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# Deficiency of gamma-ray excision repair in skin fibroblasts from patients with Fanconi's anemia

(human excision repair/DNA damage)

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**ABSTRACT** **The capacity of preparations of skin fibroblasts from normal individuals and patients with Fanconi's anemia to excise gamma-ray products of the 5,6-dihydroxydihydrothymine type from exogenous DNA was investigated. The excision capacity of whole-cell homogenates of fibroblasts from two of four patients with Fanconi's anemia was substantially below normal. This repair deficiency was further pronounced in nuclear preparations from cells of the same two patients.**

Fanconi's anemia (FA) is one of a group of autosomal recessive diseases in man which is characterized by chromosomal instability and increased frequency for the development of cancer (1, 2). For FA, additional clinical symptoms include pancytopenia with elevated levels of fetal hemoglobin, abnormal "café au lait"-type skin pigmentation, and stunted growth (2). Evidence for a defect in the repair of interstrand DNA crosslinks formed by the bifunctional, DNA-damaging agents mitomycin C and psoralen plus light has been presented, but the deficiency has not yet been characterized in molecular terms (3–6). Some observations suggest a deficiency of FA cells in repairing DNA damage induced by monofunctional agents such as ethylmethanesulfonate (6) and decarbamoyl-mitomycin C (5). It has also been reported that the exonucleolytic degradation step in the excision repair of thymine-photodimers may be deficient in skin fibroblasts from a patient with FA after exposure to relatively high doses of ultraviolet light (7).

A first indication that FA cells may have a deficiency in the repair of DNA damage induced by ionizing radiation comes from the detection of an increased sensitivity of FA cells for the formation of chromosomal aberrations by x-rays (8) while their sensitivity to killing by x-rays and methylmethanesulfonate appears to be normal (9). We have compared the capacity of preparations of skin fibroblasts from normal individuals and patients with FA to excise selectively gamma-ray products of the 5,6-dihydroxydihydrothymine type (t') from an exogenous DNA substrate and have found a significant repair deficiency in two of four FA lines. The difference in repair capacity between normal and FA cells was more pronounced for purified nuclear preparations than for whole-cell preparations (25).

## MATERIALS AND METHODS

**Source and Growth of Cells.** All skin fibroblasts were grown as monolayers in Dulbecco's modified Eagle's medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco) plus antibiotics. Normal cell strains, CRL 1121 and 1141, and Fanconi's anemia (FA) strains CRL 1196 and CCL 122 were obtained from the American Type Culture Collection. FA cell strain GM62 (identical to CCL 122 from the American Type Culture Collection) was obtained from the Institute for Medical Research, Camden, N.J., and FA strains 1802T, 1802B, and 1265T from Meloy Laboratories, through the auspices of Dr. George Todaro. The cultures were free of mycoplasma as determined by the method of Levine (10).

**Preparation of Whole-Cell and Nuclear Sonicates.** For the whole-cell sonicates, skin fibroblasts were harvested by trypsinization within 3 days of subculturing. The cells were collected by low-speed centrifugation, followed by two washes in an excess of cold, sterile 0.15 M NaCl–0.01 M potassium phosphate, pH 7.4, or Hanks' balanced salt solution. Cell yield was determined from the cell count in the second wash, using a hemacytometer. The cells were resuspended at $5 \times 10^7$ cells per ml in 0.09 M NaCl–0.01 M potassium phosphate, pH 7.6–1 mM dithiothreitol and sonicated with a Biosonik instrument at 0° by three bursts of 15 sec at a setting of 20, with mixing between each burst. Essentially all cells were disrupted, as checked by microscopic examination.

For preparation of nuclear sonicates, cells from the second wash above were resuspended at 3 to $5 \times 10^6$ cells per ml in 80 mM NaCl–0.01 M potassium phosphate, pH 7.4–0.3% Triton X-100, allowed to swell for 15 min at 0°, then disrupted by 15 strokes with the tight pestle in a Dounce homogenizer. Nuclei were essentially free of cytoplasmic contamination, as determined by inspection under a Zeiss phase contrast microscope. Nuclei were washed twice in 0.25 M sucrose–0.01 M potassium phosphate, pH 7.1–3 mM $CaCl_2$–1 mM dithiothreitol. Recovery was determined by counting stained nuclei from an aliquot of the second suspension for washing, and varied from 50 to 100%. Washed nuclei were resuspended at $5 \times 10^7$/ml in NaCl–phosphate buffer and sonicated, as described for whole cells.

**Preparation of DNA Substrates.** Bacteriophage lambda was grown and labeled with *methyl*-[$^3$H]thymidine (New England Nuclear, specific activity, 55 Ci/mmol) according to the method of McMacken *et al.* (11); bacteriophage T7 was grown in *Escherichia coli* B thy$^-$ (from Dr. R. Boyce) in M9 medium supplemented with 1.5–2.0 μg/ml of unlabeled thymidine and 10 μCi/ml of radioactive thymidine. The bacteriophages were purified as described by Yamamoto *et al.* (12), and the DNA was extracted with sodium dodecyl sulfate–phenol. Lambda DNA was passed through a hydroxyapatite column prior to use, but this step was not used with T7 DNA, since no significant purification of the DNA was achieved as judged by the $A_{260}/A_{280}$ ratio or the profile on alkaline sucrose gradients. The specific activity for lambda DNA ranged from 0.6 to $3 \times 10^7$ dpm per $A_{260}$ unit and was approximately $5 \times 10^7$ per $A_{260}$ unit for T7 DNA. ($A_{260}$ unit is that amount of material that gives an absorbance of 1 when dissolved in 1 ml of solvent with a light path of 1 cm.)

Immediately prior to use as substrate, either lambda or T7 DNA, in 5 mM potassium phosphate, pH 7.4, was irradiated under aerobic, nonprotective conditions with gamma rays from a cesium-137 source at a dose yielding from 0.05 to 0.25% of

Abbreviations: FA, Fanconi's anemia; t', gamma-ray products of the 5,6-dihydroxydihydrothymine type; Dulbecco's MEM, Dulbecco's modified Eagle's medium.

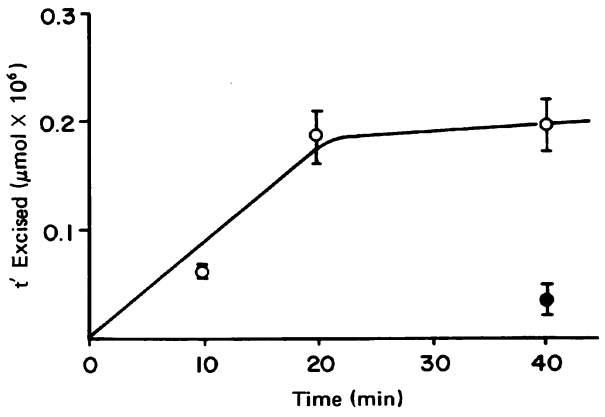


FIG. 1. Time-course of excision of gamma-ray-induced products of the 5,6-dihydroxydihydrothymine type (t') by nuclear sonicates of normal and FA skin fibroblasts. Sonicates of $5 \times 10^6$ nuclei were incubated at 37° with bacteriophage lambda DNA containing $0.6 \times 10^{-6}$ μmol of t' and excision was determined as described in *Materials and Methods*. (○) Normal CRL 1121; (●) FA-CCL 122.

products of the 5,6-dihydroxydihydrothymine type (t'), as determined by the alkali-acid degradation assay (13) (3–10 krads, depending on the radiation-chemical purity of each DNA preparation at a dose rate of 2 krads/min; the oxygen supply in the radiolysis solutions is sufficient to maintain aerobic conditions). The tritiated water produced by the irradiation (14) was removed by evaporation, and the DNA was further purified on a Sephadex G-50 column in 1 or 5 mM potassium phosphate, pH 7.4. Only the material that eluted in the void volume was used for the excision experiments.

**Excision of Gamma-Ray Products of the 5,6-Dihydroxydihydrothymine Type (t').** The following additions were made to whole-cell and nuclear sonicates: 15 mg/ml of bovine serum albumin (Armour), 1 mM dithiothreitol, 2 mM phospho*enol*-pyruvate potassium salt, 0.4 mM ATP, 0.2 mM each of the four deoxynucleoside triphosphates, and 140 units/ml of lyophilized pyruvate kinase (Worthington Biochemicals). Each sample contained 100 μl of the above plus 25 μl of irradiated phage DNA in phosphate buffer. The molar amount of t' per sample was calculated from the radioactivity added, the specific activity, and the percentage of thymine residues modified to t'. Incubations were at 37° for 0–40 min and were terminated by the addition of cold 7% trichloroacetic acid. The acid-precipitable material collected by centrifugation was assayed for t' as described by Hariharan and Cerutti (13). As shown in Fig. 1, the kinetics of excision of t' is nonlinear and is essentially complete after 30–40 min of incubation. Therefore, 40 min was selected as the time of incubation in all of our experiments measuring t' excision as a function of initial substrate concentration.

**Radiation-Induced DNA Degradation in Normal and FA Skin Fibroblasts.** Rapidly growing monolayer cultures of normal (CRL 1121) and FA skin fibroblasts (1802B and GM 62) were grown in the presence of *methyl*-[$^3$H]thymidine (1 μCi/ml in Dulbecco's MEM supplemented with $10^{-6}$ M cold thymidine) for 16 hr. The cellular pool was depleted of radioactivity by growth for 1 hr in Dulbecco's MEM and 2 hr in Dulbecco's MEM containing $10^{-6}$ M thymidine. The culture flasks were filled with cold Hanks' balanced salt solution at 0° and irradiated with 150 krads of $^{137}$Cs gamma rays at a dose rate of 9 krads/min. At this high dose, oxygen depletion may have occurred, generating hypoxic irradiation conditions. The cells were trypsinized immediately (at 0°) after irradiation or incubated for 10 or 30 min at 37° in a small amount of growth medium. The release of acid-soluble radioactivity after irradiation was determined.

## RESULTS

### Gamma-ray-induced DNA degradation in normal and FA skin fibroblasts

Monolayer cultures of normal skin fibroblasts (CRL 1121) and FA skin fibroblasts (1802B and GM 62) were first labeled in DNA with *methyl*-[$^3$H]thymidine and chased with unlabeled thymidine to remove radioactive label from the pool. The cultures were then irradiated with 150 krads at 0° and incubated at 37° in fresh medium for 0–30 min. The acid-soluble label in the culture medium and the cell fractions was determined. No significant difference was detected in the extent of DNA degradation between normal and FA cells, the final level being only 0.2–0.3%.

### Excision of gamma-ray products of the 5,6-dihydroxydihydrothymine type (t') by whole-cell sonicates of normal and FA skin fibroblasts

The capacity of whole-cell sonicates of rapidly dividing, normal skin fibroblasts CRL 1121 and CRL 1141 and FA skin fibroblasts CCL 122 (the notation used by American Type Culture Collection for GM 62), CRL 1196, 1802B, and 1265T to remove selectively t' from gamma-irradiated bacteriophage DNA was determined. The amount of t' removed from acid-precipitable DNA by sonicates of $5 \times 10^6$ cells in 40-min incubation at 37° was measured as a function of the amount of t' initially present in the reaction mixture. Maximal excision was achieved within approximately 20–30 min for preparations from normal skin fibroblasts (see Fig. 1). The different initial levels of t' were obtained by adding different amounts of exogenous DNA substrate with a constant t' content. As shown in Fig. 2A and B, the amount of t' excised increases linearly with increasing initial levels of t' for the normal fibroblasts CRL 1121 and CRL 1141 and for the FA strains CRL 1196 and 1265T. The slopes of the curves are a measure of the excision capacity and are similar for these four strains. From the data shown in Fig. 2C and D, it is evident that preparations from FA strains CCL 122 (or GM62) and 1802B possess significantly lower t' excision capacities than the normal counterparts. The curves for the FA strains, in contrast, are nonlinear, having slopes that decrease towards higher initial t' concentrations. At $3.5 \times 10^{-6}$ μmol of initial t' concentration, CCL 122 (or GM62) preparations had only approximately 50% of the excision capacity of normal skin fibroblasts, and 1802B, approximately 44%. It is concluded that whole-cell sonicates of the FA strains CCL 122 (or GM62) and 1802B are partially deficient for the excision of gamma-ray products of the 5,6-dihydroxydihydrothymine type, especially at higher initial concentrations of damage. Nonspecific degradation of the exogenous DNA substrates determined as radioactivity rendered acid-soluble by the cell sonicates was comparable for all cell lines and varied from 2 to 3% for different experiments. It follows that the excision process measured in our experiments is selective.

### Excision of gamma-ray products of the 5,6-dihydroxydihydrothymine type (t') by nuclear sonicates of normal and FA skin fibroblasts

The capacity of sonicates of hypotonic Triton X-100 nuclei to excise t' from exogenous gamma-irradiated bacteriophage DNA was determined for two normal and four FA skin fibroblast lines. The experimental conditions and the presentation of the data are the same as described above, except that $5 \times 10^6$ nuclear equivalents per reaction mixture were used in place of

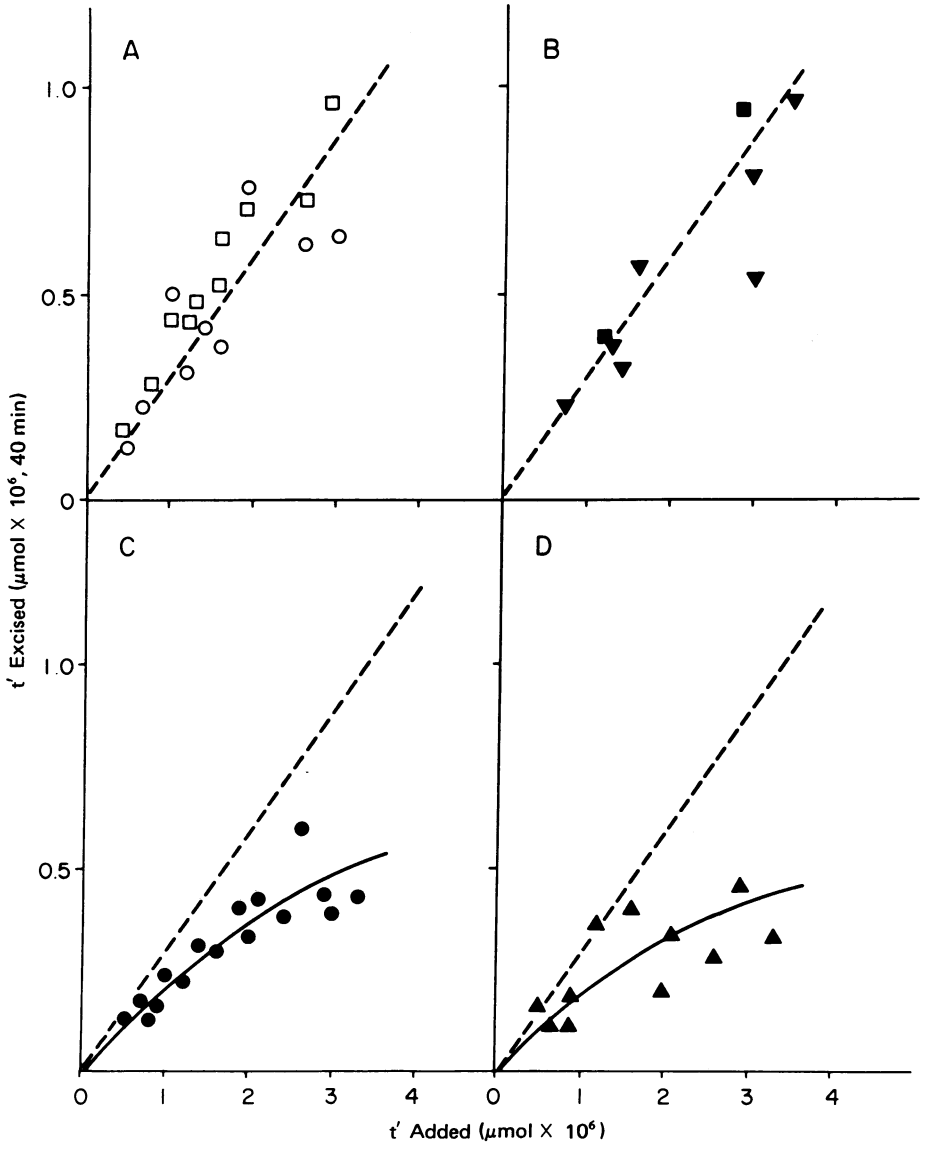


FIG. 2. Excision of products of the 5,6-dihydroxydihydrothymine type (t') by whole-cell sonicates of normal and FA skin fibroblasts as a function of the initial t' concentration. Sonicates of $5 \times 10^6$ cells were incubated for 0 and 40 min at 37° with gamma-irradiated bacteriophage DNA. Disappearance of t' from acid-precipitable material was determined as described under *Materials and Methods*. The amount of t' excised was plotted as a function of the initial t' concentration. The dashed line in all four quadrants represents the curve obtained for preparations from the normal fibroblasts. □, Normal CRL 1141; ○, normal CRL 1121; ■, FA 1265T; ▼, FA CRL 1196; ●, FA CCL 122 or GM 62; ▲, FA 1802B.

whole-cell sonicates. The t' content of the reaction mixtures varied from 0.5 to $4 \times 10^{-6}$ μmol. The completeness of t' removal varied as a function of the initial t' concentration between 25 and 35%. Acid solubilization of the DNA substrate was less than 1% for nuclear preparations from normal and FA cells. Fig. 3A contains data from several experiments with nuclear preparations from the normal strains CRL 1121 and 1141 and the FA strains CRL 1196 and 1265T. While the curves from individual experiments with normal cells were linear, there was variation among experiments in the slopes as indicated by the boundaries. The t'-excision capacity of nuclear sonicates of the FA strains CCL 122 (or GM62) and 1802B is compared with that of the normal lines in Fig. 3B. It is evident that the t'-excision capacity is substantially below normal for CCL 122 (or GM62) and virtually zero for 1802B.

As shown in Fig. 4, significantly lower excision capacities relative to normal (CRL 1121) were also determined for nuclear preparations of the FA strains CCL 122 (or GM62) and 1802B at very low t' concentrations of $0.03–0.1 \times 10^{-6}$ μmol where the completeness of t' removal was between 50 and 60% for normal cells.

## DISCUSSION

Our results with whole-cell sonicates demonstrate a partial deficiency for the excision of gamma-ray-induced thymine damage from exogenous DNA for two (CCL 122 or GM62 and 1802B) of four FA skin fibroblast lines. This repair deficiency was even more pronounced for nuclear sonicates. Nonspecific nucleases in whole-cell sonicates may partially mask the nuclear deficiency and be responsible for the difference in excision capacity *in vitro* of whole-cell and nuclear preparations. It should be noted, however, that random degradation of the DNA substrate was only slightly increased in our experiments with whole-cell sonicates relative to nuclear sonicates for both FA and normal cells. The unexpected results that homogenates of xeroderma pigmentosum and normal cells were equally capable

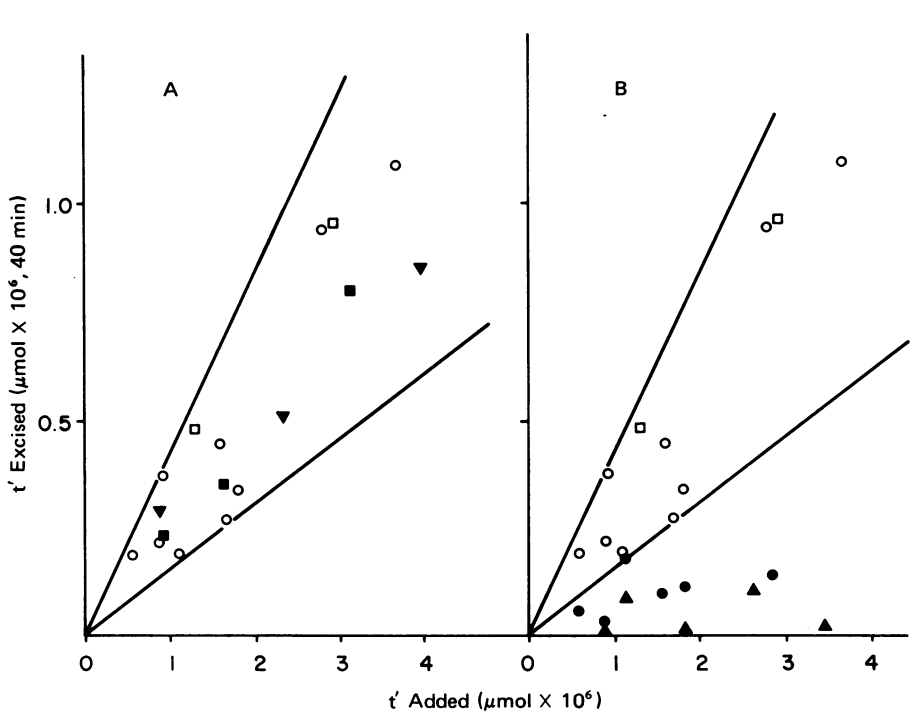


FIG. 3. Excision of products of the 5,6-dihydroxydihydrothymine type (t') by nuclear sonicates of normal and FA skin fibroblasts as a function of the initial t' concentration. Experimental conditions were as described in the legend to Fig. 2, except that the whole-cell sonicates were replaced by nuclear sonicates (see *Materials and Methods*). The boundaries indicate the variation in the data obtained in different experiments with preparations from the normal skin fibroblast strains. □, Normal CRL 1141; ○, normal CRL 1121; ■, FA 1265T; ▼, FA CRL 1196; ●, FA CCL 122 or GM62; ▲, FA 1802B.

of incising ultraviolet-irradiated exogenous DNA (15) and of removing thymine photodimers (K. Mortelmans, E. C. Friedberg, and J. Cleaver, personal communication) may be due to a similar effect.

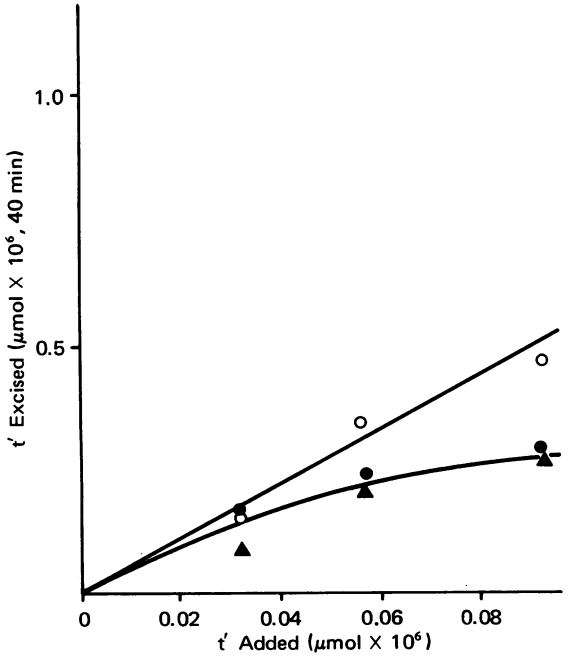


FIG. 4. Excision of products of the 5,6-dihydroxydihydrothymine type (t') by nuclear sonicates of normal and FA skin fibroblasts at low initial t' concentrations. Experimental conditions were as described in the legend to Fig. 3 except that the initial t' concentration was in the range of 0.03–0.1 X $10^{-6}$ μmol. ○, Normal CRL 1121; ●, FA CCL 122; ▲, FA 1802B.

Hypotonic Triton X-100 nuclei prepared by our procedure are essentially free of cytoplasmic contamination, but have lost part of their content. Differential loss of material during preparation of FA relative to normal nuclei could also explain our data with nuclear sonicates. It should be stressed, however, that significant excision deficiencies were detectable with whole-cell sonicates and nuclear sonicates of the same two FA strains. No significant differences were detectable in the protein content of nuclear preparations from normal and FA fibroblasts as determined according to Lowry *et al.* (16).

Several possible artifacts can be ruled out as the basis for the observed differences in t'-repair capacity between FA and normal preparations. The data presented in this report were obtained over an extended period and different shipments of serum were used for the growth media, excluding a "serum effect" in our results. Comparable results were obtained with DNA preparations from *E. coli* phage lambda and T7 and *Pseudomonas* phage PM-2. In most cases, experiments with normal and FA preparations were performed at the same time with the same DNA substrate. Differences in purity and physicochemical properties of the DNA substrates can, therefore, be eliminated as the basis for our observations. The differences in excision capacity do not appear to be a function of the age of the cells in culture (17). Preparations of normal fibroblasts, CRL 1121, showed a similar excision capacity at passages 11 through 23, as did CRL 1141 for passages 14 through 26. For GM62, the same curve was obtained for cells at passage 25 as at passage 30, the highest used experimentally. At this point, cells still looked normal and were doubling at the usual rate. For 1802B, passage 9 was essentially the same as passage 29.

The preparations of the four FA lines that were investigated possessed different levels of t'-excision capacity, suggesting that genetic complementation groups may exist in FA. Mixing experiments with nuclear preparations of the two deficient FA

lines CCL 122 (or GM62) and 1802B which might shed light on this question have been inconclusive.

FA skin fibroblasts seem to possess normal resistance to killing by ionizing radiation [Finkelberg *et al.* (9); J. Little, personal communication]. It should be kept in mind that classes of lesions such as t′ (18), which might have low killing efficiency, may be of particular importance for mutagenesis and, possibly, malignant transformation.

Assuming the existence of a t′-excision pathway in normal human skin fibroblasts similar to that in *E. coli*, the removal of t′ is expected to occur in at least two steps involving an endo- and an exonuclease. Bacchetti and Benne (19), Van Lancker and Tomura (20), and Verly *et al.* (21) have described endonuclease activities that could be involved in the incision step, and an exonuclease with properties similar to that identified in human placenta by Doniger and Grossman (22) could be responsible for the second step. The endonuclease described from calf thymus by Bacchetti and Benne (19) is a particularly attractive candidate since it recognizes unidentified, minor lesions rather than cyclobutane-type thymine photodimers in ultraviolet-irradiated DNA. We have found that t′-type products are formed not only by gamma rays, but also by ultraviolet light *in situ* in mammalian cells (Hariharan and Cerutti, unpublished results), and it may be this class of products that is recognized by this enzyme. Since apyrimidinic and, probably, apurinic sites are present in gamma-irradiated DNA (23), the participation of an apurinic-site enzyme in gamma-ray repair has to be considered. Teebor and Duker (24) found normal levels of apurinic-site endonuclease(s) in cell homogenates of FA line CRL 1196 and progeria skin fibroblasts. Our results so far do not allow any conclusions regarding the steps responsible for the t′-excision deficiency in FA.

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