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Genetics

Hypomutability in Fanconi anemia cells is associated with increased deletion frequency at the *HPRT* locus

(mutagenic repair/cross-links/monoadducts/psoralen/Southern blot analysis)

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ABSTRACT **Fanconi anemia (FA) is an inherited human disorder associated with a predisposition to cancer and characterized by anomalies in the processing of DNA cross-links and certain monoadducts. We reported previously that the frequency of psoralen-photoinduced mutations at the *HPRT* locus is lower in FA cells than in normal cells. This hypomutability is shown here to be associated with an increased frequency of deletions in the *HPRT* gene when either a mixture of cross-links and monoadducts or monoadducts alone are induced. Molecular analysis of mutants in the *HPRT* gene was carried out. In normal cells the majority of spontaneous and induced mutants are point mutations whereas in FA deletion mutations predominate. In that case a majority of mutants were found to lack individual exons or small clusters of exons whereas in normal cells large (complete or major gene loss) and small deletions are almost equally represented. Thus we propose that the FA defect lies in a mutagenic pathway that, in normal cells, involves bypassing lesions and subsequent gap filling by a recombinational process during replication.**

Cells possess various mechanisms to repair DNA lesions in order to maintain cell viability and the functional stability of DNA. Lesions unrepaired or repaired inaccurately lead to changes in the genetic information. These heritable changes range from base substitutions to large chromosomal rearrangements. Knowledge of the DNA lesions produced by a mutagen, coupled with an analysis of the molecular nature of the induced mutations, can give clues as to which lesions are biologically relevant and how these lesions are processed. In addition, the determination of mutagenic specificity in the presence or absence of a particular repair system can suggest mechanisms by which this system modulates the mutagenic process. Mutational changes are implicated in human health problems, including genetic diseases and cancer. Thus it is important to acquire information directly from human cells. Molecular techniques have made it possible to examine the molecular events underlying mutations at specific loci in mammalian cells (for reviews, see refs. 1 and 2). The following systems have been established for this purpose: shuttle vectors (for review, see ref. 3 and references therein), single integrated copies of a transfected bacterial gene (4), or analysis of mutations at endogenous loci (5–7). Among these systems, the last is the most laborious, but the advantage is that the chromosomal location, the structural conformation of DNA, and the topological constraints correspond to a natural situation. Among the various endogenous genes studied, the *HPRT* gene has several advantages: its large size (45 kilobases), its X chromosome location, and the availability of hybridization probes and of nucloeotide sequence data (8, 9). Moreover, large deletions or rearrangements can be detected since the *HPRT* locus is not close to essential genes (6, 10).

Cells from patients with defects in DNA-damage processing provide opportunities to explore the molecular mechanisms of mutagenesis operating in human cells. All of these rare recessive diseases [xeroderma pigmentosum, ataxia telangiectasia, Fanconi anemia (FA), etc.] are characterized by hypersensitivity to specific DNA-damaging agents and are likely to be related, with anomalies in repair processes (for review, see ref. 11). FA is an autosomal recessive disorder characterized by an increased cellular and chromosomal sensitivity to DNA cross-linking agents and a predisposition to cancer (12–15). At least two genetic complementation groups A and B have been identified (16) and they differ in their phenotypic response to cross-linking agents (17–21). It should be noted that group B means "different from group A" since cell lines in group B could in fact represent two separate groups B or C (16). We have shown (22) that FA lymphoblasts belonging to group A are hypomutable in comparison to normal lymphoblasts at two loci (*HPRT* and Na^+,K^+-ATPase) after treatment with two bifunctional psoralens. Herein we demonstrate that FA cells belonging to another complementation group, referred to as group B, are also hypomutable at the *HPRT* locus under conditions in which monoadducts (MAs) and DNA interstrand cross-links (CLs) or only MAs are induced by photoactivated 4,5',8-trimethylpsoralen (Me_3Pso). The hypomutability observed in FA cells is found to be associated with a substantial increase in the deletion frequency at the *HPRT* gene. In normal cells point mutations predominate in spontaneous and psoralen-photoinduced mutants.

MATERIAL AND METHODS

Cell Lines. The normal human lymphoblasts AHH-1 (23) (provided by W. Thilly, Massachusetts Institute of Technology) and FA lymphoblasts FA-HSC-62 (15), belonging to group B (provided by M. Buchwald, Hospital for Sick Children, Toronto), both Epstein–Barr virus-transformed lines, were routinely grown in suspension in RPMI 1640 medium, supplemented with 2 mM glutamine and 10% (vol/vol) fetal calf serum (Boehringer Mannheim). Cell lines were maintained in exponential growth by daily dilution to 3×10^5 cells per ml (22). Under these conditions, the doubling times were ≈16 hr for the normal and 22 hr for the FA cell lines. The cells were periodically tested for mycoplasm.

Photosensitizing Treatment. Cells in exponential growth (7×10^7 cells per experimental point) were resuspended in 10 ml of isotonic phosphate-buffered saline (PBS), containing 5 μM Me_3Pso (Sigma), placed in 90-mm Petri dishes, and incubated for 10–15 min at 37°C. Thereafter, the cells were exposed to monochromatic radiation at 365 nm or 405 nm, with stirring. The cells were then washed in PBS, centrifuged, and resus-

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Abbreviations: FA, Fanconi anemia; Me_3Pso, 4,5',8-trimethylpsoralen; CL, DNA interstrand cross-link; MA, monoadduct; PE, plating efficiency; 6-TG^R, 6-thioguanine resistant.

pended at 3.5–4.5 × 10^5 cells per ml. Irradiation sources and dosimetry were as described (19, 24).

Cell Survival and Mutagenicity Assay. The method used is derived from Furth *et al.* (25) with modifications that improve the plating efficiency (PE) of FA lymphoblasts (22). Briefly, the cell survival was determined by measuring the colony-forming ability of cells immediately after treatment. To determine the mutation frequencies induced at the *HPRT* locus [6-thioguanine-resistant (6-TG^R) mutants], the treated cells were diluted daily to 4 × 10^5 cells per ml for the time required for full phenotypic expression (7–10 days). Then, the cells were plated on feeder cells (GM-1899-A, HPRT$^-$, 10^4 cells per well) in nonselective medium (2–4 cells per well, 4 plates per point, 96 wells per plate) or in selective medium containing 6-thioguanine at 6 μg/ml (2 × 10^4 cells per well, 6 plates per point). To ensure the independent origin of mutants, for some doses the mutagenized population was split into six flasks and separately grown during the expression time. Each flask corresponded to one microwell plate in selective conditions. After 14–16 days of incubation, the colonies were scored. The mutant fraction is a ratio of the PE under selective and nonselective conditions (25).

Mutant Collections. The independent HPRT$^-$ clones, spontaneous or induced in normal or FA lymphoblasts, were isolated and expanded in selective medium to 6 × 10^7 cells per clone. Then the cells were washed twice with PBS and cellular pellets were stored at −70°C until DNA isolation.

Southern Blot Hybridization Analysis. High molecular weight nuclear DNA was isolated by proteinase K digestion and organic solvent extraction (26). Chromosomal DNA (10–15 μg) was digested with *Pst* I in the buffer specified by the supplier (BRL/GIBCO) for 12–16 hr at 37°C. After precipitation with ethanol, the digested DNAs were fractionated on 0.8% agarose gels for 16 hr at 1 V/cm. DNA fragments were then transferred (1.5 M NaCl/0.5 M NaOH) to Hybond-N$^+$ nylon membranes (Amersham) (27). The hybridization protocol essentially followed that described by Church and Gilbert (28) using a probe of 942 base pairs (bp) for human HPRT cDNA (gift from C. T. Caskey, Baylor College of Medicine, Houston) labeled by an oligolabeling reaction (29) with [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham). Autoradiography was performed on Kodak XAR 5 film at −70°C with an intensifying screen for 2–4 days.

RESULTS

Mutation Frequencies at the *HPRT* Locus in Normal and FA Lymphoblasts. To investigate the relative contribution of CLs and MAs in mutagenicity, Me_3Pso was used in combination with monochromatic irradiation at two wavelengths, 365 nm and 405 nm. Treatment of cells with Me_3Pso plus 365-nm irradiation results in a mixture of CLs, furan-side MAs, and pyrone-side MAs, whereas treatment with Me_3Pso plus 405-nm irradiation induces only MAs. Cell survival and the induction of mutations at the *HPRT* locus (6-TG^R) were compared in normal and FA complementation group B lymphoblasts.

As with previous results on FA fibroblasts (18, 19), FA lymphoblasts (HSC-62) are more sensitive to killing than normal cells (AHH-1), when both CIs and MAs or when only MAs are photoinduced (Fig. 1). The dose-modifying factor at 37% survival, estimated from survival curves, equals 1.9 and 2 after Me_3Pso plus 365-nm irradiation and Me_3Pso plus 405-nm irradiation, respectively, when FA cells are compared to normal cells. The mutation frequencies in FA group B cells are significantly lower than in normal cells when either CLs and MAs (Fig. 1*a*) or MAs alone (Fig. 1*b*) are induced. To induce the same number of 6-TG^R mutants, a 3-fold higher dose was required in FA cells as opposed to normal cells after

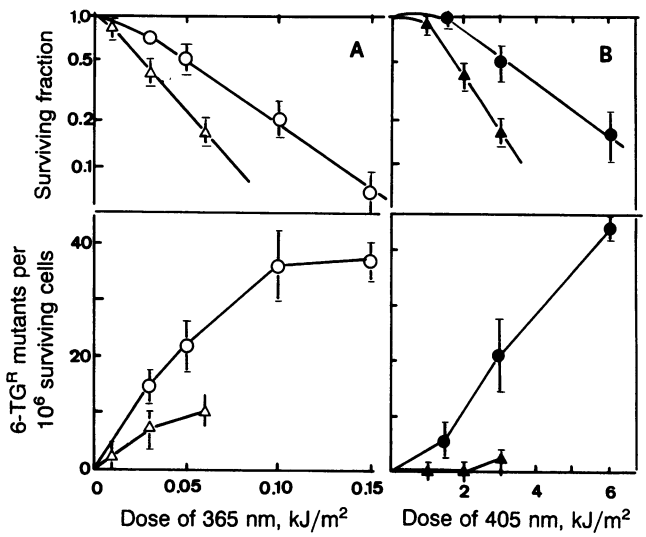

FIG. 1. Clonogenic cell survival (log scale) and induction of 6-TG^R mutants (linear scale) in normal human lymphoblasts (○, ●) and in FA complementation group B lymphoblasts (△, ▲) after Me_3Pso photoaddition. The data are expressed as a function of radiation dose at 365 nm (*A*) or 405 nm (*B*). The mean value of three to five experiments is presented. The PE of normal cells was of 50–70% and the PE of FA cells was 16–35%.

Me_3Pso plus 365-nm irradiation (10 mutants per 10^6 survivors) or plus 405-nm irradiation (2.5 mutants per 10^6 survivors). When the mutation frequency is plotted as a function of survival levels, normal human lymphoblasts are as mutable after Me_3Pso plus 365-nm irradiation as after Me_3Pso plus 405-nm irradiation (Fig. 2). Thus, in normal cells, when the amount of CLs plus MAs or MAs alone is such that the same killing effect is induced, the same number of mutants is generated. In contrast, in FA lymphoblasts the mutation frequency as a function of equitoxic doses is higher after Me_3Pso plus 365-nm than after Me_3Pso plus 405-nm irradiation. In other words, at the same survival level, the mixture

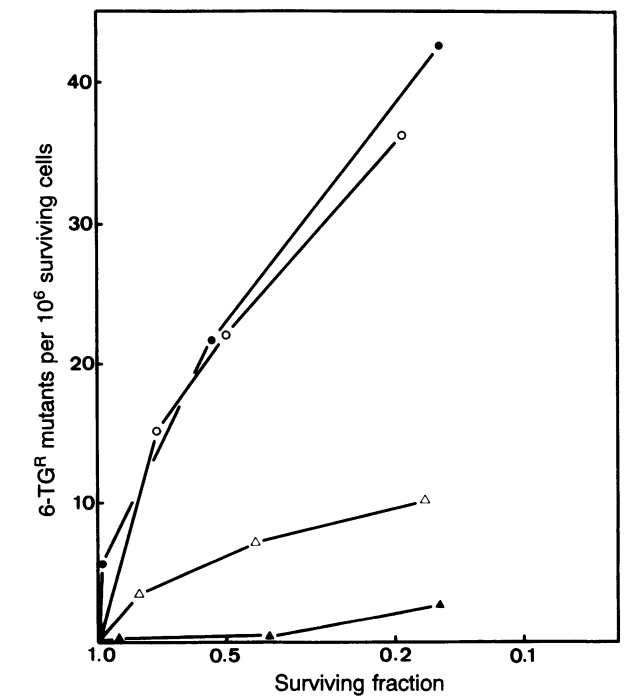

FIG. 2. Induction of 6-TG^R mutants in normal (●, ○) and FA group B (▲, △) lymphoblasts after exposure to Me_3Pso plus either 365-nm radiation (○, △) or 405-nm radiation (●, ▲).

of CLs plus MAs is more effective than MAs alone in inducing mutations at the *HPRT* locus in FA cells. Thus these observations suggest that, although MAs constitute the primary premutagenic lesions in normal cells, CLs may be more the predominant mutagenic lesions in FA cells. Moreover, the mutagenic processing of these lesions (CLs and MAs) appears to be defective in FA cells.

Molecular Analysis of HPRT⁻ Mutants Induced in Normal and FA Lymphoblasts. In view of the differences in mutation frequencies between normal and FA lymphoblasts and to understand the molecular basis underlying this phenomenon, a collection of 130 HPRT⁻ mutants has been analyzed for changes (>100 bp) in the restriction enzyme digestion patterns of the *HPRT* gene. Southern blot hybridization analysis performed on *Pst* I-cleaved DNA allowed the detection of six X chromosome-linked *HPRT* bands representing exons 1–9 plus autosomal bands deriving from pseudogenes (refs. 30 and 31 and Fig. 3). The HPRT⁻ mutants were isolated from normal and FA lymphoblasts untreated or after treatment with Me_3Pso plus 365-nm or 405-nm irradiation. Induced mutants were isolated after treatments leading either to the same number of lesions or to about the same survival level. A representative autoradiogram of a Southern blot hybridization including mutants with some of the detected rearrangements is shown in Fig. 3. The alterations detected in the *Pst* I digestion patterns in the HPRT⁻ mutants are listed in Table 1. In contrast to normal cells where point mutations predominate, in HPRT⁻ mutants derived from untreated and treated FA cells, an increase in rearrangements is observed. The majority of these alterations are small deletions of individual exons or clusters of exons localized close to each other (Fig. 4). It must be pointed out that the changes detected in *Pst* I restriction patterns by blot hybridization analysis with a full-length cDNA do not allow analysis of the individual loss of exons 7, 8, and 9 or exons 5 and 6 (32). In all 33 mutants from FA cells representing alterations, 4

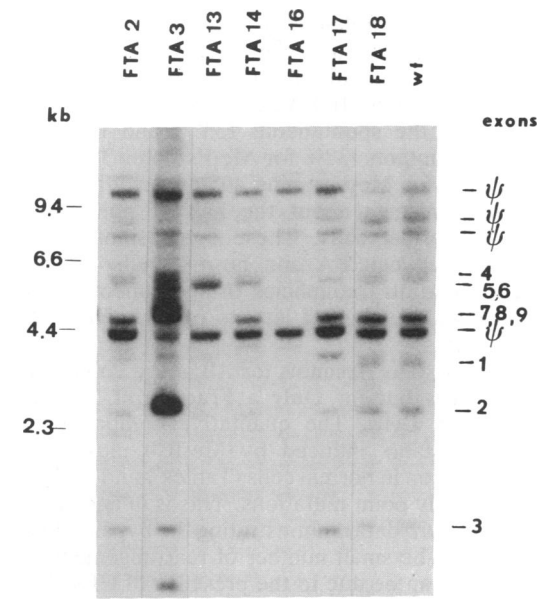

FIG. 3. Southern blot hybridization analysis of the *HPRT* gene in wild-type (wt) and psoralen-induced (Me_3Pso plus 365-nm irradiation) *HPRT*-deficient mutants of FA cells. DNA from each clone was digested with *Pst* I prior to blot hybridization analysis with a human HPRT cDNA probe. Sizes of *Hin*dIII fragments of phage λ DNA used as molecular size markers are to the left in kilobases; fragment exon or pseudogene (ψ) assignments are to the right. In mutants FTA2 and FTA18, no rearrangement was detected; in FTA3, complex rearrangement; in FTA13, exons 7–9 are missing; in FTA14, new band of 4000 bp; in FTA16, exons 1–9 missing; in FTA17, exon 4 missing.

Table 1. Description of the *HPRT* gene alteration detected in mutants derived from normal and FA cells

Source cells	Mutant	Missing sequences (exons)	Novel band, bp
Normal untreated	NSp15	7–9	—
	NSp19	1–6	—
	NSp23	—	6600
	NSp49	—	3500
Normal treated	NTA103	1–4	—
	NTA105	1, 2	—
FA untreated	FSp2, FSp4, FSp19, FSp21	4	—
	FSp3	2	—
	FSp11, FSp14, FSp17	4–6	—
	FSp12	1–6	—
	FSp15, FSp18	7–9	3500
	FSp20	1	—
FA treated	FTA1, FTA8	1–4	—
	FTA3	CR	
	FTA7	—	2000
	FTA11, FTA12	7–9	—
	FTA13, FTA35, FTA38	7–9	—
	FTA14	—	4000
	FTA16	1–9	3800
	FTA17	4	—
	FTA32	2, 3	—
	FTA37, FTA39	3	—
	FTB1, FTB6	7–9	3500
	FTB2, FTB3, FTB5	4–6	—
	FTB4	4	—
	FTB15	3	3600

Within the mutants derived from treated cells, the symbols A correspond to cells treated with Me_3Pso plus 365-nm irradiation (MA plus CL) and B to cells with Me_3Pso plus 405-nm irradiation (MA only). CR, complex rearrangement.

mutants lack a major part of the 5′ end of the gene or show complete loss of the gene and one mutant has a complex rearrangement.

Considerable differences were observed in the spectrum of spontaneous as well as induced mutants in FA cells compared to normal lymphoblasts (Table 2). Changes in restriction patterns are seen in 23% of the spontaneous mutants derived from normal cells as opposed to 67% in FA cells.

After Me_3Pso plus 365-nm irradiation (CLs and MAs), 10% of HPRT⁻ mutants derived from normal cells present a rearrangement whereas 62% of mutants from FA group B cells demonstrate a loss of individual exons or partial or total deletion of the gene.

The treatment by Me_3Pso plus 405-nm irradiation inducing only MAs did not yield changes (0/30 mutants) in the *Pst* I digestion patterns in normal cells, suggesting that these lesions are precursors of point mutations or of deletions too small to be resolved by Southern blot hybridization. However, in FA cells the treatment by Me_3Pso plus 405-nm irradiation yielded detectable rearrangements in 39% of the mutants. These data show that FA group B cells are defective in the production of point mutations that are the major molecular change observed in normal cells.

Mutants exhibiting loss of the entire gene or a major part of the gene [missing exons 1–4 or 1–6 (class 1 mutants)] can be scored separately from those showing the loss of individ-

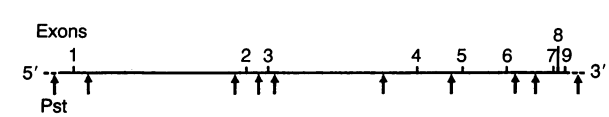

FIG. 4. HRPT gene. Exons 1–9 and *Pst* I cleavage sites are indicated.

Table 2. Proportion of HPRT$^-$ rearranged mutants in normal and FA cells and classification according to the extent of structural alteration of the *HPRT* gene

Condition(s)	Cell line	No. mutants per 10^6 survivors	No. mutants	% unrearranged clones	% rearranged clones	% class 1	% class 2
Untreated	Normal	6.3	17	77 (13/17)	23 (4/17)	6 (1/17)	17 (3/17)
	FA	7.8	18	33 (6/18)	67 (12/18)	5.5 (1/18)	61 (11/18)
Me_3Pso plus 365-nm irradiation (MA and CL)	Normal (0.1 kJ/m^2)	41.0	20	90 (18/20)	10 (2/20)	5 (1/20)	5 (1/20)
	FA (0.03 kJ/m^2)	15.9	24	38 (9/24)	62 (15/24)	12 (3/24)	46 (11/24)
Me_3Pso plus 405-nm irradiation (MA only)	Normal (3 kJ/m^2)	25.0	15	100 (15/15)	0 (0/15)	0 (0/15)	0 (0/15)
	Normal (6 kJ/m^2)	67.0	15	100 (15/15)	0 (0/15)	0 (0/15)	0 (0/15)
	FA (3 kJ/m^2)	12.1	18	61 (11/18)	39 (7/18)	0 (0/18)	39 (7/18)

Class 1 corresponds to 6-TG^R mutants missing a large part of the *HPRT* gene (exons 1–4, 1–6, and 1–9). Class 2 corresponds to 6-TG^R mutants missing individual exons or small clusters of exons. Mutants with new bands are scored in class 2. In untreated normal cells, two such mutants are included which account for the relatively high proportion (17%). For this classification, data are derived from Table 1.

ual exons [or clusters of exons representing relatively small regions (class 2 mutants)]. In normal cells, classes 1 and 2 are more or less equally represented (Table 2). In contrast, in FA cells under all conditions, class 2 is more abundant than class 1. When normal and FA cells are compared, great differences in proportions are seen only for the class 2 mutants missing individual exons, FA demonstrating an increase in this type of mutant.

DISCUSSION

The molecular analysis of spontaneous and induced mutations in cells with repair proficient and deficient genetic backgrounds should contribute to a better understanding of the mechanisms of DNA damage processing. In addition, information on DNA structural features, on cellular processes, including DNA replication and recombination, possibly involved in mutation production and predisposition to cancer can be gathered from the study of the type of mutations in an endogenous gene.

FA Group B Cells Are Defective in an Error-Prone Mechanism. Human lymphoblasts derived from patients with FA are hypomutable when compared to normal cells after photoaddition of a psoralen. A decreased mutation frequency at the *HPRT* locus is observed in FA cells (group B) when the cells are exposed to Me_3Pso plus 365-nm irradiation (CLs and MAs) or to Me_3Pso plus 405-nm irradiation (MAs). This is observed whether the mutation frequencies are expressed as a function of UVA-irradiation or as a function of survival level. FA lymphoblasts from complementation group A are also hypomutable in comparison with two normal cell lines (AHH-1 and TK6) after psoralen treatments which differ in the ratio of CLs to MAs (22). The decreased mutation frequency in FA group A cells observed for the *HPRT* and Na^+,K^+-ATPase loci suggested that these cells were essentially unable to produce base substitutions—i.e., detectable mutations in the Na^+,K^+-ATPase locus. Moreover, FA fibroblasts derived from four patients were found to be hypomutable in comparison to three normal cell lines after treatment with mitomycin C or ethyl methanesulfonate at the *HPRT* or Na^+,K^+-ATPase loci (ref. 33; M. Buchwald, personal communication). Thus the data suggest that FA cells are defective in a mutagenic process operating on specific lesions in normal human cells.

The Vast Majority of Spontaneous and Psoralen-Photoinduced Mutants Is Due to Point Mutations in Normal Cells Whereas Only a Fraction of Mutants in FA Is Due to Such Events. Southern blot hybridization analysis of restricted DNA from 6-TG^R mutants by using a human HPRT cDNA probe allows the detection of gross alterations such as translocation, deletions, and duplications. Alterations of fragments smaller than ≈100 bp do not lead to detectable changes in the restriction digestion pattern and are termed here "point mutations." We show that the majority of the spontaneous and of induced 6-TG^R mutants in normal AHH-1 cells are due to point mutations. The response of these cells is similar to other normal human and hamster cells in that the majority of spontaneous mutants in nonessential endogenous genes are also due to point mutations (31, 34–37). The same is true for mutations induced by a variety of mutagens including UV radiation and alkylating and intercalating agents (38–42). In contrast, treatment with ionizing radiation yields mostly substantial deletions and rearrangements in the endogenous loci (7, 34, 43, 44). After photoaddition of psoralen, 90% of the mutations induced by a mixture of CLs and MAs and 100% of the mutations induced by MAs alone do not cause detectable changes in the restriction patterns of the *HPRT* gene in normal human cells (Table 2). This predominant induction of point mutations after photoaddition of a bifunctional psoralen is in accord with the reported mutation spectra in the *SupF* gene carried by a shuttle vector plasmid mutated in human cells (45) as well as in the *lacI* gene in *Escherichia coli* (46). In FA cells, point mutations are also present among the spontaneous (33%) and the psoralen-photoinduced mutants (38% for Me_3Pso plus 365-nm irradiation and 61% for Me_3Pso plus 405-nm irradiation). Their proportion represents about the half of point mutations observed in normal cells. These conclusions hold when comparisons between FA and normal cells are made at equivalent mutation frequencies of spontaneous mutations and at an equal number of lesions or of lethal events for induced mutants.

Processing of MAs Accounts for All of the Point Mutations in Normal Cells but for Only a Fraction of This Type of Mutants in FA Cells. The qualitative comparison of the changes in mutants induced by Me_3Pso plus 365-nm or 405-nm irradiation in normal cells (Tables 1 and 2) shows that MA induces only point mutations. This is in agreement with *in vivo* and *in vitro* data demonstrating the bypass of psoralen MAs (47–49). The small number of rearrangements seen at 365 nm (10%) can be due to the presence of CLs. From the total number of lesions per genome (24) and from the size of the *HPRT* locus (45 kilobases), it is estimated that at most one lesion per locus is induced under our conditions. Consequently, the presence of two overlapping lesions is unlikely.

In contrast to normal cells, in FA cells, 62% and 39% of structural rearrangements are present in 6-TG^R mutants induced at 365 nm and 405 nm, respectively (Table 2). This implies that, in FA cells, a fraction of the induced MA in both conditions lead to mutagenic events that are not point mutations (i.e., that are deletions and large rearrangements). It is generally assumed that point mutations are predominantly

produced either during the process of mismatch correction of spontaneous errors or when the replicative complex acts on a damaged template. Thus the partial defect in an error-prone pathway postulated above for FA cells may concern the production of point mutations by replicative errors (spontaneous) and by the processing of MA (induced). The differences between the proportion of rearranged mutants at 365 nm (62%) vs. 405 nm (39%) in FA cells could be due to either the higher proportion of furan-side MAs or the combined presence of CLs and MAs.

Possible Mechanism Resulting in Deletions in Human Cells and Nature of the FA Defect. In normal cells, the two classes of deletion mutants, with small (class 2) and large (class 1) deletions, are almost equal. When the same classification is applied to the mutants derived from normal human cells, the same conclusions hold true. For instance, classes 1 and 2 represent 16% and 22% among the 85 mutants analyzed in TK-6 lymphoblasts (31), 22% and 18% of the 23 mutants derived from simian virus 40 normal fibroblasts (10), and 5% and 8% among the 319 mutants from human T lymphocytes (37). It is possible that, in normal cells, the small fraction of unexcised or nonexcised lesions that cannot be bypassed give rise to gaps. Filling in gaps by recombination between parental and daughter strands can result in untargeted or nontargeted point mutations whereas recombination between termini of palindromic structures can lead to deletions.

In contrast to normal cells, a clear-cut predominance of class 2 mutants is observed in all conditions in FA group B cells. It is unlikely that the under-representation of the large deletion class of mutants in FA is due to their inviability since in normal cells this class is present in spontaneous and induced mutants in the same proportion as the small deletion class. Moreover, in Werner syndrome (10), the large deletion class is predominant. The hypomutability associated with a reduction in the frequency of point mutations in FA cells may suggest that these cells are deficient in an error-prone pathway operating during replication by the bypass of unexcised lesions. This deficiency results in the formation of gaps that are not correctly filled by a recombinational process. The increased proportion of small deletion mutants in FA suggests that this deficiency might be strand specific. It is of interest to note that a hamster cell mutant sensitive to killing by a variety of agents has been found to be hypomutable in association with a defect in post-replication repair (50). The involvement of an alteration in post-replication repair in FA cells is suggested also by the effect of caffeine, a known inhibitor of this process in eukaryotic cells (51).

In the course of DNA metabolism related to replication, recombination, and repair, deletions can be generated by different pathways. The study of mutants affecting the distribution of deletions may help to understand how deletions originate. In this respect FA can serve as a model human system.

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