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Genetics

Ultraviolet light induction of diphtheria toxin-resistant mutants in normal and xeroderma pigmentosum human fibroblasts

(carcinogenesis/somatic mutations/DNA repair)

THOMAS W. GLOVER*†, CHIA-CHENG CHANG*, JAMES E. TROSKO*, AND STEVEN S.-L. LI§‡

*Department of Human Development, College of Human Medicine, Michigan State University, East Lansing, Michigan 48824; and §Laboratory of Environmental Mutagenesis, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

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ABSTRACT **The UV induction of diphtheria toxin-resistant (DT^r) mutants in normal and xeroderma pigmentosum human fibroblasts has been quantitatively characterized. A concentration of diphtheria toxin at which DT^r cells are cross-resistant to *Pseudomonas aeruginosa* exotoxin A was determined and used in the selection of resistant mutants. Recovery of mutants was not influenced by the presence of wild-type cell densities of $1–8 \times 10^5$ per 9-cm plate, indicating no metabolic cooperation exists, in contrast to what is seen in the selection of some other variant phenotypes. Expression periods for UV-induced mutations differed with the severity of mutagen treatment and cell strain used. A relatively long (10–15 days after UV treatment) expression period was required for the maximum recovery of DT^r mutants. Maximum recovery was followed by a decrease in mutation frequency on subsequent days evaluated. An apparent linear dose response within the dose range used was observed for UV-induced mutations in both normal and xeroderma pigmentosum fibroblasts. Our results indicate that xeroderma pigmentosum fibroblasts have higher UV-induced mutation frequencies per unit UV dose but similar frequencies per unit survival compared to normal cells within the range of UV doses tested.**

Xeroderma pigmentosum (XP) is a rare genetic disease predisposing affected individuals to sunlight-induced skin damage, pigmentation changes, and multiple skin carcinomas on areas exposed to the sunlight and, in some forms of the disease, developmental and neurological disorders (1, 2). All XP cell strains are more sensitive than normal cells to killing by UV irradiation or a number of chemical mutagens, as measured by colony-forming ability *in vitro*. Andrews *et al.* (3) have shown that the manifestation of neurological abnormalities is correlated with the greatest sensitivity to UV-induced cytotoxicity *in vitro* among XP patients.

Since the original observation that cells from classical XP were deficient in the ability to repair UV-induced DNA damage (4–6) when compared to fibroblasts from normal individuals (7), it was hypothesized that the clinical manifestation of this cancer-prone syndrome might be the result of their hypermutability due to a reduced ability to perform error-free repair of DNA damage. Maher and McCormick, using resistance to 8-azaguanine as a genetic marker, demonstrated that fibroblasts of several XP strains, when compared to normal fibroblasts, had increased cytotoxicity and mutation frequencies induced by UV irradiation or a number of chemical carcinogens (8).

The question remains as to whether the higher induced mutation frequencies at the hypoxanthine phosphoribosyltransferase (HPRT) locus in XP cells holds for other genetic loci. Quantitative mutagenesis studies in human cells have been limited by the availability of reliable genetic markers (9). Most investigations of this kind have used the HPRT mutation system. Because of problems inherent in this mutation system, such as a cell density effect, drug concentration effect, the influence of serum components, and the possibility of an epigenetic change resulting in the selected phenotype (10), we endeavored to develop a better mutation system for human cells. On the basis of the finding of Moehring and Moehring (11) that mutants with altered elongation factor 2 of protein synthesis could be isolated in Chinese hamster ovary (CHO-K1) cells by selection with high concentrations of diphtheria toxin, we undertook a series of studies to characterize the induction of similar mutants in human fibroblasts systematically. The induction of diphtheria toxin-resistant (DT^r) mutants by chemical mutagens in normal human fibroblasts has recently been described (9). Quantitative dose response, however, was not demonstrated in those experiments. Using this marker, we quantitatively measured UV-induced mutations in normal and XP fibroblasts in order to test the hypothesis that higher UV-induced mutation frequencies would be found in excision repair-deficient XP cells than in normal cells.

MATERIALS AND METHODS

Cell Strains. Diploid fibroblasts from human skin were used throughout all experiments. Cell strain 73-6NF was derived from the foreskin of a normal human male and was provided by David J. Segal (University of Alberta, Canada). Cell strain XP7BE was derived from the skin biopsy specimens of a female patient with classical XP including neurological abnormalities, and was obtained from the American Type Culture Collection (Rockville, MD). XP7BE cells have been shown to belong to XP complementation group D (12).

Culture Conditions. Cells were grown in modified Eagle's minimal essential medium (13) with Earle's salts, supplemented with a 100% increase of all nonessential amino acids, 50% increase of all vitamins and essential amino acids except glutamine, 1 mM sodium pyruvate, and 10% fetal calf serum (GIBCO). The bicarbonate concentration was decreased to 1 g/liter. Stock cell cultures were grown in static culture attached to the surface of 75-cm^2 plastic flasks. Cells were incubated at 37°C in humid air supplied with 5% CO_2. Under these conditions cells not contact inhibited have a generation time of about 24 hr. Cells were removed from plastic surfaces by using 0.01% crystalline trypsin in phosphate-buffered saline without calcium and magnesium ions.

Toxins. Diphtheria toxin (DT) was the product of Connaught Medical Research Laboratories (Toronto, Canada), lot D343, 2000 floculating units (Lf) per ml, 19,000 guinea pig minimum

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Abbreviations: DT, diphtheria toxin; DT^r, diphtheria toxin-resistant; Lf, floculating units; HPRT, hypoxanthine phosphoribosyltransferase; XP, xeroderma pigmentosum.

† Present address: University of Hawaii School of Medicine, Department of Anatomy and Reproductive Biology, Honolulu, HI 96822.

‡ To whom reprint requests should be addressed.

lethal dose (MLD) per mg protein, and about 3 μg protein per Lf. Highly purified *Pseudomonas aeruginosa* exotoxin A was originally a gift of Stephen Leppla of the U.S. Army Medical Research Institute of Infectious Diseases and has 5000 mouse lethal doses per mg of protein. DT was sealed in glass ampules in concentrated (2000 Lf/ml) form and was stored frozen in liquid nitrogen until needed. *Pseudomonas* exotoxin A was stored frozen at −20°C until needed.

Cell Survival. The cytotoxicity of DT was determined by plating 73-6NF fibroblasts at various cell densities into 9-cm plastic dishes in 10 ml of medium. After 6.5 hr for cell attachment, medium was changed and DT was added at appropriate concentrations. After 48 hr, toxin-containing medium was removed from the plates and 10 ml of fresh medium was added. After about 3 weeks of growth, surviving colonies were fixed in 95% ethanol, stained with Giemsa stain, and scored. Relative survival was determined from the average percentage of survivors in six plates per toxin concentration.

To determine the cross-resistance of DT^r colonies to *Pseudomonas* exotoxin, normal cells and DT^r colonies selected at various concentrations of DT were isolated with a glass cylinder, trypsinized, and seeded into 4 wells of a 24-well culture dish. After an overnight incubation the medium was changed and cells in one well were exposed to *Pseudomonas* exotoxin at 0.1 μg/ml for 48 hr, and cells in one other well were similarly treated with DT at 0.1 Lf/ml. Cells in the remaining two wells were not treated and served as controls. Wells were scored for cell survival at various times up to 2 weeks thereafter.

To determine UV survival, various numbers of cells (200–2000 cells per each of six 9-cm plate) were plated in toxin-free medium. Relative percent of survival was determined by dividing the total number of colonies developed by the total number of cells plated, multiplied by 100, and divided by the percent plating efficiency of untreated cells.

Mutation Experiments. Cells were trypsinized and plated for attachment 6.5 hr before UV irradiation and medium change. In typical expression time and dose–response experiments, from 3.2×10^6 to 3×10^7 cells were plated for each UV dose, depending on the severity of UV treatment. With the medium removed from the plates, the attached cells were exposed to a germicidal lamp (GE25T8-25W) positioned to deliver 20 ergs/mm^2 per sec (2 J/m^2 per sec) or, in comparative experiments using normal and XP cells, to a short-wave UV lamp (Mineralight, UVS11, Ultra-Violet Products, San Gabriel, CA) positioned to deliver 2.5 ergs/mm^2 per sec for normal cells and raised to deliver 1.25 ergs/mm^2 per sec for XP cells. Growth medium (10 ml) was added to each plate for various expression times indicated in the tables and figures. Cells were subcultured into 75-cm^2 plastic flasks when confluent or at about every third day during expression periods. Cells from all UV treatments were tested alike during the expression periods. At the end of these periods, cells were trypsinized and replated for survival and mutation assays. In typical experiments, the number of cells per plate and the number of plates (9 cm) used were 4×10^5 cells in each of 12–18 plates and 200 cells in each of 6 plates, respectively, for each mutation and plating efficiency assay. After 6.5 hr for attachment, the medium was changed and attached cells were exposed to selective medium containing DT at 0.1 Lf/ml for 48 hr, at which time toxin-containing medium was replaced with 10 ml of fresh toxin-free medium. One additional medium change was done 8 days later. Colonies were grown for about 3 weeks before they were stained and scored. The mutation frequency was calculated by dividing the total number of DT^r mutants recovered by the total number of cells plated corrected by the plating efficiency determined for these cells.

RESULTS

Establishment of Selection Conditions. Initial experiments were designed to determine a concentration of DT to use for selection whereby all surviving cells would be cross-resistant to *Pseudomonas* exotoxin A. The cytotoxicity of DT toward normal human fibroblasts is shown in Fig. 1. A similar low frequency of survivors was observed at toxin concentrations of 0.001–1 Lf/ml with 48-hr toxin exposure. The range 0.001–0.1 Lf/ml was chosen for further study. The effect of toxin concentration on the frequency of recovery of DT^r variants in both untreated and UV-treated cell populations and the cross-resistance of these surviving colonies to a highly toxic dose of *Pseudomonas* exotoxin A, determined to be 0.1 μg/ml applied for 48 hr, are shown in Table 1. Initial selection with DT at 0.001 Lf/ml for 24 or 48 hr, or with DT at 0.01 Lf/ml for 24 hr resulted in recovery of 139–1130 colonies per 10^6 survivors in the UV-treated population and as high as 1510 colonies per 10^6 survivors in the nonirradiated population. However, under these conditions only 5/30 (16.7%) of all isolated colonies were cross-resistant to *Pseudomonas* exotoxin A or showed stable resistance to more stringent selection with DT (0.1 Lf/ml for 48 hr). Initial selection with DT at 0.01 Lf/ml for 96 hr or at 0.1 Lf/ml for 24, 48, or 96 hr resulted in a lower frequency of DT^r colonies, but all isolated colonies tested (24/24) were cross-resistant to *Pseudomonas* exotoxin and showed stable resistance to DT at 0.1 Lf/ml applied for 48 hr. These results indicate that cells resistant to both DT and *Pseudomonas* exotoxin A can be efficiently recovered by selection with high concentrations of DT in human fibroblasts. For further experimentation, DT at 0.1 Lf/ml for 48 hr was chosen for selection of DT^r mutants.

It is worth noting that in determining selection conditions, DT was removed from the cells after specific time periods. The basis for this procedure was that it was noticed in preliminary experiments with human and Chinese hamster V79 fibroblasts that, when DT is added to resistant cells throughout the entire growth period of DT^r colonies, the growth rate of some cells is very much reduced, representing a practical disadvantage. We therefore endeavored to establish conditions whereby sensitive cells would be quickly killed and DT could be removed from the growth medium.

The effect of cell density on the recovery of UV-induced DT^r mutants was investigated. As shown in Fig. 2, the frequencies of recovery of DT^r mutants remain the same in the presence of wild-type cell densities of $1–8 \times 10^5$ per 9-cm plate. Furthermore, because sensitive cells are quickly killed and detached from the plates after addition of DT, there was no noticeable

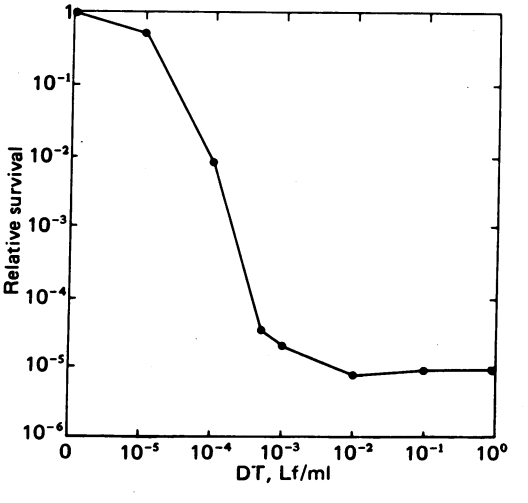

FIG. 1. Cytotoxicity of DT in normal human fibroblasts. The cells were exposed to various concentrations of DT for 48 hr.

Table 1. Effect of DT concentration on DT^r colonies and cross-resistance of isolated colonies to *Pseudomonas* exotoxin A

UV*	DT for original selection, Lf/ml	Toxin exposure, hr	DT^r colonies per 10^6 survivors	Isolated colonies resistant to† DT at 0.1 Lf/ml, 48 hr	Isolated colonies resistant to† Exotoxin at 0.1 μg/ml, 48 hr
−	0.001	24	1510	0/6	0/6
−	0.001	48	421	1/6	1/6
−	0.001	96	9.5	—	—
−	0.01	24	28.5	—	—
−	0.01	48	6.3	—	—
−	0.01	96	3.2	—	—
−	0.1	24	3.2	—	—
−	0.1	48	3.2	—	—
−	0.1	96	3.2	—	—
+	0.001	24	1130	1/6	1/6
+	0.001	48	245	1/6	1/6
+	0.001	96	68.0	5/6	5/6
+	0.01	24	139	2/6	2/6
+	0.01	48	34.0	5/6	5/6
+	0.01	96	44.2	6/6	6/6
+	0.1	24	37.4	6/6	6/6
+	0.1	48	27.2	6/6	6/6
+	0.1	96	34.0	6/6	6/6

* Mutagenized cells received UV irradiation at 200 ergs/mm^2 and were allowed 6.5 days for mutation expression.

† In every case resistant colonies were resistant to both toxins.

difference in cell morphology or colony size with the different cell densities tested. These results indicate that under these selective conditions there is no cross-feeding effect (metabolic cooperation) resulting in the loss of mutant recovery at high cell densities as is observed with the 6-thioguanine or 8-azaguanine selective system, nor is the effective toxin dose diluted at cell densities up to 8×10^5 per plate. A cell density of 4×10^5 per 9-cm plate was chosen for subsequent experiments on the basis of these results and the efficiency of scoring DT^r colonies.

Expression and Dose Response of UV-Induced DT^r Mutants. The recovery of DT^r mutants in normal human fibroblasts as a function of expression time and UV dose is shown in Fig. 3 (experiment 1). Mutation expression was found to be earlier for cells treated with lower doses of UV; however, for all UV doses evaluated, a maximum recovery of UV-induced mutations was observed at 10 days after UV treatment. Mutation frequencies at all UV doses steadily declined after 10 days, presumably due to a reduced fitness of mutant cells compared to wild-type cells in the populations. Eight representative colonies isolated from 5-, 10-, and 14-day replatings all showed resistance to both DT at 0.1 Lf/ml and *Pseudomonas* exotoxin A at 0.1 μg/ml.

The highest mutation frequencies observed at 10-day expression time and the corresponding UV survival curve for the cells irradiated in this experiment are shown plotted as a function of UV dose in Fig. 4. These results showed that the DT^r

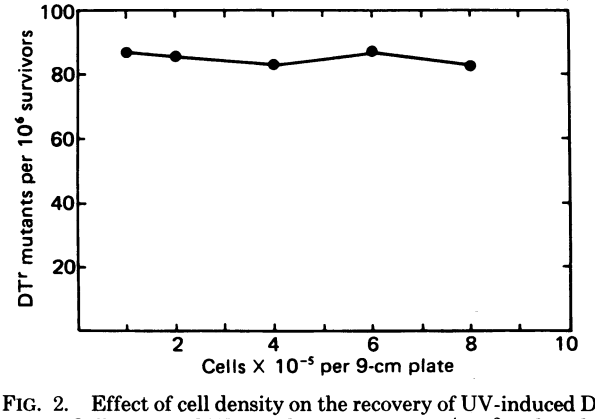

FIG. 2. Effect of cell density on the recovery of UV-induced DT^r mutants. Cells received UV irradiation at 113 erg/mm^2 and 15 days were allowed for mutation expression. Replating efficiency of cells was 28.7%.

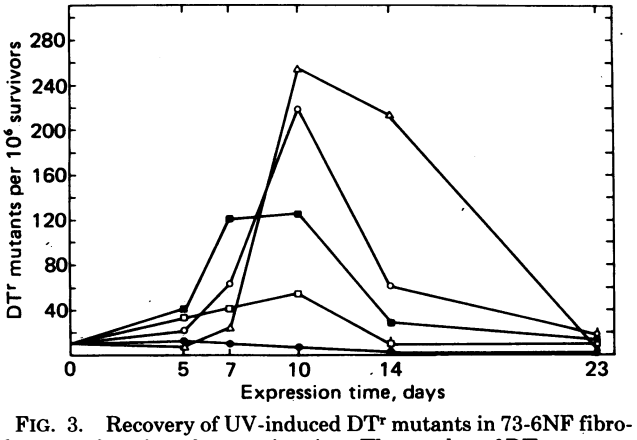

FIG. 3. Recovery of UV-induced DT^r mutants in 73-6NF fibroblasts as a function of expression time. The number of DT^r mutants, the replating efficiency, and the total number of cells assayed at 10-day expression time for each UV dose are given in parentheses. ●, 0 ergs/mm^2 (10, 31%, 4.8×10^6); □, 50 ergs/mm^2 (81, 30.6%, 4.8×10^6); ■, 100 ergs/mm^2 (186, 30.5%, 4.8×10^6); ○, 150 ergs/mm^2 (413, 31.4%, 6.0×10^6); △, 200 ergs/mm^2 (429, 32.3%, 5.2×10^6).

mutation frequency increased linearly with increasing doses of UV irradiation. The results show that UV radiation is effective in inducing DT^r mutations in human fibroblasts.

Comparative Mutagenesis between Normal and XP Fibroblasts. An experiment was performed to compare the UV-induced DT^r mutation frequencies in 73-6NF and XP7BE fibroblasts under the same experimental conditions (experiment 2). The recovery of DT^r mutants as a function of expression time and UV dose is shown in Fig. 5 (*top* and *middle*). The results for 73-6NF cells are essentially the same as those obtained in a previous experiment (described above) using a different UV source. XP7BE cells exhibited a longer period of time for the expression of the DT^r phenotype. The highest mutation frequencies for the expression times chosen were not observed until 15 days after UV irradiation. Because of the size of such experiments, only a limited number of expression times could be evaluated and therefore it is not known whether the highest observed frequencies are the precise maximum frequencies attainable. In light of the peaked expression time curves observed for normal cells, this could explain why the maximum observed mutation frequency at 17.5 ergs/mm^2 UV irradiation in XP7BE cells was no higher than that for 12.5 ergs/mm^2 irradiation.

The mutation experiment was repeated for XP7BE (experiment 3), using expression times of 8, 11, 14, and 18 days. The recovery of DT^r mutants as a function of expression time and UV dose is shown in Fig. 5 *bottom*. The experiment showed that the highest mutation frequencies were detected at 14-day expression time. As for normal human fibroblasts, the mutation frequencies for XP cells declined after extended growth. Four DT^r XP clones were grown for more than 20 passages in toxin-free medium. When retested, they were all resistant to DT.

The highest mutation frequencies observed at 10-day expression time for normal cells and a 14- or 15-day expression for XP cells and the corresponding UV survival curves for the cells irradiated in these experiments are plotted as a function of UV dose in Fig. 6. The linear dose–response curve for UV-induced DT^r mutations in 73-6NF cells is consistent with previous results, and a similar dose–response curve is seen with XP7BE cells at much lower UV doses. These results indicate that XP7BE cells have higher induced mutation frequencies per unit UV dose than normal fibroblasts. The mutation frequencies shown in Fig. 4 and Fig. 6 are overlapping when plotted as a function of UV survival (Fig. 7.) and appear similar in the normal and XP cell strains.

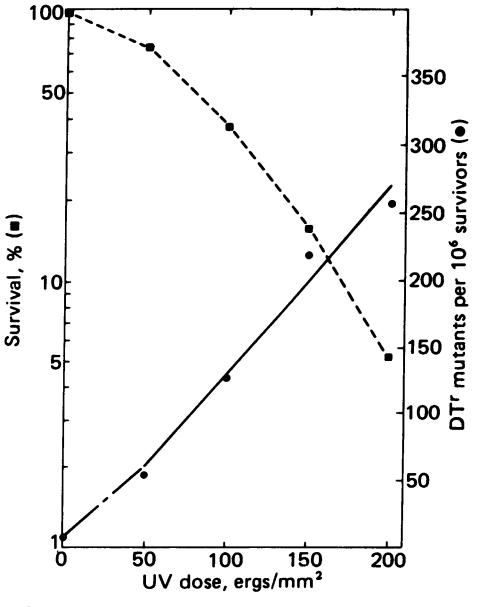

FIG. 4. Dose response of UV-induced cytotoxicity (■, left ordinate) and DT^r mutation frequencies (●, right ordinate) in 73-6NF fibroblasts. Solid line through induced mutation frequencies was determined by least squares linear regression analysis.

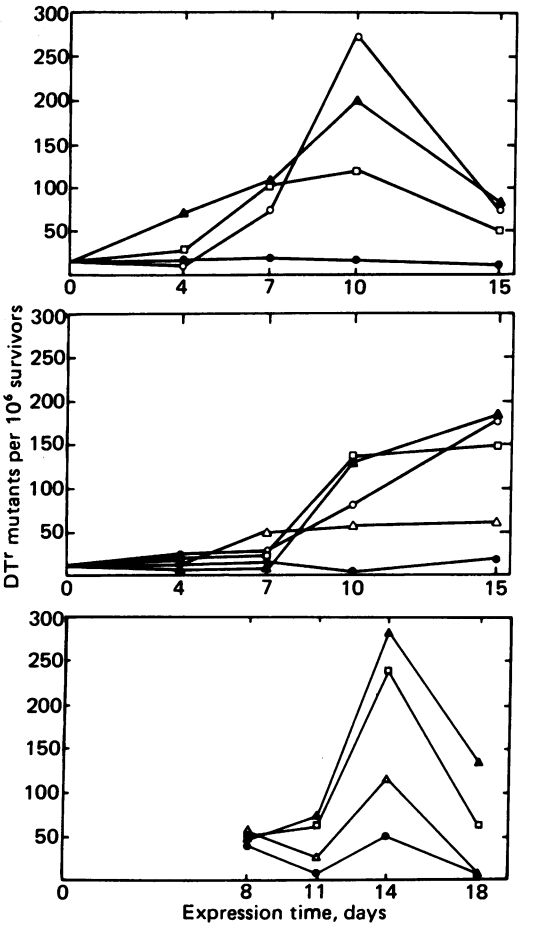

FIG. 5. Recovery of UV-induced DT^r mutants in 73-6NF (experiment 2, *Top*) and XP7BE (experiment 2, *Middle;* experiment 3, *Bottom*) fibroblasts as a function of expression time. Doses are given in ergs/mm^2. (*Top*) ●, 0; □, 75; ▲, 113; ○, 150. (*Middle*) ●, 0; △, 2.5; □, 7.5; ▲, 12.5; ○, 17.5. (*Bottom*) ●, 0; △, 2.5; □, 7.5; ▲, 15.

DISCUSSION

The purpose of this study was twofold. First, we were interested in developing a simpler and more reliable quantitative mutation assay system for human fibroblasts; second, we were interested in applying this mutation system to the comparative study of UV-induced mutations in normal and XP fibroblasts. From results obtained, some prominent features of DT^r mutations in human fibroblasts were observed: (*i*) lack of a cell density effect; (*ii*) high inducibility by the mutagen; (*iii*) apparent linear UV dose response; (*iv*) long expression time; and (*v*) apparent reduced fitness for DT^r mutants. Features *i* and *ii*, observed also by Gupta and Siminovitch (9), together with the apparent linear dose response observed in these experiments, may constitute the major advantage of this mutation system, because they provide a simpler and more sensitive assay than the HPRT system. Features *iv* and *v*, considered to be disadvantages, are also found with the HPRT mutation system. It therefore appears important to evaluate induced DT^r mutation frequencies at multiple expression times for accurate results. Gupta and Siminovitch (9) reported a shorter expression time and a high induced mutation frequency with certain mutagen treatments.

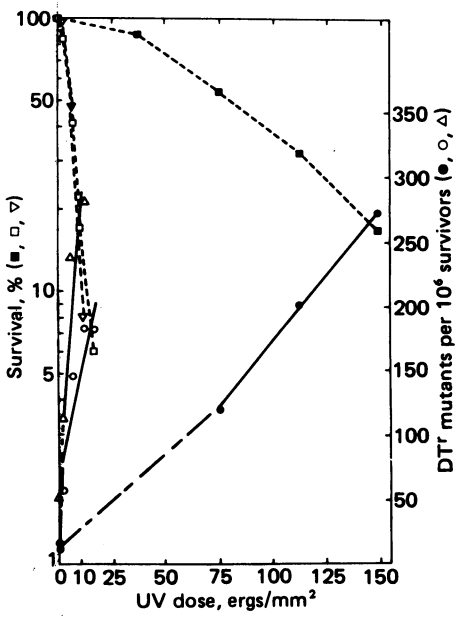

FIG. 6. Dose response of UV-induced cytotoxicity (■, □, ▽; left ordinate) and DT^r mutation frequencies (●, ○, △; right ordinate) in 73-6NF (■, ●, experiment 2) and XP7BE (□, ○, experiment 2; ▽, △, experiment 3) fibroblasts. Solid line through induced mutation frequencies was determined by least-squares linear regression analysis.

The discrepancy could be due to the different mutagen treatments. Alternatively, it may be due to the more stringent selective conditions employed in our experiments.

Concerning the nature of induced DT^r mutants, the following observations are relevant: (*i*) the DT^r phenotype appears stable and heritable in the absence of the selective agent, because toxin resistance was retained in all of four clones tested even after 20 passages; (*ii*) the variants are inducible by mutagens with a linear dose response; and (*iii*) DT^r mutants selected at higher concentration of DT are cross-resistant to *Pseudomonas* exotoxin A. These properties are consistent with genetic change, although not sufficient to rule out an epigenetic origin. In Chinese hamster ovary (CHO-K1) cells, two types of DT^r mutants have been described (11); permeability variants in which uptake of toxin was impaired and translational variants with altered elongation factor 2. The translational mutants were also found to be cross-resistant to *Pseudomonas* exotoxin A,

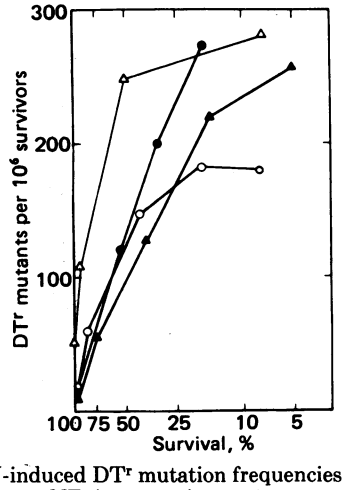

FIG. 7. UV-induced DT^r mutation frequencies as a function of cytotoxicity in 73-6NF (▲, experiment 1; ●, experiment 2) and XP7BE (○, experiment 2; △, experiment 3) fibroblasts.

which has been shown to ADP-ribosylate elongation factor 2 in an identical fashion as DT does (14, 15) but which enters the cell through a different mechanism (11, 14–16). If these observations hold true for human cells, and because under our selective condition (DT at 0.1 Lf/ml for 48 hr) DT^r mutants selected are also cross-resistant to *Pseudomonas* exotoxin A, the mutants selected may be considered as presumptive translational mutants. Further biochemical characterizations are needed to verify if this analogy is true for both cell systems.

The comparative mutagenesis study using this new assay system indicates that excision repair-deficient XP7BE cells have higher induced mutation frequencies per unit UV dose but similar mutation frequencies per unit survival compared to normal cells. These results agree with those of Maher and McCormick (8), who used resistance to 8-azaguanine as a genetic marker for the study of induced mutations in XP complementation group A and C fibroblasts, and support the hypothesis that the excision repair function lacking in XP7BE cells is error-free, otherwise a higher frequency of induced mutations per unit survival would be expected in normal cells. Furthermore, these results support a prediction of the mutation theory of cancer, namely certain humans with syndromes that predispose the individual to cancer will have higher induced mutation frequencies than nonsusceptible individuals (17).

The results from this study may be significant because they could demonstrate that XP cells are more sensitive to the mutagenic effects of UV light than are normal cells at a genetic locus requiring, presumably, a mutation resulting in an altered, but still functional, gene product (as opposed to a deleted gene product) in order to manifest the phenotypic expression of the mutants. This genetic marker should be useful in other quantitative mutagenesis studies in human cells.

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