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Microinjected photoreactivating enzymes from Anacystis and Saccharomyces monomerize dimers in chromatin of human cells

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Summary

Photoreactivating enzymes (PRE) from the yeast *Saccharomyces cerevisiae* and the cyanobacterium *Anacystis nidulans* have been injected into the cytoplasm of repair-proficient human fibroblasts in culture. After administration of photoreactivation light, PRE-injected cells displayed a significantly lower level of UV-induced unscheduled DNA synthesis (UDS) than non-injected cells. This indicates that monomerization of the UV-induced pyrimidine dimers in the mammalian chromatin had occurred as a result of photoreactivation by the injected PRE at the expense of repair by the endogenous excision pathway. Purified PRE from yeast is able to reduce UDS to 20–25% of the UDS found in non-injected cells, whereas the in vitro more active PRE from *A. nidulans* gives a reduction to only 70%. This suggests that the eukaryotic enzyme is more efficient in the removal of pyrimidine dimers from mammalian chromatin than its equivalent purified from the prokaryote *A. nidulans*.

Several DNA-repair pathways are known whereby living cells remove pyrimidine dimers induced after exposure to ultraviolet light. In the dark a multi-enzyme process results in the excision of the dimers (excision repair), whereas exposure to near-UV or visible light facilitates the action of a single protein, the photoreactivating enzyme (PRE), which binds to the dimer and monomerizes this DNA lesion (see for reviews Hanawalt, 1979; Lehman and Karran, 1981; Kraemer, 1983). PRE has been demonstrated in many organisms (Rupert, 1962; Sutherland, 1981; Eker, 1983). However, studies of PRE activity in rodent and primate cells yielded conflicting results (Ananthaswamy and Fisher, 1981; Harm, 1980). Sutherland and coworkers (1974a, b, 1980b, 1982) and Henderson (1978) demonstrated photoreactivation of dimers in human cells. In contrast, other investigators failed to detect PRE activity in cells of placental mammals (Cleaver, 1966; Cook and McGrath, 1967; Trosko and Isoun, 1970; Paterson, 1978). Evidence has been presented that PRE activity is sensitive to as yet unidentified cell culture condi-

Abbreviations: PR light, photoreactivating light; PRE, photoreactivating enzyme (photolyase, EC 4.1.99.3); TdR, deoxythymidine; UDS, unscheduled DNA synthesis; XP, xeroderma pigmentosum.

tions, which may explain these contradictory results (Mortelmans et al., 1977).

In a series of experiments ultimately aiming the introduction and expression of prokaryotic and primitive eukaryotic PRE genes in human cells, we tested the ability of the products of these genes to remove pyrimidine dimers from the chromatin of human fibroblasts in culture. The technique of microneedle injection (Graesmann and Graesmann, 1976; Anderson et al., 1980; de Jonge et al., 1983) has been used to introduce the PREs into the cells. The injected enzymes significantly reduced the UV-induced unscheduled DNA synthesis (UDS), of repair-proficient human fibroblasts, thus competing with the endogenous excision repair for the removal of dimers.

Materials and methods

Preparation of PRE

(a) Crude extracts from S. cerevisiae

Crude extracts were prepared from *S. cerevisiae* strain Ya 1-27a (pJDB207) i.e. strain Ya 1-27a (MATa rad1-1 phr1-1 ade2-1 his3 leu2) containing the vector pJDB207 (Beggs, 1978) further referred to as PRE^- extract, and from GRF18 (pA8-3) i.e. strain GRF 18 (MATα his3 leu2) transformed with the recombinant plasmid pA8-3, which consists of vector pJDB207 with a cloned yeast phr-gene (Yasui and Chevallier, 1983) further referred to as PRE^+ extract. Cells were grown to the late stationary phase, in medium containing 10 g yeast extract, 5 g peptone and 20 g glucose per liter resuspended in 0.1 M K_2HPO_4, 5 mM β-mercaptoethanol, 1 mM EDTA, pH 7.5 and homogenized using a cell homogenizer (Braun Melsungen, FRG). The PRE^--extract and the PRE^+-extract were obtained by adding ammonium sulphate to the homogenized suspensions to 25% saturation (v/v). The precipitates were removed by centrifugation for 1 h at $5000 \times g$. Ammonium sulphate was added to saturation to the respective supernatants, the precipitated proteins were collected by centrifugation for 30 min at $5000 \times g$, dissolved in 50 mM K_2HPO_4, 2 mM β-mercaptoethanol, 1 mM EDTA, pH 7.5 and dialyzed overnight against this buffer. The dialyzed material was cleared by centrifugation at $3000 \times g$, glycerol was added to the supernatant to a final concentration of 5% and aliquots were stored at $-70°C$ (Herriott, 1979).

(b) Purification of PRE from S. cerevisiae

Yeast PRE was purified from *S. cerevisiae* strain Ya 1-27a (pA8-3). Due to the high copy number of pA8-3 the level of photolyase is more than 15 times that in wild-type strains (Yasui and Chevallier, 1983). Cells were grown as described above and disintegrated by repeated passage through a 1-inch diam. french pressure cell. After centrifugation to remove cell debris, photoreactivating enzyme was purified by chromatography on porous silica beads, UV-irradiated DNA–cellulose, heparine–sepharose and CM–cellulose (Eker and Yasui, in preparation). The final preparation was at least 65% pure as judged from the absorption spectrum. On the basis of molecular weight, chromophore identity and absorption spectrum this enzyme may be identical with the yeast photoreactivating enzyme isolated by Iwatsuki et al. (1980).

(c) Purification of PRE from A. nidulans

The cyanobacterium *Anacystis nidulans* (strain 1402-1 SAUG) was grown in a mineral medium adapted from Kratz and Myers (1955) with CO_2 as carbon source and illuminated with white fluorescent lamps. The cells were destroyed by passing them twice through a sonic oscillator equipped with a flow vessel. Cell debris was removed by centrifugation and photoreactivating enzyme was purified by chromatography on porous silica beads (sferosil type D), UV-DNA covalently linked to cellulose, heparine–sepharose and DEAE–cellulose (Eker, in preparation). According to polyacrylamide gel electrophoresis the final preparation was apparently homogeneous. The purified enzyme showed, besides protein absorption, an absorption band in the visible region with a maximum at 438 nm.

In vivo assay of PRE activity, microinjection in repair proficient fibroblast

In all microinjection experiments the repair-proficient human fibroblast cell strain C5RO was used. The fibroblasts were cultured in Ham's F10 medium supplied with 7.5% fetal (FCS) and 7.5% newborn calf serum (NCS), penicillin 100 I.E./ml

and streptomycin 100 μg/ml. Prior to injection polykaryons were made by cell fusion using inactivated Sendai virus at a concentration of 500–1000 HAU/ml (de Weerd-Kastelein et al., 1972), the cells were seeded onto 0.6 cm × 1.0 cm glass slides provided with a grid and cultured for 4–5 days. Cells were fused to facilitate the identification and the analysis of the treated cells since only multinucleated fibroblasts were injected (de Jonge et al., 1983) and to prevent confusion of early or late S-phase cells with cells performing UDS (Jaspers et al., 1981). Crude extracts and purified PREs were injected into the cytoplasm of a homopolykaryon with the aid of a microneedle (Graessmann et al., 1980; Cappecchi, 1980; Anderson et al., 1980; de Jonge et al., 1983). The procedure was carried out in the dark to prevent photoreactivation; the microscope was supplied with a red filter. Immediately after injection cells were washed with PBS (phosphate-buffered saline), irradiated with various UV-doses and either illuminated with photoreactivating light for 1 h or kept in the dark. Either 4 Blacklite/Blue lamps F20/T12BLB (Sylvania) or a TLADK 30 W/03 lamp (Philips), at a distance of 10 cm for both *S. cerevisiae* and *A. nidulans* PRE were the source of PR light. No differences with respect to reduction in UDS level was noticed, between the two types of lamp. Illumination was carried out in culture medium as described above through a 6-mm thick glass-plate; the temperature varied between 34°C and 38°C for both the illuminated and non-illuminated cells. The medium was kept under a constant flow of 5% CO_2. After illumination, the UDS assay was performed in the dark. Cells were washed with PBS and cultured for 2 h in Ham's F10 medium supplemented with 8–10% dialyzed FCS and 10 μCi/ml [^{3}H]thymidine (^{3}H-TdR) (46 Ci/mmole) (Zelle and Bootsma, 1980). Dialyzed FCS was used to obtain a high ^{3}H-TdR-incorporation. After incubation the cells were fixed and the slides covered with stripping film (Kodak stripping plates AR10). After exposure in the dark at 4°C for 4 or 5 days the slides were developed, stained with Giemsa and analyzed. The number of grains per nucleus or per fixed square of a nucleus was counted and the average of 25–50 nuclei calculated. The number of grains is a measure of the UDS level.

In vitro test of photoreactivation

The activity of PRE-preparations was tested in vitro with a *Haemophilus influenzae* transformation system (Rupert et al., 1958, for experimental details see Piessens and Eker, 1975). Appropriately diluted enzyme solutions were mixed in the dark with UV-irradiated DNA (580 J/m^2 at 254 nm) isolated from a streptomycin-resistant *H. influenzae* donor strain. One half of the mixture was incubated in the dark while the other half was illuminated with a blue fluorescent lamp (Philips TL ADK 30W/03) for 30 min at 35°C. Then competent cells of a streptomycin-sensitive *H. influenzae* strain were added followed by incubation with gentle agitation for 2.5 h at 37°C to transform the recipient cells.

Samples of the transformation mixture were plated on streptomycin-containing agar and after at least 48 h of incubation at 37°C streptomycin-resistant colonies were counted. The photoreactivating activity is expressed as $DF \times (N_L - N_D)/N_D$ where N_D and N_L are the numbers of transformants of dark incubated and photoreactivated samples, and DF is the dilution factor of the enzyme solution. The specific activity is the photoreactivating activity per mg protein.

Protein concentrations were measured with the Coomassie Brilliant Blue method (Bradford, 1976).

Results

In order to examine whether PRE from yeast or from *A. nidulans* is active on UV-induced pyrimidine dimers in the chromatin of mammalian cells the microinjection technique was employed. PRE was injected into the cytoplasm of fused repair-proficient fibroblasts (C5RO), the injected cells were UV-irradiated, illuminated with PR-light and incubated in [^{3}H]thymidine (^{3}H-TdR) for the UDS assay. Fig. 1 shows a C5RO homodikaryon injected with PRE purified from an *S. cerevisiae* strain. As evident from Fig. 1 the injected polykaryon displays only a fraction of the number of grains observed above the neighbouring monokaryon which was treated in exactly the same way except for the PRE injection. To discriminate between true photoreactivation and possible artefacts of the procedure, a number of control experiments was carried out, the results of which are sum-

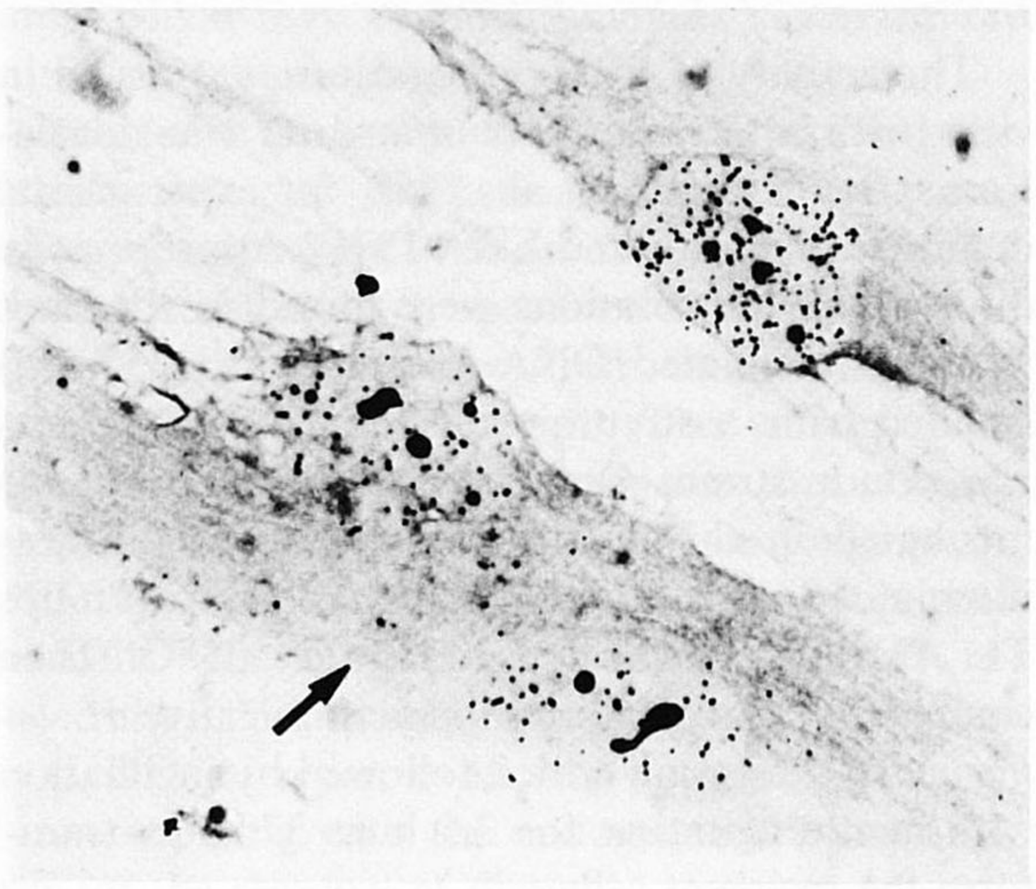

Fig. 1. Unscheduled DNA synthesis in human cells after microinjection of PRE from yeast. A repair-proficient, UV-irradiated (20 J/m^2) homodikaryon (see arrow) shows a reduced UDS level after injection with PRE from yeast and illumination with PR light. The monokaryon has not been injected.

marized in Table 1. Firstly the reduction in UDS is PR-light-dependent: purified PRE from *S. cerevisiae* reduces the UDS level (at a UV dose of 10 J/m^2) to about 20%. When the PRE-injected fibroblasts are kept in the dark after UV-irradiation a slight decrease in UV-induced UDS is observed. Secondly a crude PRE$^+$-extract (Table 1) was compared to an extract from a PRE$^-$ yeast strain. Injection of the PRE$^+$ extract decreased UDS to approximately 30% (at a UV dose of 10 J/m^2), no significant reduction was observed with the PRE$^-$ extract. The reduction in UDS is caused by a protein since it was no longer observed when purified PRE was treated with proteinase K prior to injection. In this experiment proteinase K was covalently linked to Sepharose beads and removed from the PRE solution before injection. A parallel PRE sample incubated for 2 h at 37°C without proteinase K did not exhibit this inactivation, indicating that the activity responsible for UDS reduction is sensitive to proteolytic degradation

TABLE 1

EFFECT OF INJECTED PRE ON THE UV-INDUCED UDS OF REPAIR-PROFICIENT (C5RO) HUMAN FIBROBLASTS

Source (strain/plasmid)	Experimental details [a]	UDS grains/nucleus (±SEM) [b]		UDS % (±SEM)
		Injected	Non-injected	Inj./non-injected
S. cerevisiae	either TLADK or Blacklite Blue as PR-light source	10±1	51±3	18±2
S. cerevisiae	no PR light	44±2	51±3	86±6
S. cerevisiae	30 min PR light instead of 60 min	10±1	50±3	20±2
S. cerevisiae [c] (GRF18/pA8-3)	PRE$^+$ extract	13±1	42±3	30±3
S. cerevisiae [c] (Yal-27a/pJDB207)	PRE$^-$ extract	55±3	52±3	106±8
S. cerevisiae	PRE was treated with proteinase K (2 h) prior to injection [d]	57±3	58±3	98±8
S. cerevisiae	PRE was incubated at 37°C during 2 h	8±1	42±2	19±4
A. nidulans	either TLADK or Blacklite Blue as PR-light source	40±3	56±3	69±6
A. nidulans	no PR light	49±3	46±3	107±9

[a] Standard procedure: fused fibroblasts are injected with PRE, irradiated with a UV dose of 10 J/m^2 and illuminated with PR light for 1 h. Afterwards the cells are assayed for UDS. Differences in and supplements to the standard procedure are registered in this column. Purified PRE was used unless otherwise indicated.

[b] In each case the average number of grains per nucleus is determined by counting 25–50 nuclei.

[c] Strain Yal-27a(phr$^-$); strain GRF 18 (phr$^+$); plasmid pA8-3 is vector pJDB207 containing the cloned yeast *phr* gene.

[d] Proteinase K treatment was carried out according to de Jonge et al. (1983).

and is not inactivated by the incubation itself. A reduction in UDS caused by the microinjection itself was never noticed. We conclude that both PR light and PRE are required to cause reduction of UDS. The in vitro activities of the purified and crude PRE preparations are determined in a *H. influenzae* transformation assay (Table 2). Purified PRE is about 30-fold as active in this test as the crude PRE preparation, whereas in the in vivo microinjection experiments only a minor difference in the reduction of UDS is registered. Illumination with PR light using different lamps or during a shorter period of time (30 min instead of 60 min) had no effect on the level of residual UDS (Table 1). These results indicate that neither PRE nor the PR light is the limiting factor, but the damage causing the residual UDS may be inaccessible for PRE. Injected cells were irradiated with increasing UV doses (up to 35 J/m^2) to investigate at which UV dose the enzyme is limiting. The effect on UDS is depicted in Fig. 2. It appears that even at low UV doses a considerable residual UDS exists, which is relatively constant up to 20 J/m^2. Only at doses of 25 J/m^2 and higher does the relative proportion of the residual UDS increase, suggesting that the injected PRE is limiting at these doses. For non-injected fibroblasts a UV dose of approximately 15 J/m^2 is saturating whereas for polykaryons injected with purified PRE and illuminated with PR light this saturation is reached at UV doses of 30–35 J/m^2. A similar observation was made with the crude PRE^+ extract: at doses below 6 J/m^2 no difference was noticed between the purified and crude enzymes, as both caused a reduction in UDS to approximately 20% of the control value (data not shown). At higher UV doses, the UDS reduction caused by PRE in the crude preparation is less evident than with the purified enzyme (Table 1). These data indicate that even in the low UV-dose range, where PRE is present in excess, a constant proportion of the damage is resistant to photoreactivation.

Purified PRE from *A. nidulans* was injected into the cells to examine whether PRE from prokaryotic origin exerts a similar effect on the DNA in the mammalian nucleus as the eukaryotic yeast PRE. The injected cells were assayed as described above. PRE from *A. nidulans* is 2 times more active in the in vitro assay (Table 2) than purified

TABLE 2

PRE-ACTIVITY DETERMINED IN VITRO USING THE *Haemophilus influenzae* TRANSFORMATION ASSAY

Source [a] (strain/plasmid)	Quality	Photoreactivating activity [b]	Specific activity
S. cerevisiae (Yal-27a/pA8-3)	highly purified	5.2×10^5	8.7×10^5
S. cerevisiae (GRF18/pA8-3)	PRE^+ extract	1.6×10^4	790
S. cerevisiae (Yal-27a/pJDB207)	PRE^- extract	<1	<0.5
A. nidulans (1402-1 SAUG)	highly purified	1.1×10^6	7.5×10^6

[a] Strain Yal-27a (phr^-); strain GRF 18 (phr^+); plasmid pA8-3 is vector pJDB207 containing the yeast *phr* gene.

[b] Expressed as $DF\times(N_L - N_D)/N_D$ where N_L represents the number of transformants after treatment with PR light and N_D without PR light. Specific activity is the photoreactivating activity per mg protein.

PRE from *S. cerevisiae*, however the UDS level in cells injected with *A. nidulans* PRE is reduced to only 70% of the control value, whereas purified

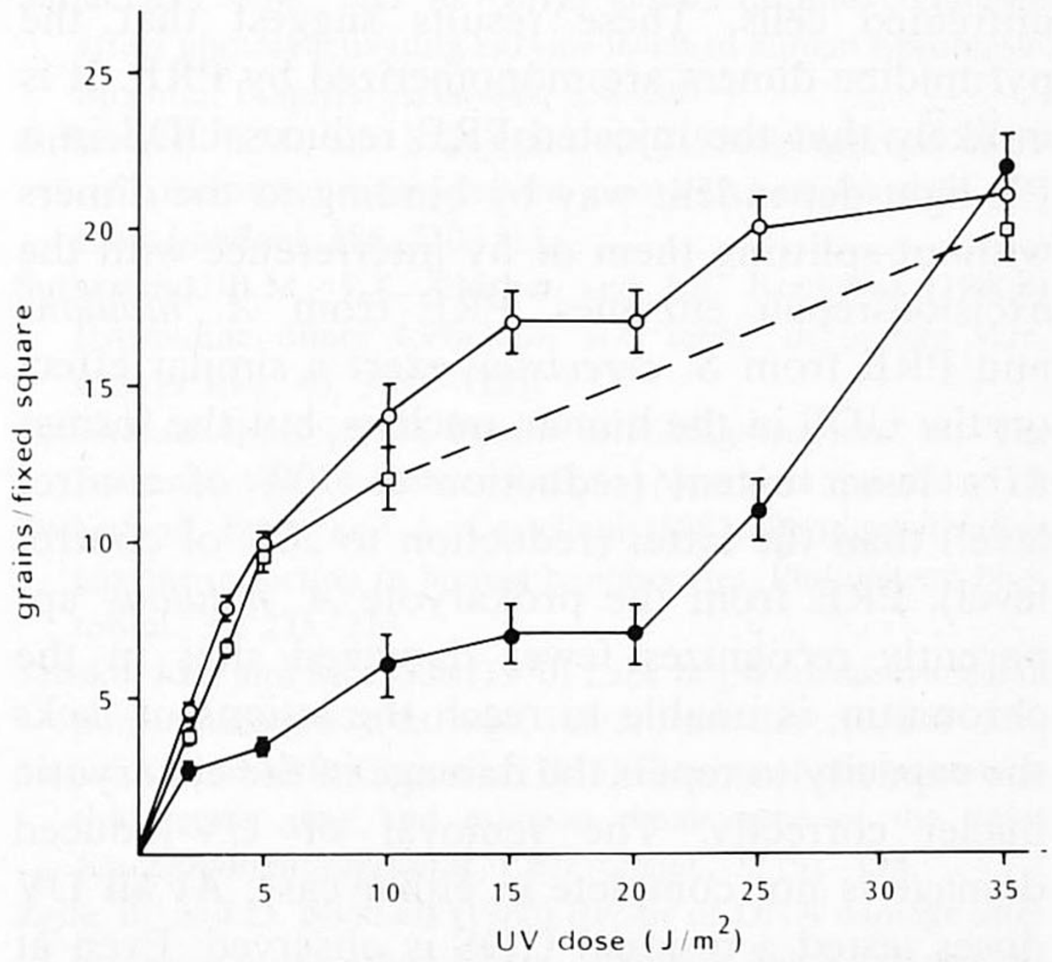

Fig. 2. The effect of PRE from yeast on the UDS in repair-proficient human fibroblasts after irradiation with different UV doses. The number of grains per fixed square of a nucleus was counted. Cells were injected with PRE and illuminated with PR-light (●); injected with PRE and kept in the dark (□); not injected (○), (average of the UDS of illuminated and unilluminated cells).

PRE of yeast gives a reduction to ±20% (Table 1). To exclude premature inactivation of the *A. nidulans* PRE in human cells, the assay for photoreactivation was carried out 3 h after the microinjection. In this experiment the photoreactivating enzymes exert the same effect on UDS compared to the effect immediately after the injection, indicating that the difference in in vivo activity is not due to the instability of PRE from *A. nidulans*. These results suggest that *A. nidulans* PRE monomerizes fewer dimers in human chromatin than PRE from *S. cerevisiae*. It will be interesting to investigate if this phenomenon holds for other prokaryotic and eukaryotic enzymes.

Discussion

Microinjection of PRE from eukaryotic (*S. cerevisiae*) and prokaryotic (*A. nidulans*) origin into the cytoplasm of human fibroblasts results in a significant reduction of UV-induced UDS. This reduction is observed when the cells are injected with active PRE followed by illumination with PR light. Fibroblasts injected with PRE that are kept in the dark or fibroblasts injected with inactivated PRE display almost the same UDS level as the untreated cells. These results suggest that the pyrimidine dimers are monomerized by PRE. It is unlikely that the injected PRE reduces UDS in a PR-light-dependent way by binding to the dimers without splitting them or by interference with the excision-repair enzymes. PRE from *A. nidulans* and PRE from *S. cerevisiae* exert a similar effect on the UDS in the human nucleus, but the former to a lesser extent (reduction to 70% of control level) than the latter (reduction to 20% of control level). PRE from the prokaryote *A. nidulans* apparently recognizes fewer damaged sites in the chromatin, is unable to reach the lesions or lacks the capacity to repair the damage in the eukaryotic nuclei correctly. The removal of UV-induced damage is not complete in either case. At all UV doses tested a residual UDS is observed. Even at low UV doses UDS is not further reduced with excess of PRE or with extension of the PR-light exposure. This residual UDS may be caused by repair of non-dimer lesions (6–4′-pyrimidine lesions, glycol adducts) and/or a category of dimers that is inaccessible to repair enzymes in general or PRE in particular (Pendrys, 1983). These observations confirm the experiments described by Sutherland and Hausrath (1980) who introduced the *E. coli* PRE into V79 Chinese hamster cells by permeabilizing the cell membrane with polyethylene glycol. A considerable loss of *M. luteus* endonuclease-sensitive sites in the DNA of these cells, illuminated with PR light, was reported.

The presence of human PRE, claimed by Sutherland and coworkers (1974a, b, 1980b, 1982), Henderson (1978) and D'Ambrosio et al. (1981) could not be confirmed.

A PR-light reduction in UDS, caused by monomerization of pyrimidine dimers was never detected in human cells under the conditions described. Since the photoreactivating capacity of human PRE seems to depend on growth phase and culture medium (Sutherland and Oliver, 1976; Mortelmans et al., 1977) the inability to detect the enzyme under the conditions described is no prove against its existence. The data presented in this paper provide the basis for experiments attempting to introduce cloned *phr*-genes into the genome of mammalian cells. This may enable the study of the role of UV-induced pyrimidine dimers on mutagenesis, carcinogenesis and cell survival.

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