·mg^{-1}·min^{-1} after transfection with 10 μg of the plasmids pSV2catSVgpt, pSV2cat, and pRSVcat per plate, respectively.

Table 2. Plasmids used in DNA repair studies of CAT inactivation

Plasmid	Plasmid size, bp	CAT inactivation D_0,* J·m^{-2}	mRNA size, bases†	UV target size,‡ bases per dimer
pSV2catSVgpt	7268	56 (22, −0.98)	1640	1817
pSV2cat	5020	50 (12, −0.96)	1640	2008
pRSVcat	5027	50 (7, −0.99)	1648	2011

*The number of samples and the correlation coefficient are shown in parentheses.
†Calculated from sequence data to give putative mRNA size (B. Howard, personal communication, and ref. 6).
‡Calculated from data in Figs. 3 and 4 as described in the text.

plasmids and are similar to those observed for SV40 DNA *in vitro* (21).

From the data of Figs. 3 and 4 we can calculate that 56 J·m^{-2} induces one T4 endonuclease V-sensitive site (i.e., pyrimidine dimer) per [7268/(56/7)] = 908.5 bp or per 1817 bases of pSV2catSVgpt. Similar calculations give a target size of 2008 bases for pSV2cat and 2011 bases for pRSVcat (Table 2). Transcription is blocked by dimers in the DNA coding strand (22–25) and, at least in *Escherichia coli in vitro*, not by dimers in the complementary strand (W. Sauerbier, personal communication). The size of the putative *cat* gene mRNAs, as predicted from sequence data on the basis of start sites and possible termination signals (6), is listed in Table 2. Predicted values are close to those determined by inactivation data for the size of the CAT transcription unit and imply that one pyrimidine dimer is sufficient to inactivate expression of the *cat* gene in the xeroderma pigmentosum group A and D cells. To our knowledge, it has not been demonstrated previously that one pyrimidine dimer blocks gene expression in xeroderma pigmentosum cells *in vivo*, most probably by blocking transcription (22–25). The difference of 10–20% in target size between the theoretical and experimental values may represent experimental variability, inhibition of transcription by minor (i.e., nondimer) photoproducts, a variability in the size of the mRNA population transcribed, or slightly nonuniform distribution of pyrimidine dimers along the transcription unit.

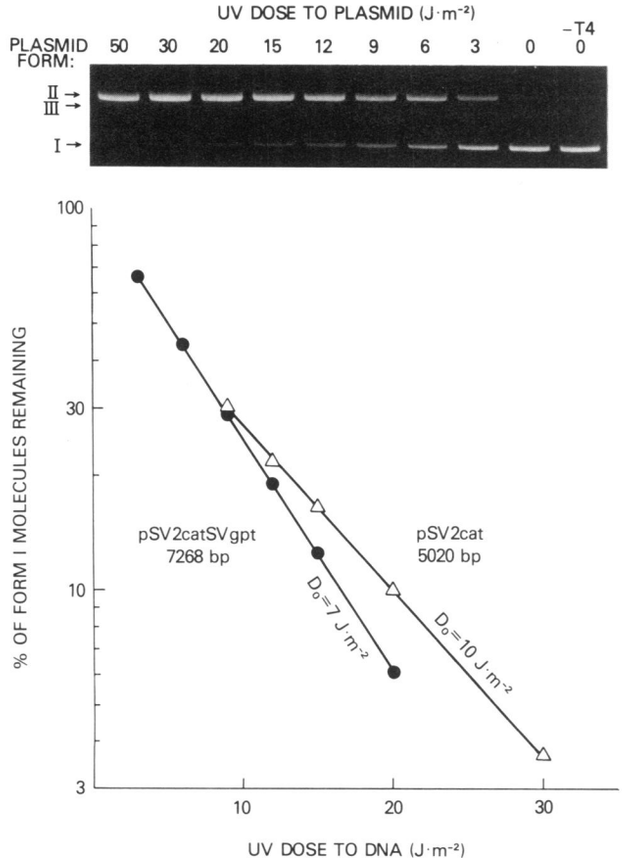

FIG. 4. Induction of T4 endonuclease V-sensitive sites by UV radiation in plasmids pSV2cat and pSV2catSVgpt. (*Upper*) Agarose gel pattern indicating increasing sensitivity to nicking of form I plasmid pSV2cat by treatment with T4 endonuclease V after UV radiation. (*Lower*) Percentage of form I molecules remaining in T4 endonuclease V-treated pSV2cat and pSV2catSVgpt as a function of UV dose.

The specificity of the xeroderma pigmentosum DNA repair defect was examined by single-strand nicking of >99% of the form I molecules of pRSVcat or pSV2catSVgpt by DNase I. This damage resulted in a similar reduction of *cat* expression in the normal (GM0637) and the xeroderma pigmentosum group A (XP12Be) cells to 10–30% of control CAT activity. This result indicates that single-strand nicks are dealt with differently from pyrimidine dimers since there was similar inhibition of CAT activity in repair-proficient and repair-deficient cells. Additional studies showed that treatment of DNase I-nicked plasmid pRSVcat with 50–300 J·m^{-2} UV showed no further inhibition of CAT activity in the normal line, GM0637. In the XP12Be cells CAT activity was further inhibited by UV with the same kinetics ($D_0 \approx 50$ J·m^{-2}) as with the unnicked plasmid shown in Fig. 3. This result demonstrates that the DNA repair defect is equally manifest on supercoiled or open circular transfecting DNA.

This plasmid host cell reactivation assay measuring inactivation of CAT activity is highly sensitive and reproducible. The assay permitted measurement of a 12-fold difference in UV inactivation slopes between repair-deficient xeroderma pigmentosum and repair-proficient SV40-transformed human cells. We have also studied UV inactivation of *cat* gene expression in xeroderma pigmentosum group D (XP6Be) and normal (GM1652) primary fibroblasts. We found that the control CAT activity was lower by a factor of about 10–20 than in the SV40-transformed XP6Be and GM0637 cells but there was the same 12-fold difference in inactivation slopes (Fig. 2). Sodium butyrate-stimulated xeroderma pigmentosum group A and normal primary fibroblasts recently were reported to show only a 2.8-fold difference in UV dose producing 50% inactivation of *cat* gene expression (26).

This assay differs from that described by Spivak *et al.* (27), in which UV treatment of plasmids resulted in enhanced stable integration during 2–3 weeks in selective medium. This assay also differs from host cell reactivation assays that measure plaque formation by intact viruses (2, 17) or infectious center production by viral DNA (3) in that replication of input DNA is not required. We have looked for the presence of episomal replicating pSV2catSVgpt and pRSVcat DNAs by performing Southern blots of Hirt extracts of transfected normal human (GM0637) and XP12Be cells obtained 48 hr after transfection. *Dpn* I digestion (an endonuclease that cuts at methylated adenines incorporated in plasmids replicated in bacteria but not in mammalian cells) abolished 99.9% of the plasmid-size DNA in the Hirt extracts. Thus, the vast majority of intracellular plasmids at the time of peak CAT activity is unreplicated. This assay thus is similar to UV transcription inhibition mapping in bacterial and mammalian systems (22).

This plasmid host cell reactivation assay, involving use of recombinant DNA vectors, affords the possibility of studying excision repair independently of postreplication repair since these plasmids do not replicate as episomes (4) in human cells and permits the study of the effect of defined types of DNA damage on plasmid expression. The UV-inactivation target size for other genes contained in transfecting plasmids may

be measured. We have determined that the target size for the *gpt* gene contained in pSV2catSVgpt is smaller than the size of the *cat* gene. Induction of other DNA lesions, such as apurinic sites, also leads to inactivation of *cat* expression (unpublished data). The effect of purified or highly specific repair enzymes on UV- or carcinogen-damaged expression vectors may provide a means for dissecting human DNA repair systems *in vivo*.

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