DNA single-strand breaks during repair of UV damage in human fibroblasts and abnormalities of repair in xeroderma pigmentosum

(alkaline elution of DNA/DNA crosslinking/x-ray sensitivity/excision repair)

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ABSTRACT   The method of DNA alkaline elution was applied to a study of the formation and resealing of DNA single-strand breaks after irradiation of human fibroblasts with ultraviolet light (UV). The general features of the results were consistent with current concepts of DNA excision repair, in that breaks appeared rapidly after UV, and resealed slowly in normal fibroblasts, whereas breaks did not appear in those cells of patients with xeroderma pigmentosum (XP) that are known to have defects in DNA repair synthesis. The appearance of breaks required a short post-UV incubation, consistent with the expected action of an endonuclease. Cells of the variant form of XP characterized by normal DNA repair synthesis exhibited normal production of breaks after UV, but were slower than normal cells in resealing these breaks. This difference was enhanced by caffeine. A model is proposed to relate this finding with a previously described defect in post-replication repair in these XP variant cells. DNA crosslinking appears to cause an underestimate in the measurement of DNA breakage after UV.

The major photochemical damage produced in DNA by ultraviolet light (UV) consists of pyrimidine dimers. The efficiency of dimer production by UV appears to be the same in all mammalian cells (1). Major differences, however, exist in the DNA repair events that follow UV damage [recently reviewed by Cleaver (2)]. Of particular interest are the defects in DNA repair that have been noted in patients with xeroderma pigmentosum, a rare inherited disease characterized by extreme photosensitivity and the formation of multiple skin cancers [recently reviewed by Robbins et al. (3)].

Pyrimidine dimers are thought to be removed from the DNA of mammalian cells by the process of excision repair (2). This process begins with a single-strand scission near the site of the dimer. The dimer is then removed, along with a substantial segment of the adjacent DNA. The resulting gap in the DNA strand is filled by synthesis of new DNA which is finally joined to the end of the preexisting strand (4, 5). The occurrence of DNA repair synthesis, the filling of gaps, and the joining to preexisting strands have all been demonstrated in mammalian cells (2).

The excision model predicts the transient appearance of single-strand breaks in the DNA (2). Such breaks have been difficult to detect in eukaryotes, however, because their frequency is so low that the resulting single-strand segments are too long to be readily measured by standard techniques. The technique of DNA alkaline elution, recently developed in our laboratory, has helped us to approach this problem (6).

We have examined several lines of xeroderma pigmento-

sum (XP) cells. Most XP cell lines are known to be deficient in dimer excision and DNA repair synthesis (3). A few patients with the clinical symptoms of XP, however, have been found whose cells have normal capacities for dimer excision and DNA repair synthesis (7-10). The search for a defect in DNA repair in these cells has been a recent challenge (11, 12). These cells were of particular interest to us because it seemed possible that their defect might be in strand rejoining.

MATERIALS AND METHODS

Cell Lines and Cell Labeling. Human fibroblast cell lines were obtained from the American Type Culture Collection, Rockville, Md. The cells were grown in Dulbecco's medium with 10% fetal calf serum plus penicillin and streptomycin at 37° under 5% CO_2. Six days prior to the experiments, 2 X 10^5 fibroblasts were plated on 160 mm^2 petri dishes with [2-^{14}C]thymidine (0.02 μCi/ml, 57 mCi/mmol); 3 days later, the medium was replaced with nonradioactive medium. On the day of experiments, the cells were in a confluent monolayer.

L1210 mouse leukemia cells were grown in suspension culture in RPMI 1630 medium supplemented with 20% fetal calf serum plus penicillin and streptomycin (13). Exponentially growing L1210 cells were labeled with [^{3}H]thymidine (0.1 μCi/ml, 10^{-6} M thymidine) for 20 hr.

Irradiation. Confluent fibroblast monolayers were irradiated with a GE 254 nm germicidial lamp calibrated with a short wave UV intensity meter (J-225 UV meter, UV Products, Inc., Calif.). Prior to irradiation, the cells were washed with warm phosphate-buffered saline (PBS). The PBS was decanted and the cells were irradiated at 37°. Fresh nonradioactive medium, containing 10% serum and buffered with 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer, was quickly added. The cells were incubated at 37° for various times ("repair" time). At the end of this incubation, the cells were washed with warm PBS for 40 sec, incubated with 0.25% trypsin at 37° for 60 sec, and suspended in cold PBS. X-irradiation of fibroblasts was carried out on cells suspended in PBS at 0°. The dose rate was 140 rad/min at 200 kV.

Alkaline Elution. The procedure used is a modification of that described by Kohn et al. (6). Cells were diluted in cold PBS and filtered onto a 25 mm diameter, 2 μm pore-size polyvinylchloride filter (Millipore Corp., Bedford, Mass.) and washed several times with cold PBS. The cells were then lysed at room temperature with 5 ml of 2 M NaCl, 0.02 M Na_3EDTA, and 0.2% Sarkosyl (pH 10.2), which was allowed to flow slowly through the filter without suction. The filters were then washed with 3 ml of 0.02 M Na_3EDTA (pH 10.2) and eluted in the dark with 0.10 M tetrapropylammonium
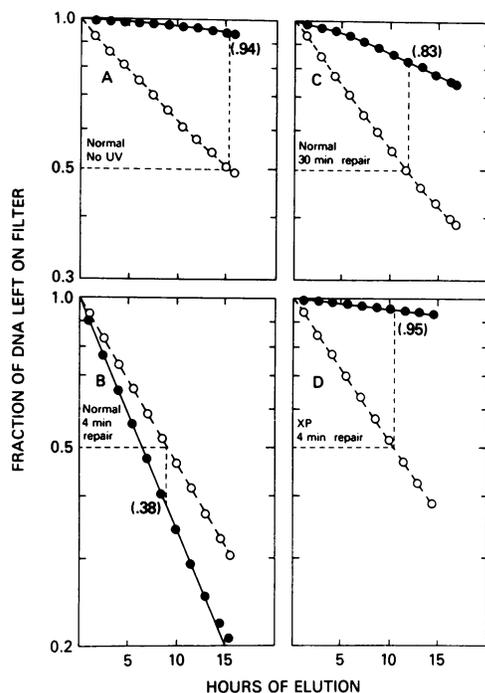
FIG. 1. Response of human fibroblasts to 15 erg/mm² of 254 nm radiation shown by alkaline elution of DNA. Confluent monolayers of normal (CRL1119) or xeroderma pigmentosum (XP12BE) fibroblasts, prelabeled with [2-¹⁴C]thymidine, were exposed to UV and then incubated for 4 min or 30 min ("repair"). An additional 1 min of incubation was required to suspend the cells by trypsinization. Further repair was stopped by cooling to 0°. Prior to filtration for the elution assays, 2 × 10⁵ ¹⁴C-labeled fibroblasts (●———●) were mixed with 4 × 10⁵ [³H]thymidine-labeled L1210 cells which had been exposed only to 150 rad of x-ray and which served as internal standard in the assays (O- - - -O). The fraction of the fibroblast DNA retained on the filter when 50% of the standard L1210 cell DNA had eluted is called "relative retention," and is indicated in parentheses.

hydroxide–0.02 M H₄EDTA (pH 12.2) at a pump speed of 0.04 ml/min. Fractions were collected at 90-min intervals for 15 hr. The fractions were mixed with 10 ml of Aquasol (New England Nuclear) containing 0.3% acetic acid, and counted in a liquid scintillation counter. Radioactivity remaining on the filters was determined as previously described (6).

In order to improve quantitation, an internal standard was used in most of the assays. The internal standard consisted of [³H]thymidine-labeled L1210 cells which had received 150 rad of x-ray in medium at 0°. Approximately 4 × 10⁵ L1210 cells were mixed with 2 × 10⁵ fibroblasts for each assay. The effect on the fibroblast DNA was quantitated as the fraction of the fibroblast DNA that was retained on the filter when 50% of the L1210 cell DNA had eluted. This quantity will be termed "relative retention" (Fig. 1).

Mixed Cell Culture. In several experiments, mixed cultures were prepared in order to make critical comparisons between two fibroblast lines. Cells for these experiments were set up on day 0 in plastic bottles (Falcon) and labeled with either 0.02 μCi/ml of [¹⁴C]thymidine or 0.06 to 0.12 μCi/ml of [³H]thymidine, both at 3.5 × 10⁻⁷ M thymidine. On day 3, the medium was replaced with nonradioactive medium. On day 4, the cells were trypsinized and suspended. The respective ³H-labeled and ¹⁴C-labeled cells were mixed and put on 160 mm² petri dishes at 4 × 10⁵ cells per plate. On day 5, the plates were irradiated and 2 × 10⁵ cells were analyzed by alkaline elution.
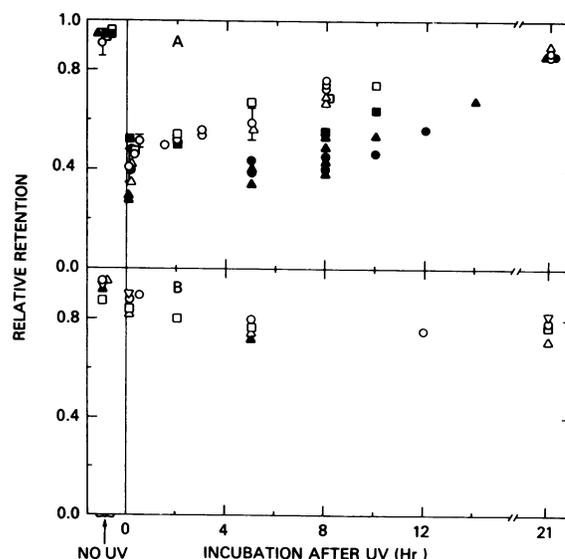

FIG. 2. Response of various human fibroblast lines to 100 erg/mm² of 254 nm radiation. "Relative retention" is defined in *Materials and Methods* and the legend of Fig. 1. (A) Normal lines: O, CRL1119; △, CRL1229; □, CRL1220. XP variant lines: ●, XP4BE; ▲, XP13BE; ■, XP7TA. (B) XP lines: O, XP12BE; △, XP1BE; □, XP5BE; ▽, XP11BE; ▲, XP2BE. (When a point represents four or more determinations, standard deviation is shown; otherwise the individual values are shown.)

RESULTS

X-ray-Induced Breaks. In order to calibrate the elution assay, cells were assayed after various doses of x-ray. Elution (measured as "relative retention"—see *Materials and Methods*) was found to approximate a first-order relation with respect to x-ray dose (i.e., a plot of logarithm of relative retention versus x-ray dose was nearly linear up to 300 rad). The dose at which relative retention was reduced to 37% of control was 165 rad. Assuming an efficiency for DNA single-strand breakage of 2.3 × 10⁻¹² per dalton per rad (14), this dose would correspond to 1 break per 2.6 × 10⁹ daltons (8 × 10⁶ nucleotides). There was no significant difference between the x-ray sensitivity of DNA in the different cell lines.

Breaks As a Result of Excision Repair. A typical elution experiment showing the effect of UV is shown in Fig. 1. The DNA elution from the reference cells (L1210 cells exposed to 150 rad but not UV) is shown by the open circles. In unirradiated normal fibroblasts (Fig. 1A), 94% of the DNA was retained when 50% of the L1210 cell DNA had eluted (i.e., the relative retention was 0.94). In normal fibroblasts that received 15 erg/mm² of UV followed by 5 min of incubation at 37° (Fig. 1B), the elution rate was markedly increased; the relative retention was 0.38. Further incubation of these cells showed recovery, with relative retention increasing to 0.83 after 30 min (Fig. 1C). In XP cells, the UV-induced increase in elution did not occur (Fig. 1D).

When cells were incubated for various times after 100 ergs/mm² of UV, maximum DNA elution occurred within 5 min (Fig. 2A). In three normal cell lines tested (open symbols), elution then returned towards normal with a half-recovery time of approximately 8 hr. In three XP variant lines tested, the initial increase in DNA elution was similar to that in the normal lines, but the recovery phase was significantly delayed, and the half-recovery time was over 12 hr. Both the normal and XP variants recovered to within the range of
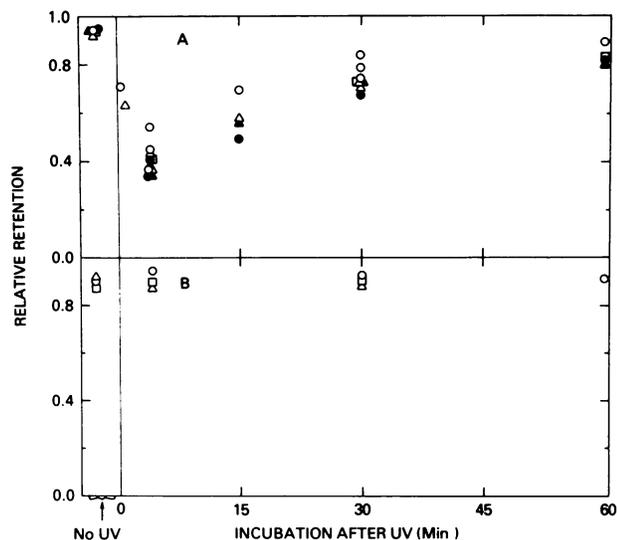
Biochemistry: Fornace *et al.*

Proc. Nat. Acad. Sci. USA 73 (1976)   41

FIG. 3. Response of various human fibroblast lines to 15 erg/mm^2 of 254 nm radiation. Symbols are the same as in Fig. 3. (A) Normal and XP variant lines. (B) XP lines.

FIG. 4. Difference between normal (N) and XP variant (XPV) cells after exposure of mixed cultures to 100 erg/mm^2. Cells were labeled with either [^{3}H]- (O- - - -O) or [^{14}C]- (●—●) thymidine. Oppositely labeled pairs of normal (CRL1229) and XP variant (XP13BE) cultures were mixed and plated. On the following day, the mixed cultures, which had grown to a confluent monolayer, were UV-irradiated and incubated for various repair times. The cells were then analyzed by alkaline elution, and the results were plotted as fraction of DNA left on the filter versus time of elution.

unirradiated cells by 21 hr. In XP cells of complementation groups A, B, C, and D (3) (Fig. 3B), only a slight effect was seen at 5 min incubation after 100 erg/mm^2. A further small increase in elution was seen after 5 hr of incubation, but there was no recovery, even after 12 or 21 hr.

With 15 ergs/mm^2, a similar damage and recovery pattern was seen as with the larger UV dose, except that the recovery time was much shorter (Fig. 3A, note expanded time scale). DNA elution was maximal within 5 min after UV, and returned nearly to normal by 60 min. There was little or no difference between normal (open symbols) and XP variant (closed symbols) cells at this dose. In XP cells of complementation groups A, C, and D, this low UV dose had no detectable effect (Fig. 3B).

In Figs. 2A and 3A, it is seen that the effect on DNA elution is largest within 5 min of incubation after UV. If the effect on DNA is due to repair-endonuclease activity, a period of incubation should be required for the appearance of this change. Immediately after UV, there should be no DNA breaks and hence no change in elution. It was not possible to

Table 1. Effect of UV on x-ray sensitivity of cell DNA as measured by alkaline elution

UV dose (erg/ mm^2)	Incuba- tion after UV (min)	X-ray dose		
		0	110 rad	440 rad
A	0		0.922	0.475	0.132
B	100	0	0.894	0.535	0.215
C	400	0	0.838	0.613	0.359
D	100	5	0.354	(Trypsinized prior to UV)
E	100	5	0.405	(Trypsinized after UV)

Normal cells (CRL1220) were treated with trypsin for 1 min and washed with PBS at 37° while still remaining attached to the plate. The cells were exposed to UV at 37° for 20 sec and immediately suspended in PBS at 0°. The cells were then exposed to x-ray at 0°. The values in the table represent "relative retention," determined as described in Fig. 1. The sample in row D was incubated in medium at 37° for 5 min after UV. The sample in row E was exposed to trypsin beginning 4 min after UV; total time at 37° after UV was 5 min.
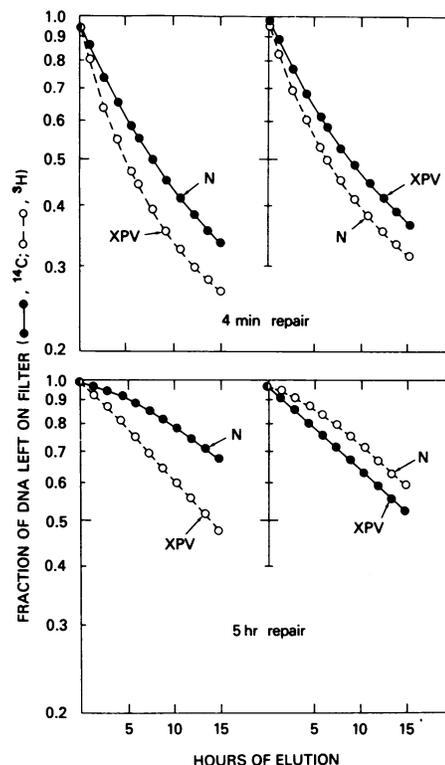
test this in the experiments of Figs. 2 and 3, because of the incubation during trypsin treatment after UV. Experiments were therefore carried out in which trypsin treatment preceded UV. A comparison between the two procedures showed that similar results were obtained for a given UV dose and repair-incubation time, regardless of whether trypsinization preceded or followed UV (Table 1, entries D and E). Trypsinization prior to UV allowed the time between the start of UV exposure (at 37°) and chilling to 0° to be reduced to about 30 sec. When this was done, UV produced only slight increases in elution. (Table 1, column under 0 x-ray dose). Thus incubation is required for the appearance of high DNA elution after UV.

Normal Versus XP Variants. The difference between normal and XP variant cells was confirmed in mixed cell culture experiments, in which the two cell types were labeled differently, and then grown and irradiated on the same plate (Fig. 4). Dual experiments were carried out with reverse labeling. When examined 5 or 8 hr after UV, DNA from XP variant cells consistently eluted faster than DNA from normal cells. This was true regardless of the order of isotopes, although ^{3}H labeling caused slightly faster elution than ^{14}C labeling. The two cell types showed no significant differences when examined 5 min after UV or when not irradiated. These results were obtained with the XP variant lines XP13BE and XP4B3 in mixed cultures with the normal line CRL1229. Similar experiments showed no difference between the two normal lines CRL1229 and CRL1119.

Table 2. Effect of caffeine on UV repair of DNA in XP variant cells

Additional treatment	No UV		UV*	
	Normal‡	XPV§	Normal‡	XPV§
None	0.91	0.90	0.82	0.79
Caffeine†	0.89	0.87	0.80	0.58

Numbers are relative retention of DNA on the filter, determined as in Fig. 1.
* 100 erg/mm² followed by incubation for 15 hr.
† 1.6 mM caffeine for 15 hr. In UV-irradiated cells, caffeine was added immediately after UV.
‡ CRL1220.
§ XP variant line XP7TA.

Crosslinking Effect.

Ultraviolet light is known to reduce the extractability of DNA from cells, possibly due to formation of crosslinks between DNA and protein (15–19). Crosslinking could reduce the elution of DNA containing single-strand breaks. If UV caused significant crosslinking in our experiments, we would expect that x-ray should produce less DNA elution from UV-irradiated cells than from unirradiated cells (20). X-ray was delivered to the cells at 0° just before elution so as to impart to the DNA a controlled extent of strand breakage. Significant crosslinking effects were consistently found, both immediately after UV (Table 1), and in cells incubated after UV (data not shown). These results indicate that the alkaline elution measurements after UV may underestimate the extent of DNA breakage.

The crosslinking effect in normal and XP variant cells diminished with time of incubation after UV. This effect, however, did not account for the difference between the two cell types shown in Fig. 2A.

Effect of Caffeine. Lehmann *et al.* (11) reported recently that caffeine inhibits post-replication repair in XP variant cells but not in normal human fibroblasts. We, therefore, tested whether caffeine would also show such a selective effect in the rejoining phase after UV. The results in Table 2 indicate that this is the case. Confluent normal or XP variant cells were exposed to 100 erg/mm² of UV and incubated for 15 hr in the presence or absence of 1.6 mM caffeine. Caffeine alone had no effect either in normal or in XP variant cells. In normal cells exposed to UV and allowed to repair for 15 hr, DNA relative retention had recovered nearly to normal, and this recovery was unaffected by caffeine. In XP variant cells in the absence of caffeine, the recovery from the DNA damage was nearly as good as in normal cells, but in the presence of caffeine the recovery was impaired (relative retention 0.58, as opposed to 0.79 in the absence of caffeine).

DISCUSSION

The interpretation of our findings rests on the assumption that alkaline elution measures DNA single-strand breaks. Evidence supporting this assumption was reported in ref. 6, and further support will be presented elsewhere. The argument briefly is as follows. (*1*) Alkaline elution is sensitive to very low doses of x-ray, indicating a very large target. (*2*) Alkaline elution is unaffected by enzymatic removal of nearly all of the protein and RNA from the filter lysate or by treatment with sodium dodecyl sulfate prior to or during elution. This indicates that the elution kinetics are governed by the DNA itself and argues against non-DNA targets for the x-ray effect. (*3*) Elution kinetics are independent of
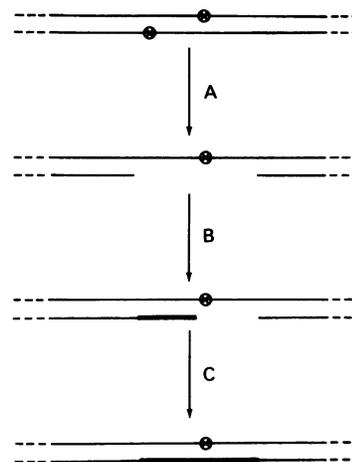

FIG. 5. Proposed mechanism for retardation of strand rejoining during UV repair in XP variant cells, based on an assumed defect in the ability of these cells to cope with template damage. According to this scheme, XP variant cells would be slowed at step C. The symbol ⊗ represents a pyrimidine dimer. The heavy line segments represent DNA generated by repair synthesis. (Although the scheme depicts the complete removal of the excised DNA segment as occurring in step A, the actual time when this occurs is not known and is not relevant to the proposed mechanism. Degradation of the excised segment might be linked with synthesis of the repair segment, so that no large gap ever exists.)

number of cells applied to the filter. This excludes an intercellular aggregate or gel as a governing factor. (*4*) The eluted DNA is almost exclusively single-stranded and the elution of DNA crosslinked by nitrogen mustard (HN2) is impaired. Thus strand-separation precedes elution. (*5*) The kinetics and x-ray sensitivity of elution are consistent with the possibility that elution depends on the time required for the unwinding of very long DNA helices (21–23).

Single-strand breakage, however, is not the only factor determining elution rate. This is apparent from the finding that in cells treated with HN2 or UV the ability of x-ray to increase elution rate is diminished. This effect may be due to the introduction of crosslinks, either between DNA and protein (15–19) or between DNA strands (20, 24, 25). Either type of crosslink could retard elution and lead to underestimation of strand breakage.

With this understanding of our method, we can proceed to interpret our data in more detail.

After UV in normal cells we observed an increase in DNA elutability that required a short incubation period to develop. In XP cells, however, UV produced little or no change, although x-ray produced the normal increase in DNA elutability. This is consistent with the possibility that the defect in XP cells is related to an endonuclease reaction.

The crosslinking effect immediately after UV was similar in normal and XP cells. Hence, although crosslinking tends to hide part of the strand scission effect, it is not responsible for the observed difference between normal and XP cells.

The maximum DNA elutability upon incubation after UV was nearly independent of UV dose, in agreement with Cleaver's recent findings by alkaline sedimentation (26). The maximum effect after high doses, however, is underestimated because of crosslinking.

The time required for the return of DNA elutability to normal upon further incubation after UV was strongly dependent on UV dose. The return towards normal after 15 erg/mm² was gradual over a period of about 45 min, where-

as after 100 erg/mm², the time required extended to about 15 hr. This long time required for recovery is in accord with the slowness of dimer excision after doses of this magnitude (2). Our results in normal cells do not conform to a steady-state level of breaks over a period of time, as suggested by Cleaver (26). Instead, we find that the level of DNA breakage appears to decrease steadily with time.

XP variant cells, which have normal DNA repair synthesis, showed the same increase in DNA elutability after UV as did normal cells. However, these cells differed from normal cells in that the return of DNA elutability toward normal after 100 erg/mm² was significantly slower (Fig. 2A). A possibly related result was reported recently by Lehmann *et al.* (11), who found that the rate of joining of short DNA segments replicated after UV is slower in XP variant cells than in normal cells. The distinction between the two observations is that Lehmann's pertains to newly replicated DNA in proliferating cells, whereas ours deals with repair of preexisting DNA in nonproliferating cells. In both cases, the deficient rejoining rate in XP variant cells was exaggerated by caffeine. Lehmann *et al.* (11) suggested that the abnormality they observed may be due to an impairment in the ability of XP variant cells to bypass defects in the template strand during replication. A similar mechanism could account for our finding of retarded joining in the repair of preexisting DNA (Fig. 5). This defect in XP variant cells would be expressed when two pyrimidine dimers exist near each other on opposite strands. One of the dimers could be excised in the normal way, since XP variant cells have no abnormality in excision or repair synthesis. The critical event would arise when the DNA repair synthesis process encounters a dimer on the template. We may suppose, in analogy with Lehmann (11), that a special mechanism is required for repair synthesis to cope with a template lesion, and that this mechanism is defective, as well as caffeine-sensitive, in XP variant cells. DNA repair synthesis in XP variant cells therefore would be blocked at step C (Fig. 5).

Is this model quantitatively reasonable? The expected frequency of pyrimidine dimers in human cells exposed to 100 erg/mm² is 1 per 13,000 nucleotides (27). The relevant size of the DNA single-strands measured by alkaline elution was of the order of 10⁷ nucleotides (6). Therefore, there were of the order of 1000 dimers per measurable strand length. Since the gap size following excision of a dimer is about 100 nucleotides, the total number of potentially excised nucleotides per measurable strand length was about 10⁵. There would, therefore, be of the order of 10 dimers opposite potential excision gaps per measurable strand length. This number of opposing dimers can easily account for the observed effect on the DNA alkaline elution measurement of XP variants. The incidence of opposed dimers should be proportional to the square of the UV dose. This would ex-

plain why we found little or no difference between the two cell lines when the dose was reduced to 15 erg/mm².

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