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Effects of Microinjected Photoreactivating Enzyme on Thymine Dimer Removal and DNA Repair Synthesis in Normal Human and Xeroderma Pigmentosum Fibroblasts[1]

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ABSTRACT

UV-induced thymine dimers (10 J/m^2 of UV-C) were assayed in normal human and xeroderma pigmentosum (XP) fibroblasts with a monoclonal antibody against these dimers and quantitative fluorescence microscopy. In repair-proficient cells dimer-specific immunofluorescence gradually decreased with time, reaching about 25% of the initial fluorescence after 27 h. Rapid disappearance of dimers was observed in cells which had been microinjected with yeast photoreactivating enzyme prior to UV irradiation. This photoreactivation (PHR) was light dependent and (virtually) complete within 15 min of PHR illumination. In general, PHR of dimers strongly reduces UV-induced unscheduled DNA synthesis (UDS). However, when PHR was applied immediately after UV irradiation, UDS remained unchanged initially; the decrease set in only after 30 min. When PHR was performed 2 h after UV exposure, UDS dropped without delay. An explanation for this difference is preferential removal of some type(s) of nondimer lesions, *e.g.*, (6-4)photoproducts, which is responsible for the PHR-resistant UDS immediately following UV irradiation. After the rapid removal of these photoproducts, the bulk of UDS is due to dimer repair. From the rapid effect of dimer removal by PHR on UDS it can be deduced that the excision of dimers up to the repair synthesis step takes considerably less than 30 min.

Also in XP fibroblasts of various complementation groups the effect of PHR was investigated. The immunochemical dimer assay showed rapid PHR-dependent removal comparable to that in normal cells. However, the decrease of (residual) UDS due to PHR was absent (in XP-D) or much delayed (in XP-A and -E) compared to normal cells. This supports the idea that in these XP cells preferential repair of nondimer lesions does occur, but at a much lower rate.

INTRODUCTION

Cyclobutane-type pyrimidine dimers are the major photoproducts induced in DNA by UV. Evidence has been collected that these lesions initiate the process of UV-induced mutagenesis and carcinogenesis (1, 2). Other photoproducts in DNA induced by UV comprise the (6-4)photoproducts, which are mutagenic as well (3, 4). In living cells, UV-induced DNA lesions may be repaired by a multienzyme process (excision repair) or via a light-dependent enzymatic reaction known as PHR,[3] which is specific for pyrimidine dimers (see Ref. 5 for a review). PHR has been found to occur in a wide range of organisms (6, 7); studies on the occurrence of PHR in mammalian cells, however, have yielded conflicting results (8, 9). A correlation between unrepaired DNA damage and carcinogenesis in humans was established when it was shown that the cancer-prone hereditary disease XP involves a defect in the excision-repair mechanisms acting on UV-induced DNA lesions (10).

We have investigated the kinetics of dimer removal in UV-irradiated cultured normal human and XP fibroblasts, which had been microinjected with purified yeast PRE. Thymine dimers were determined at the single-cell level by quantitative immunofluorescence microscopy based on the application of a dimer-specific monoclonal antibody and computer-assisted image processing and analysis (11).

Injection with PRE and subsequent illumination resulted in a rapid disappearance of dimers: within 15 min virtually all dimers (induced by 10 J/m^2) were removed in normal as well as in XP fibroblasts, whereas noninjected repair-proficient cells still retained 25% of their dimers after 27 h. A comparison was made between the effect of PHR by microinjected PRE on dimer removal, as measured with immunofluorescence microscopy, and the indirect effect on UV-induced UDS. This was done in parallel experiments with normal human as well as XP fibroblasts. The time interval between UV irradiation of the cells, PHR, and UDS measurement was varied.

MATERIALS AND METHODS

Antibodies. The monoclonal antibody H3, specific for thymine dimers in single-stranded DNA, has been described (11). Culture medium of hybridoma cells or ascites fluid was used diluted 1:20 and 1:500, respectively. Fluorescein-labeled goat anti-mouse IgG (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) was used in 1:150 dilution.

Cell Culture. Normal human fibroblasts (C5RO) were cultured in Dulbecco's modified Eagle's medium (Flow Laboratories, Irvine, United Kingdom) supplemented with 10% FCS (Flow), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells used in microinjection experiments were cultured in Ham's F-10 medium (Flow) containing FCS and antibiotics as above. H3 hybridoma cells were cultured in RPMI 1640 (Gibco Ltd., Paisley, United Kingdom) supplemented with 10% FCS and antibiotics.

Photoreactivating Enzymes. Purified PRE preparations from *Saccharomyces cerevisiae* were prepared as described (12, 13). Enzyme concentration was approximately 8 μM as deduced from protein determination (14) and the absorbance at 377 nm (M_r 56,000 from sodium dodecyl sulfate-polyacrylamide gel electrophoresis, ϵ_{377} 27,850).[4] PRE from *Anacystis nidulans* and *Escherichia coli* was purified as described (12). The enzymes are highly active in the *in vitro Haemophilus influenzae* transformation assay (see Refs. 13 and 15 for experimental details).

Microinjection of PRE and Photoreactivation. Cell fusion was accomplished with inactivated Sendai virus, generating polykaryons with 2–10 nuclei in addition to nonfused monokaryons. Only homopolykaryons were injected. The microinjection has been described earlier (12). After injection, usually taking about 20 min, the cells were UV irradi-

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[3] The abbreviations used are: PHR, photoreactivation; FCS, fetal calf serum; PRE, photoreactivating enzyme; UDS, unscheduled DNA synthesis; XP, xeroderma pigmentosum; TBS, 20 mM Tris-HCl-150 mM NaCl, pH 7.4.

[4] A. P. M. Eker *et al.*, manuscript in preparation.

ated (see below) and either illuminated with photoreactivating light [4 Blacklite/Blue lamps F20/T12BLB (Sylvania) at a distance of 10 cm through a 6-mm-thick glass plate] or kept in the dark for periods varying from 30 to 120 min as indicated in the legends to the figures. During illumination the cells were kept in culture medium under constant 5% CO_2 flush, at 34–38°C. These conditions are the same as those used earlier (12, 13).

UV Irradiation. The UV-C source was a single low-pressure mercury vapor lamp (Philips, The Netherlands; 15-W TUV). The incident dose rate was 0.4 J/m^2/s at 254 nm as determined with an International Light IL1500 dosimeter equipped with a SEE400 detector (International Light, Newburyport, MA). The UV dose rate in microinjection experiments was 0.6 J/m^2/s. Cells were irradiated while attached to glass at room temperature in a darkened room, directly after rinsing with phosphate-buffered saline.

Immunostaining of the Cells. The procedure as described by Muysken-Schoen *et al.* (16) was followed with minor modifications. Briefly, cells were fixed while attached to glass with 70% ethanol or with methanol and acetone (microinjection experiments). After fixation the slides were stored at −20°C. For immunostaining, slides were thawed and incubated for 0.5 h in 50 mM Tris-HCl (pH 7.2)-1 M KCl-0.3% Triton X-100, washed with TBS, and treated for 1 h with RNase A (100 μg/ml; Sigma Chemical Co., St. Louis, MO) in TBS at 37°C. To denature DNA *in situ*, slides were incubated for 2 min in freshly prepared 0.07 N NaOH in 70% ethanol, dehydrated in graded ethanol series, and air dried. Then they were treated with proteinase K (10 μg/ml; Merck, Darmstadt, Federal Republic of Germany) for 10 min in 20 mM Tris-HCl-2 mM $CaCl_2$, pH 7.4. After washing, anti-thymine dimer antibody solution (40 μl/cm^2) in TBS containing 5% FCS and 0.05% Tween 20 (Sigma) was added; incubation was for 60 min at 37°C. After being washed with TBS, the cells were incubated for 60 min at 37°C with FITC-GaM (40 μl/cm^2) in TBS containing 5% FCS and 0.05% Tween 20. Unbound antibodies were washed away with TBS, nuclear DNA was stained with propidium iodide (5 min, 40 ng/ml in TBS), and the slides were mounted in 5 mM Tris-HCl-10 mM NaCl-75% glycerol (pH 8.1), coverslipped, and sealed with paraffin. All treatments were done at room temperature unless stated otherwise. A Orthoplan microscope (Leitz, Wetzlar, Federal Republic of Germany) was used to examine the slides. The filter combinations (Zeiss, Oberkochen, Federal Republic of Germany) used were BP 485/20 and BP 515-560 for fluorescein excitation and emission, respectively, and BP 515-560/KP 555 and LP 590 for propidium iodide excitation and emission, respectively. Photographs were taken on 35-mm 400 ASA Kodak Ektachrome film (Kodak Eastman, Rochester, NY).

Quantitative Immunofluorescence. The equipment used to analyze the fluorescence images from the microscope consists of an image intensifier (Philips XXTV1500) placed in front of a normal light level TV camera (Philips; 0.3 lux for maximum video signal), which records the images (1 frame = 1 image). The images are passed to a home-made A/D converter and disk subsystem (FLEX system), to be digitized in a format of 256 × 256 pixels, the gray values of which range from 0 to 255 (8 bits). Prior to recording the images, a test procedure is carried out yielding a correction matrix (image) of 256 × 256 pixels, with which each recorded image is corrected for uneven illumination due to the HBO 100-W mercury arc lamp and other factors.

The overall procedure followed requires the recording of twin images. The first one, recorded with the propidium iodide filter combination, serves to localize the nuclei in the image. The second image of the same nuclei, recorded with the fluorescein filter combination, is used to determine the dimer content, as indicated by the signal from the fluorescein-labeled secondary antibodies. From the first image the exact position of the nuclei within the computer image is calculated, which then is used to calculate the amount of fluorescein fluorescence within these nuclei from the second image. To determine dimer-specific fluorescence, background fluorescence of cells incubated without anti-thymine dimer antibody but with fluorescein-labeled goat anti-mouse IgG is subtracted. The image-processing software package (TCL image) has been developed by TNO-TPD, Delft, The Netherlands, in collaboration with the Technical University of Delft, The Netherlands.

Unscheduled DNA Synthesis. UDS was performed as described (12, 13, 17) by pulse-labeling cells in Ham's F-10 medium (without thymidine), supplemented with 10% dialyzed FCS, 1 μM 5-fluoro-2′-deoxyuridine (Sigma), and 20 μCi/ml [^{3}H]thymidine (Amersham, Buckinghamshire, United Kingdom; 117 Ci/mmol), for 15 or 30 min. Dialysis of the serum and the addition of 5-fluoro-2′-deoxyuridine increase the sensitivity of the assay by lowering exogenous and endogenous thymidine concentrations.

RESULTS

Quantitative Detection of Thymine Dimers and Dimer-Removal by Immunofluorescence. Monoclonal antibodies specific for thymine dimers were used to detect and quantitate dimers at the single-cell level with an immunofluorescence assay. To test whether the intensity of immunofluorescence is a reliable reflection of the amount of dimers present, a dose-response curve was determined. As shown in Fig. 1*A*, linear increase of dimer-specific fluorescence with UV dose was observed in normal human fibroblasts, over a dose range from 0 to 16 J/m^2 (254 nm UV). Increase of UV exposure up to 40 J/m^2 resulted in still increasing fluorescence intensity (not shown).

To determine whether repair of dimers can be measured at the single-cell level, normal human fibroblasts were exposed to 10 J/m^2 UV and incubated for different periods of time prior to fixation and immunostaining. A gradual decrease of fluorescence was measured (Fig. 1*B*). In view of the specificity of the antibodies, this decrease can be attributed to thymine dimer removal. The results are in agreement with data published earlier about kinetics of dimer removal assessed with immunochemical methods (18, 19) or with the UV-endonuclease assay (20).

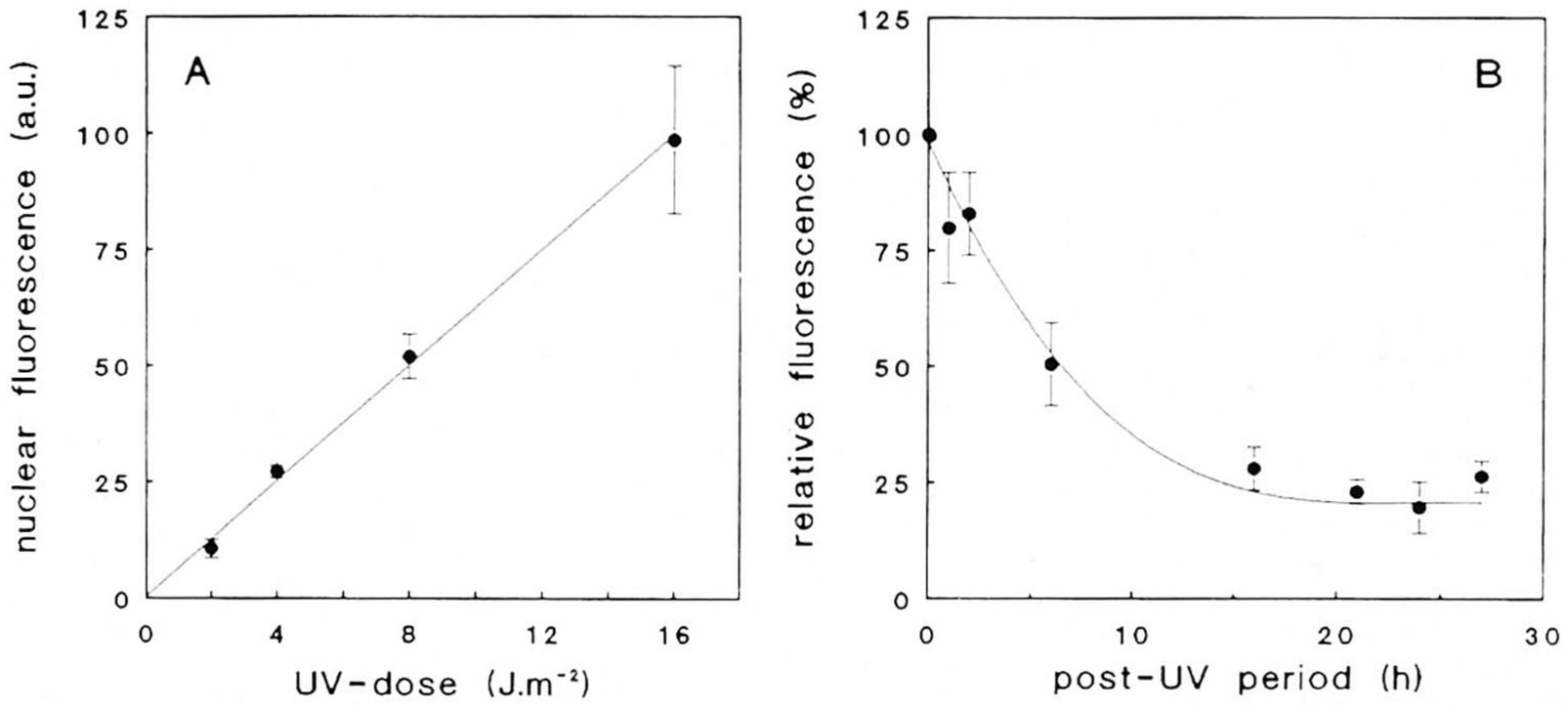

Fig. 1. Detection of thymine dimers in UV-irradiated human fibroblasts with monoclonal antibodies by means of immunofluorescence microscopy. Cells were either exposed to different dosages of UV (*A*) or UV irradiated at 10 J/m^2 followed by repair incubation for various time intervals (*B*). Thereafter cells were fixed and prepared for immunostaining. Subsequently, they were incubated with thymine dimer-specific antibodies, followed by fluorescein-labeled anti-mouse antibodies. Nuclear fluorescence was quantitated by applying computer-assisted image processing and analysis. *Points*, mean nuclear fluorescence (*A*) and relative mean nuclear fluorescence (*B*) ± SEM (*bars*) of 4 slides. Per slide, about 50 nuclei divided over 8–10 images were studied.

On the basis of these results we conclude that the immunofluorescence assay used is suitable for quantitative detection of dimers at the single-cell level.

Kinetics of Dimer Removal by Microinjected Photoreactivating Enzymes. Photoremoval of thymine dimers was studied in normal human fibroblasts, microinjected with purified yeast PRE. To facilitate reidentification of the injected cells, a subpopulation of multinuclear fibroblasts was generated by cell fusion, and only those were injected. Then, cells were UV irradiated (10 J/m^2) and either exposed to PHR-light or kept in the dark for 1 h at 37°C in culture medium. Microinjected cells illuminated with PHR-light showed strongly reduced nuclear fluorescence in comparison to the highly fluorescent noninjected cells on the same slide (Fig. 2). Quantitative immunofluorescence data are summarized in Table 1. In microinjected cells incubated in the dark, the level of fluorescence was not significantly different from that in cells not injected. Also in microinjected fibroblasts with larger amounts of pyrimidine dimers, induced by UV doses of 20 or 40 J/m^2, 1 h of PHR-light reduced the fluorescence to background values (Table 1). Microinjection with purified PRE from *A. nidulans* or *E. coli* also resulted in a PHR-light-dependent reduction of UV-induced immunofluorescence, to 4 and 56%, respectively.

To determine the kinetics of PHR of dimers by PRE in injected UV-irradiated fibroblasts (10 J/m^2), the duration of illumination with PHR-light was varied. As shown in Fig. 3,

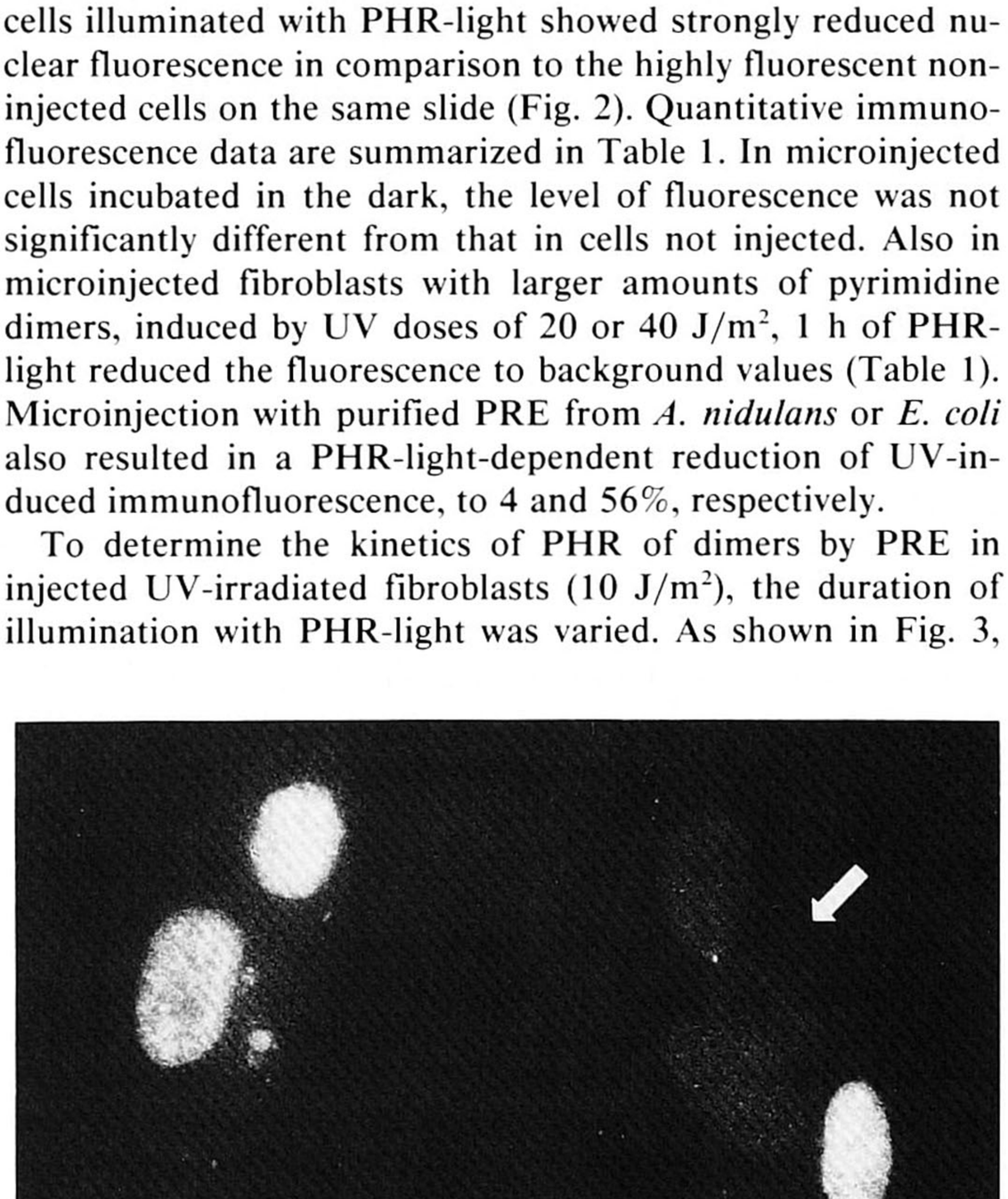

Fig. 2. Effect of photoreactivation, mediated by microinjected PRE, on thymine dimers in UV-irradiated (10 J/m^2) human fibroblasts, as detected by means of immunofluorescence microscopy. Cells were subjected to the immunostaining procedure as in Fig. 1 (*A*). Counterstaining of cell nuclei was with propidium iodide (*B*). Only multinuclear cells had been injected with PRE; after UV, all cells were illuminated with PHR-light for 1 h. The nuclei in the trinuclear injected cell are well stained with propidium iodide but are negative with regard to immunostaining of dimers (*arrow*). × 1000.

Table 1 *Effect of photoreactivation in UV-irradiated human fibroblasts on immunofluorescence associated with thymine dimers and on UDS*

Immediately after UV irradiation, cells were illuminated or incubated in the dark for 1 h. Thymine dimer-specific immunofluorescence was measured. Data are given as nuclear fluorescence ± SEM of cells injected with PRE relative to that of noninjected cells. In each group about 30 nuclei were assayed.

UV dose (J/m^2)	PHR-light	Immunofluorescence (%) injected/noninjected	UDS (%)[a] injected/noninjected
10	−	111 ± 9	86 ± 6
10	+	8 ± 5	18 ± 2; 20 ± 2[b]
20	+	0 ± 6	41 ± 3
40	+	0 ± 6	105 ± 9[c]

[a] The effect of photoreactivation on UV-induced UDS (1–3 h after UV irradiation; data from Ref. 12).

[b] Result from recent repetition of the experiment performed to test reproducibility.

[c] UDS induced by 35 J/m^2 instead of 40 J/m^2 UV (12).

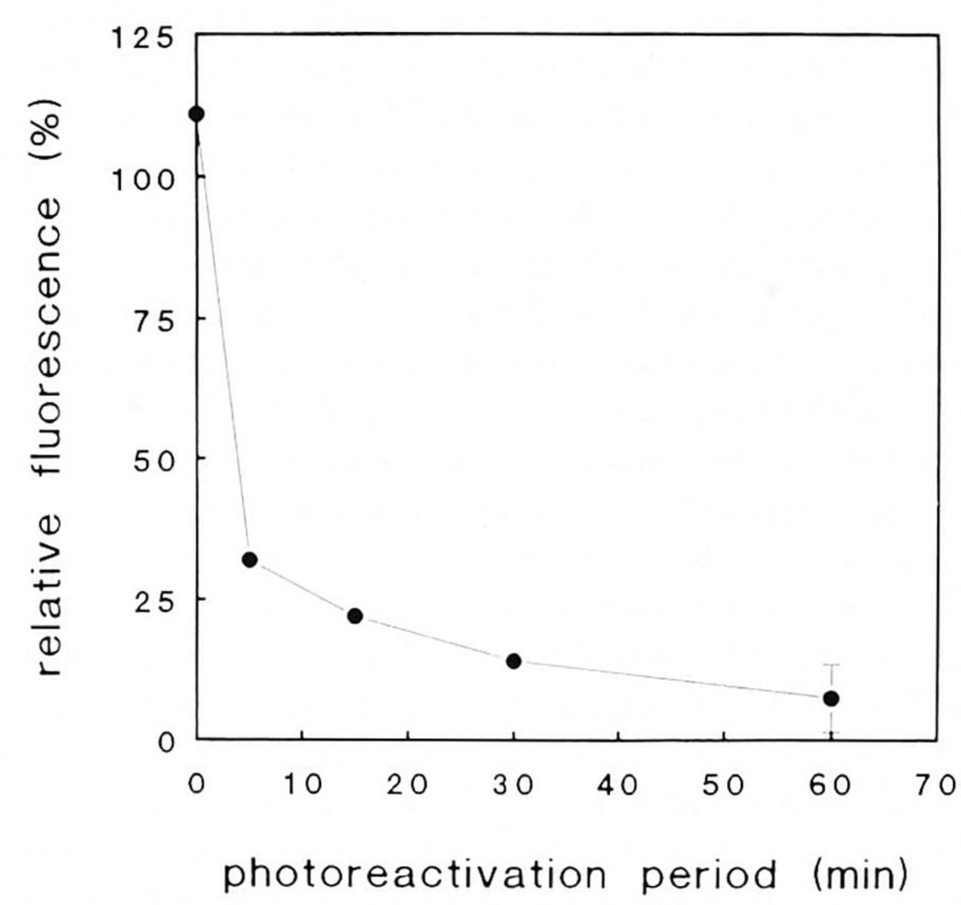

Fig. 3. Photoreactivation of thymine dimers in human fibroblasts by microinjected PRE. Cells were microinjected, exposed to UV (10 J/m^2), and subsequently incubated and illuminated with PHR-light for various time intervals just before fixation (1 h after UV irradiation). Thymine dimers were quantitated by means of immunofluorescence microscopy. Relative fluorescence was determined as the ratio of nuclear fluorescence of injected cells and noninjected cells on the same slide. *Bar*, SEM from 3 experiments. Other data are from single slides.

after a 5-min treatment with black light a pronounced reduction (about 75%) of immunofluorescence was observed, followed by a gradual further reduction after longer periods of illumination. In other experiments we have found that, when a UV dose of 40 J/m^2 was given, the bulk of dimers were removed within 15 min of exposure to PHR-light. These results show that injected PRE is present in sufficient amount and is able to efficiently reach and rapidly monomerize dimers in virtually all parts of the genome.

Kinetics of Reduction of UDS after PHR of Dimers by Microinjected PRE. As has been shown by Zwetsloot *et al.* (12), UV-induced UDS in cultured human fibroblasts can be reduced by PHR occurring after injection of PRE. This reduction of UDS, measured over the period of 2 h immediately following the 1 h illumination with PHR-light, has been interpreted as the consequence of the sudden disappearance of dimers, which then no longer contribute to the repair synthesis by the endogenous excision repair system. Results on the effect of PHR of dimers on UDS obtained under conditions essentially identical to those of our immunochemical dimer detection experiments have been included in Table 1. Obviously, the degree of reduction in UDS shows a UV dose dependency not found in the dimer removal: after a UV dose of 10 J/m^2, PHR reduced UDS to about 20% of the value in noninjected cells; at higher doses,

this percentage increased, to 100% after 35 J/m^2, *i.e.*, PHR virtually had no more effect on UDS. Microinjection of PRE without PHR illumination did not affect UV-induced UDS in these cells (Table 1).

Apparently, removal of dimers by PHR does not necessarily result in a reduced UDS. To obtain more information on the relationship between the two phenomena, a kinetic experiment was performed. In injected, UV-irradiated, and "photoreactivated" fibroblasts, UDS was measured over short time intervals (15 or 30 min), at various moments after the illumination with PHR-light (15 or 30 min). Fig. 4*A* shows that when PHR is performed immediately following UV-irradiation, UDS is not reduced instantaneously. Only after a period of 30 min does a rapid decrease set in, and at 2 h after UV irradiation UDS is reduced to 17% of the level in cells not photoreactivated, in good agreement with our previous findings (12). These data show that there is a delay in the decrease of UDS after the disappearance of dimers (*cf.* Fig. 3). This delay was not detected in earlier experiments, because in these studies UDS was measured at later time points and over a 2-h time interval (12).

Several interpretations for the observed delay in drop of UDS are possible: (*a*) it can be due to a certain duration needed to complete the excision repair process from the initial recognition or removal of dimers until the actual repair synthesis; (*b*) it may be caused by preferential excision repair of nondimer lesions occurring immediately after UV irradiation. In the former case, one expects that the delay is also observed when PHR is performed at later time points after UV irradiation. In the latter case, the delay is expected to be absent once the preferential repair of nondimer lesions is finished. To discriminate between these two main possibilities, the kinetics of UDS was also determined when PHR was performed at 2 h after UV (*i.e.*, well beyond the period of the delay observed immediately following UV irradiation). As shown in Fig. 4*B*, UDS was strongly reduced already over the first time interval studied (15 min) after PHR.

PHR of Dimers and Effect on Residual UDS in XP Fibroblasts. Previously we have found that PHR does not reduce the residual UDS of XP fibroblasts of complementation groups A, D, E, and H, whereas it does lower the residual UDS of all 4 XP-C strains tested, of XP-I (which now turns out to belong to XP-C as well), of XP-variant and (to a lesser extent) of XP-F (13). One of the explanations raised for the absence of PHR-induced reduction of (residual) UDS in XP cells of the complementation groups A, D, E, and H is that dimers in these cells are inaccessible to the injected PRE. To directly test this hypothesis, dimer removal was determined in PRE-injected fibroblasts of XP-A, -C, and -E using the immunochemical method. After UV irradiation (10 J/m^2) and 1 h illumination, microinjected cells showed background levels of thymine dimer-specific fluorescence (Table 2). Apparently, microinjected PRE is able to reach and monomerize dimers in the genome of XP fibroblasts of these complementation groups.

An alternative explanation for the absence of a PHR-induced decrease in (residual) UDS of XP-A, -D, -E, and -H cells could be an extended lag phase between PHR and the onset of its effect on UDS, in these cells compared to normal fibroblasts (and XP-C, -variant, and -F). In the earlier experiments UDS was measured over a 2-h time interval, 1–3 h post-UV irradiation. We therefore performed a kinetic experiment with UV-irradiated (10 J/m^2) XP-A, -D, and -E fibroblasts after PHR by microinjected PRE, in which UDS was studied over 30-min time intervals at later time points after UV irradiation (Fig. 5). Complementation group A cells normally have very low levels of a residual UDS (17); in this respect XP8LO fibroblasts are atypical because they have a residual UDS of about 50% compared to normal cells (13). This level of UDS offered the possibility to include XP-A cells in this kinetic study. In XP8LO (A) and XP2RO (E) a PHR-induced slow reduction of UDS was observed, which started 4–5 h after UV irradiation, in contrast to XP1BR (D) fibroblasts, in which at 9 h after UV irradiation in photoreactivated cells still the same level of UDS was measured as in noninjected cells (Fig. 5).

DISCUSSION

The results presented here illustrate the validity of the immunostaining technique for detection and quantitation of thymine dimers at a single-cell level. Immunostaining of UV-induced DNA damage *in situ* had already been described by Lucas (21). A monoclonal antibody directed toward thymine dimers has been used also to detect these lesions in UV-irradiated mouse skin (22) although without quantitation. The specificity for thymine dimers of the monoclonal antibody we used was demonstrated in enzyme-linked immunosorbent assays (11). In agreement with this, the experiments with microinjection of PRE show that binding of the antibody is specific for lesions that can be photoreactivated, *i.e.*, pyrimidine dimers. In

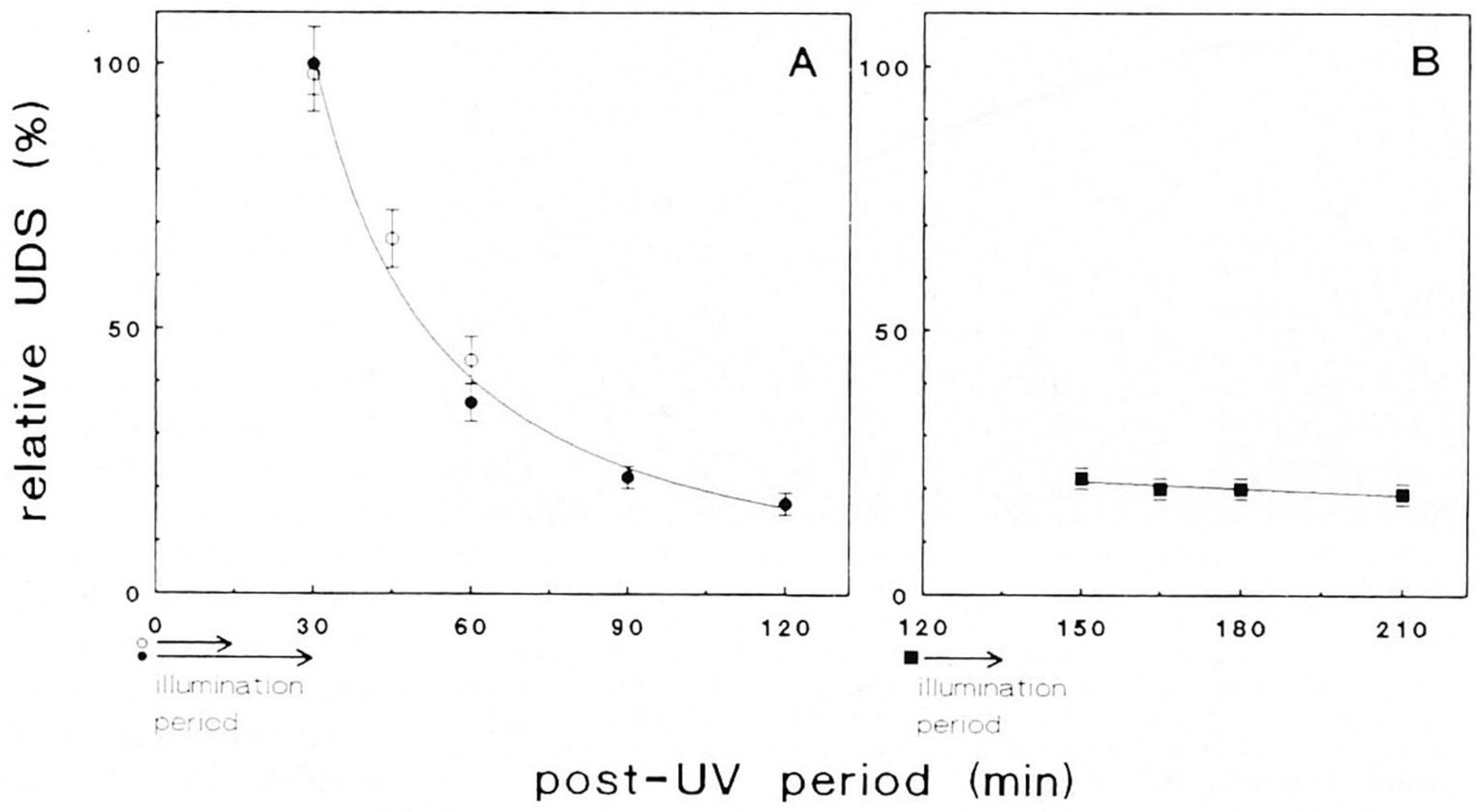

Fig. 4. Effect of PHR by microinjected PRE on kinetics of UV-induced UDS. *A*, cells microinjected with PRE, exposed to UV (10 J/m^2) and to PHR-light for 15 min (○) or 30 min (●) and subsequently incubated in the dark. *B*, cells treated as in *A* except after UV irradiation they were incubated for 2 h in the dark, subsequently exposed to PHR-light for 15 min. UDS was assayed by pulse-labeling with [^{3}H]thymidine for 15 min (○) or 30 min (●, ■). Cells were fixed immediately after pulse-labeling at the times indicated on the *abscissa*. Slides were processed for autoradiography and relative UDS was determined as the ratio of the grain number over nuclei of injected and noninjected cells. *Bars*, SEM.

Table 2 *Effect of photoreactivation on UV-induced immunofluorescence associated with thymine dimers in normal and XP fibroblasts*

After microinjection, cells were UV irradiated (10 J/m^2) and illuminated for 1 h. Thymine dimer-specific immunofluorescence was measured in injected and noninjected cells on the same slide. Data are given as relative nuclear fluorescence ± SEM. Per group about 25 nuclei were assayed.

Cell strain[a]	Immunofluorescence (%) injected/noninjected	UDS (%)[b]
C5RO (normal)	4 ± 4	100 ± 5
XP25RO (XP-A)	2 ± 4	3 ± 3
XP8LO (XP-A)	2 ± 3	49 ± 4
XP21RO (XP-C)	8 ± 6	18 ± 1
XP2RO (XP-E)	10 ± 9	44 ± 4

[a] Information in parentheses, XP complementation group.

[b] Residual UDS relative to that in repair-proficient fibroblasts accumulated over a 2-h period immediately following UV irradiation (10 J/m^2). Data from Ref. 13 (XP25RO from Ref. 17).

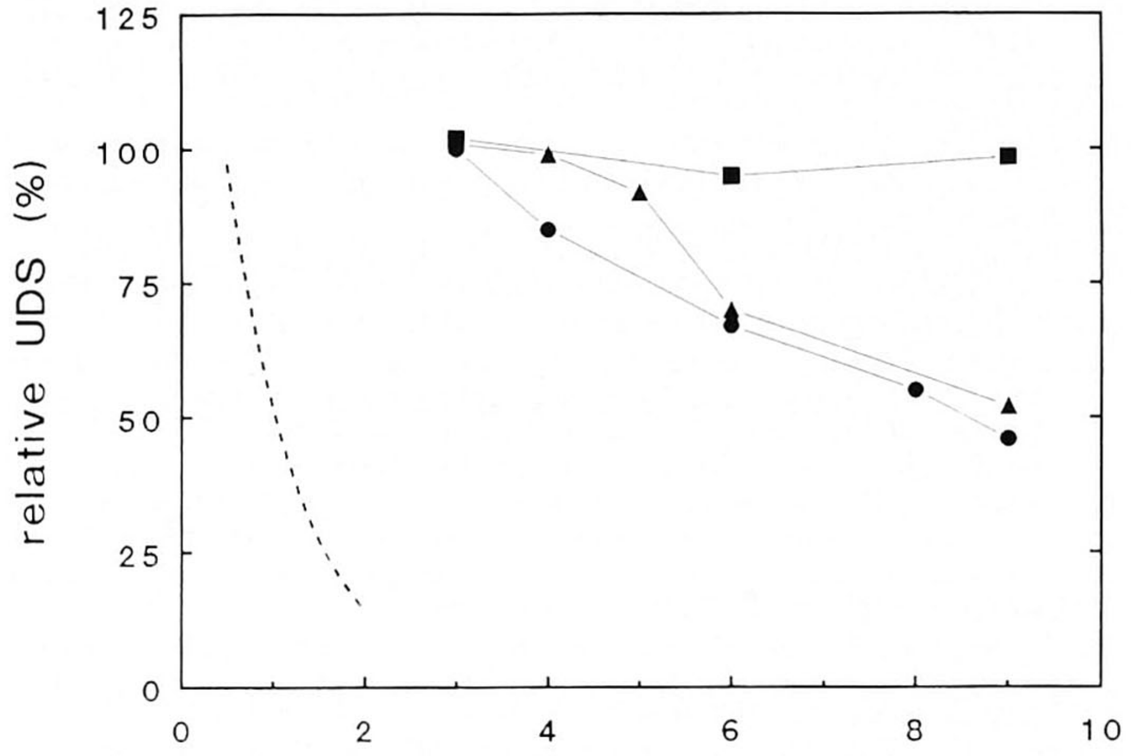

Fig. 5. Kinetics of UV-induced UDS after PHR by microinjected PRE in xeroderma pigmentosum fibroblasts. Experimental procedure as in Fig. 4: PHR-light exposure time 30 min, applied immediately after UV irradiation; [^{3}H] thymidine pulse-labeling 30 min. – – – –, data of normal fibroblasts from Fig. 4*A*. ●, XP8LO (XP-A); ■, XP1BR (XP-D); ▲, XP2RO (XP-E).

addition, these experiments show that the injected PRE efficiently monomerizes dimers in human chromatin, confirming conclusions reached earlier (12) on the basis of the indirect effect of PRE injection on UV-induced UDS.

The efficiency of PHR is indicated by the disappearance of more than 80% of the dimers upon illumination for periods as short as 5–10 min after a moderate dose of UV (10 J/m^2), and complete photoremoval within 1 h in fibroblasts irradiated with a UV dose as high as 40 J/m^2. The latter dose induces about 7 × 10^6 dimers/diploid nucleus (23). On the basis of an estimated injection volume of 1–5 × 10^{-14} liter (24), we calculate that under these conditions each PRE molecule must monomerize 10–100 dimers, assuming, rather optimistically, that all molecules injected are active and are transported into the nucleus.

In the foregoing it is assumed implicitly that the immunochemical assay detects all thymine dimer lesions in the nucleus. We cannot exclude the possibility that a fraction of dimers escapes detection by the antibody. This possibility appears not very likely, however, in view of the fact that the DNA is denatured (in alcohol and alkali) and subjected to treatment with high salt, non-ionic detergents, and proteinase K prior to microscopy. These treatments remove a major fraction of the proteins attached to the DNA and make DNA readily accessible to antibody molecules. Moreover, the same procedures are used in *in situ* hybridization techniques in which probes are visualized by immunohistochemistry, and these procedures are known to be very efficient.

Our kinetic experiments show that dimer removal by PHR carried out immediately following UV irradiation has no immediate consequence for UDS; it results in a decrease but only after a delay. This finding can be explained in various ways. One interpretation of this delay is that the repair synthesis, seen in the period directly following UV irradiation, is the result of repair of nondimer-type lesions, such as (6-4)photoproducts which are known to be removed from DNA relatively rapidly. After moderate UV irradiation of normal human cells, 80% of the antibody-binding sites associated with (6-4)photoproducts were shown to be removed within the first 3 h after irradiation (25, 26). This repair proceeds considerably faster than the dark repair observed for pyrimidine dimers (*cf.* Fig. 1). Other evidence for the preferential removal of (6-4)photoproducts stems from the characterization of a XP-A revertant cell strain that exclusively repairs (6-4)photolesions. The level of repair replication in cells of this strain resembles that of normal human cells over a period of 4 h after irradiation (27).

If the explanation given here for the lag phase in UDS decrease is correct, one would expect that the reduction of UDS due to PHR is UV dose dependent when studied over a fixed interval after irradiation. Data in Table 1 show this to be the case. The lack of PHR-induced reduction of UDS over the period 1–3 h after UV irradiation at 35 J/m^2 might be attributed to the fact that the capacity of the repair system responsible for UDS is limited; initial saturation is reached at UV doses exceeding 10–15 J/m^2 (23). At still higher UV doses the fraction of nondimer lesions induced apparently is large enough to saturate UDS for a number of hours even after PHR of dimers. Also the observation that PHR of dimers at 2 h after UV irradiation (10 J/m^2) results in a sharp decrease of UDS can be explained, if we accept that at that time most of the preferential repair of (6-4)photoproducts has been completed (which is in agreement with the 82% reduction in UDS over the period 1–3 h after UV; see Table 1). Furthermore, when most of the UDS occurring at 2 h after 10 J/m^2 UV is correctly attributed to removal of dimers, this fast decrease of UDS upon PHR at this time point (Fig. 4*B*) means that the time needed to repair a dimer lesion, *i.e.*, from the onset of dimer removal up to the repair synthesis, is (considerably) less than 30 min.

Residual UDS after PHR at 2–3 h after UV irradiation might be due to the repair of a few (6-4)photoproducts not repaired yet or to removal of other types of nondimer UV photoproducts. The fact that a low level of nonphotoreactivable UDS persists for at least 90 min (Fig. 4*A*) indicates that as a consequence of the disappearance of dimers the excision repair of the remaining nondimer lesions is not accelerated to such an extent that repair synthesis of these lesions fully compensates for the loss of UDS due to dimers.

Indications have been found that in *E. coli* and *Drosophila* photolyase stimulates dark (excision) repair (28–30). It appeared possible that also in human cells injection of PRE *per se* enhances excision repair of UV-induced lesions. In our control experiments in which PRE-injected fibroblasts were kept in the dark, thereby preventing photoreactivation of dimers, we have not obtained evidence for this; the injected cells did not display significantly higher UDS levels in the time periods measured (*i.e.*, from 1–3 h after a UV dose of 10 J/m^2, which induces subsaturating levels of UDS; see Table 1) than noninjected neighboring fibroblasts, nor was the immunofluorescence with the antithymine dimer antibody significantly lower (measured 1 h after UV irradiation; see Table 1).

Reduction of UDS upon PHR of dimers was observed earlier

in chicken embryo fibroblasts (31). Also in these cells removal of dimers seemed to be more complete than was suggested by the reduction of UDS, especially at early times after UV irradiation. According to the interpretation given above, also in these cells preferential repair of nondimer lesions early after UV irradiation could explain this observation.

Xeroderma pigmentosum fibroblasts, which are (partly) deficient in excision repair, show reduced levels of UDS after UV irradiation (10, 13). In some XP complementation groups, PHR of dimers by microinjected PRE results in hardly any or in a limited decrease of the residual UDS (13). One of the explanations proposed was an inaccessibility of dimers to the yeast PRE, *e.g.*, due to an altered chromatin structure or to a defective excision-repair protein that shields dimers. However, our experiments on immunofluorescence detection of thymine dimers clearly show complete dimer removal by microinjected PRE in UV-irradiated XP fibroblasts of various complementation groups.

Furthermore, reduction of UDS upon PHR of dimers was observed in XP8LO (A) and XP2RO (E) fibroblasts, albeit only as late as 4–5 h after UV irradiation. This suggests that the repair of pyrimidine dimers contributes to the late UDS but that the majority of UDS early after UV irradiation should be attributed to repair of nondimer lesions. Because no PHR-dependent reduction of UDS is observed in XP1BR (D) fibroblasts, in these cells UDS might be due to (impaired) repair of nondimer lesions exclusively. It has been shown that in XP-D fibroblasts removal of pyrimidine dimers is absent and that of (6-4)photoproducts is very slow, while in XP-E fibroblasts repair of both lesions is intermediate (26, 32), which correlates with our data.

In conclusion, microinjected yeast photoreactivating enzyme is able to reach the active genome and to rapidly monomerize the dimers in normal human as well as in XP fibroblasts. Early after UV irradiation UDS appears to be due to repair of nondimer lesions, whereas in normal cells at 2 h post-UV the majority of UDS (80%) is photoreactivable and, therefore, due to repair of pyrimidine dimers. Certain nondimer lesions are repaired preferentially, which repair might proceed also in XP fibroblasts, although at a lower rate.

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