PHOTOREACTIVATION OF TEMPLATE ACTIVITY OF UV–IRRADIATED DNA IN AN RNA–POLYMERASE SYSTEM. A RAPID ASSAY FOR PHOTOREACTIVATING ENZYME*

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1. Introduction

Ultraviolet irradiation of a DNA template diminishes the ribonucleotide incorporation by RNA-polymerase [1,2]. The main effects found are a lowering of the total RNA synthesis by production of shorter RNA chains and a decrease of the polymerisation rate [3–5]. It has been postulated that cyclobutane-type pyrimidine dimers, which are induced by the action of ultraviolet light on DNA, are important lesions since they are thought to act as transcription terminating sites [3].

As has been reported here, part of the reduction of template activity can be photoreactivated with DNA photoreactivating enzyme and visible light. This finding has resulted in a simple assay for photoreactivating activity which is much more rapid that the commonly used *Haemophilus influenzae* transformation assay.

2. Materials and methods

2.1. *Photoreactivation*

Highly purified DNA photoreactivating enzyme was isolated from *Streptomyces griseus* vegetative cells according to a procedure described in detail elsewhere [6]. In most experiments a preparation was used with a specific activity of 44 400 PRA** units/mg protein and a protein content of 81 μg/ml. PRE was diluted in buffer containing 0.1 M NaCl, 0.01 M K_2HPO_4, 5×10^{-3} M β-mercaptoethanol and 0.1% BSA, pH 7.4.

A mixture was prepared in the dark, consisting of 0.3 ml UV-DNA (see below) in SSC and 0.3 ml of suitably diluted PRE, distributed over two test tubes and incubated for 30 min in a waterbath at 35°C. One test tube was kept in the dark while the other was illuminated with a fluorescent lamp (Sylvania F15T8 cool white) at a distance of 2 cm, through 1 cm window glass. When not tested immediately, samples were kept frozen at −20°C.

2.2. *Ultraviolet irradiation*

Ultraviolet irradiations were performed with a germicidal lamp (General Electric G4T4.1). The dose rate at 254 nm was measured with an ultraviolet meter (Ultra-violet Products Inc. San Gabriel).

2.3. *RNA-polymerase assay*

DNA-dependent RNA-polymerase from *E. coli* K 12 was either bought (Miles Laboratories Inc.) or isolated according to Burgess [7] including the DEAE-cellulose step. Calf thymus DNA (Sigma type V) in SSC (344 μg/ml) was irradiated in a stirred cuvette with an optical pathlength of 1 cm.

The incubation mixture contained: 12.5 μmol NaCl 0.5 μmol K_2HPO_4, 0.75 μmol sodiumcitrate, 10.5 μmol Tris, 7.5 μmol KCl, 1.5 μmol $MgCl_2$, 0.25 μmol $MnCl_2$, 5 nmol EDTA, 2.5 μmol β-mercaptoethanol, 2.5 μl glycerol, 80 nmol each of 4 ribonucleotide triphosphates, 1 μCi 2-[^{3}H]ATP (20–50 Ci/mmol), 17.2 μg (UV-)DNA, 2.8 μg RNA-polymerase and 75 μg BSA. In photoreactivating experiments a mixture

* Part one in the series 'Photoreactivating enzyme from *Streptomyces griseus*'.

** *Abbreviations used*: PRE photoreactivating enzyme, BSA bovine serum albumin, BHI brain heart infusion medium, SDS sodium dodecylsulphate, TCA trichloroacetic acid, SSC 0.15 M NaCl–0.015 M sodium citrate (pH 7.0).

of UV-DNA and PRE (see 2.1) was used yielding the same DNA concentration. Total volume was 0.25 ml, pH 7.8. After incubation at 36°C for 30 min the reaction was stopped by addition at 0°C of 2.5 ml 3.5% TCA containing 10 mM $Na_4P_2O_7$. Acid insoluble material was filtered off (Schleicher & Schüll BA 85 membrane filters), washed 3 times with the TCA solution, dried and counted for radioactivity in a liquid scintillation counter.

2.4. *Transformation assay*

Transforming DNA from a streptomycin resistant *Haemophilus influenzae* strain was isolated according to the procedure of Marmur [8] supplemented with pronase deproteinization. DNA (10 μg/ml in SSC) was UV irradiated (5800 erg/mm^2), diluted with SSC to a final concentration of 0.5 μg/ml and kept frozen at −80°C. The remaining transforming activity was 0.13%.

Competent streptomycin sensitive *H. influenzae* cells were grown in a medium containing 37 g BHI, 10 mg NAD, 20 mg hemin and 20 mg L-histidine (the last two previously dissolved at 1 mg/ml in 4% triethanolamine) per litre by the method of Barnhart and Herriott [9] with omission of the second agitation period, and stored after addition of 15% glycerol at −80°C.

For transformation, 2.8 ml of competent cells diluted 1:50 in growth medium was added to 0.2 ml of a photoreactivated mixture (see 2.1) containing transforming DNA and incubated with gentle agitation at 37°C for 2 1/2 hr in the dark. Then, in duplicate, 0.1 ml samples were mixed in plastic dishes with 10 ml agar medium containing per litre 24.7 g BHI, 10.1 g Eugon broth, 15 g bacto agar, 0.75 g streptomycin and supplements as mentioned before.

Streptomycin resistant colonies were counted after at least 24 hr of incubation at 37°C. PRE activity was expressed in units of photoreactivating activity PRA = $(N_L - N_D)/N_D$ according to Muhammed [10], where N_D is the number of transformants after dark treatment and N_L after photoreactivating treatment.

3. Results and discussion

The dose-effect curve for the UV induced inactivation of template activity of calf thymus DNA is shown in fig.1. When UV-DNA was treated with PRE and visible light, part of the template activity was restored. However, even under conditions of maximal photoreactivation, when nearly all of the pyrimidine dimers are removed, considerable inactivation remained. The maximal photoreactivable sector PRS_{max} [11] showed a slow decrease with incident UV dose, indicating an increase of non-photoreactivable lesions proportional to the UV dose. The [^{3}H]AMP incorporation turned out to be the same with UV-DNA template whether or not PRE was present (in the dark), revealing that under the conditions used RNA-polymerase is not inhibited by the complex formed between PRE and the pyrimidine dimer sites on UV-DNA.

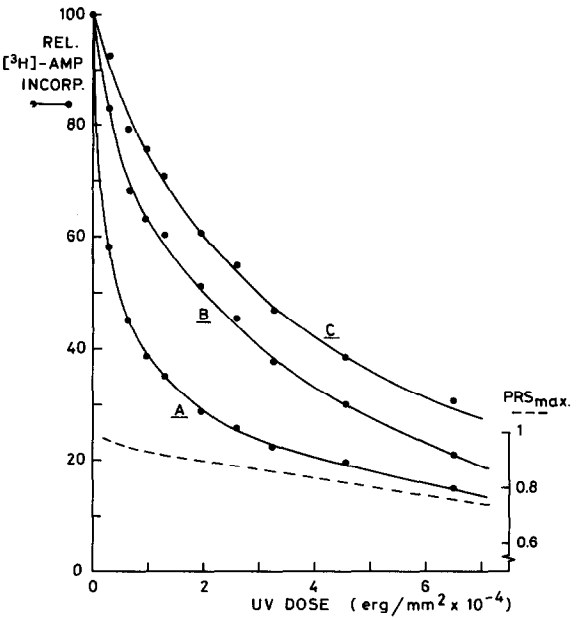

Fig. 1. Dose-effect curves for [^{3}H]AMP incorporation with differently treated UV irradiated DNA templates. A: UV-DNA kept in the dark, B: UV-DNA photoreactivated with PRE diluted 1:20, C: UV-DNA maximally photoreactivated; after normal photoreactivation 50 μl of undiluted PRE was added and the photoreactivating treatment was continued for 10 min. Dashed curve: maximal photoreactivable sector PRS_{max} calculated from curves A and C.

Hagen et al. [4] described a decrease in the chain length of RNA synthesized on UV-DNA, so the influence of photoreactivation on chain length was studied by sucrose gradient centrifugation. The sedimentation patterns given in fig.2 show a distinct shift of the maximum to a longer chain length after

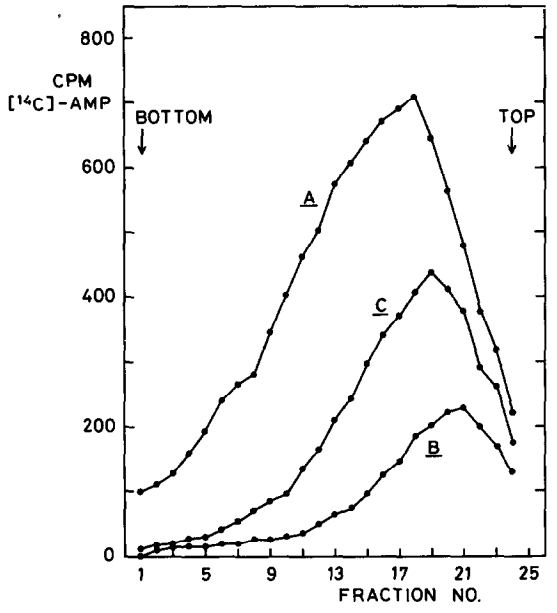

Fig. 2. Zone sedimentation in sucrose gradient of RNA synthesized with different templates. A: unirradiated DNA, B: UV-DNA (33 000 erg/mm^2), C: photoreactivated UV-DNA, PRE was diluted 1:3. The RNA polymerisation mixture (0.5 ml contained twice the amounts mentioned in 2.3 and 8-[^{14}C]ATP instead of [^{3}H]ATP. The reaction was terminated by addition of 20 µl 5.4% SDS and 20 µl 0.27 M EDTA both in sedimentation buffer (0.1 M NaCl, 0.01 M Tris and 0.2% SDS, pH 7.5). A sample of 0.2 ml was layered on a 5 ml sucrose gradient (10–30% RNase-free sucrose in sedimentation buffer) and centrifuged for 4 hr at 50 000 rev/min at 20°C (Spinco L2-65B, SW 50L rotor). Fractions of 8 drops were collected and treated after addition of 0.1 ml PRE dilution buffer as normal polymerisation mixtures.

photoreactivation, although the contribution of larger RNA molecules remains relatively low.

Weight-average molecular weights were calculated from various sedimentation experiments, see table 1.

It is clear that photoreactivation with respect to chain length was also incomplete. Still, it may be concluded that at least part of the pyrimidine dimers act as terminating sites for RNA synthesis, supporting the idea put forward earlier [3–5] and also proposed for a DNA-polymerase system [13].

The nature of the non-photoreactivable lesions is uncertain. Breakage of DNA chains as a result of UV irradiation causes a decrease of priming activity [2,4]. It is also known that certain non-cyclobutane type

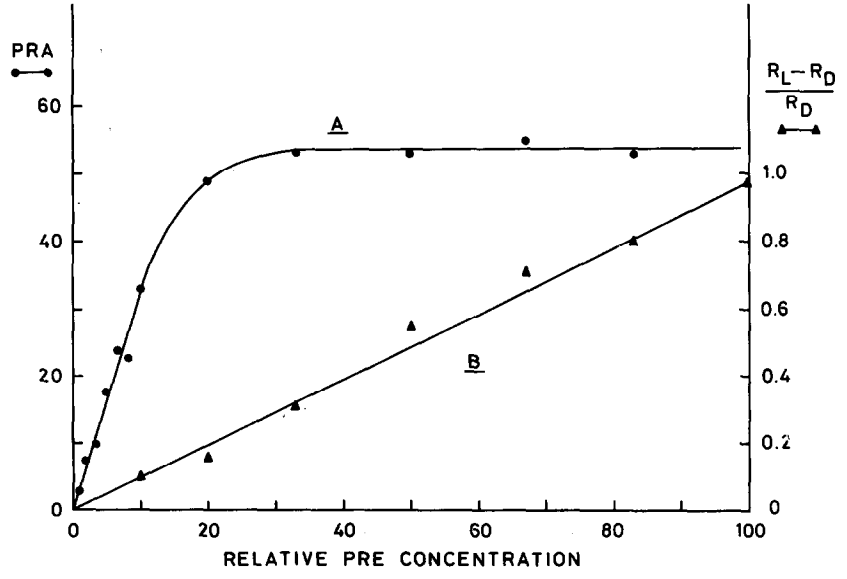

Fig. 3. Photoreactivating activity was measured as a function of the PRE concentration. Curve A represents the results of the transformation assay and curve B represents the RNA-polymerase assay. Template DNA was irradiated with 33 000 erg/mm^2.

Table 1
Chainlength of RNA synthesized after photoreactivation

UV dose erg/mm²	PR-treatment	$M_{max} \times 10^{-5}$	$M_W \times 10^{-5}$
0	–	0.73	2.4
1.9×10^4	D	0.49	1.4
1.9×10^4	L	0.73	1.8
0	–	0.84	2.8
3.3×10^4	D	0.23	1.4
3.3×10^4	L	0.60	1.7
0	–	0.73	2.3
6.5×10^4	D	0.17	1.1
6.5×10^4	L	0.49	1.4

D was incubated in the dark and L was photoreactivated. M_{max} denotes values calculated for the peak of the sedimentation pattern. Sedimentation constants were converted to molecular weights according to $M = 1435\ S^{2.12}$ which is almost identical to Spirins's relation [12]. Number-average molecular weights were not calculated owing to uncertainties introduced by the relatively large contribution of low molecular weight fractions.

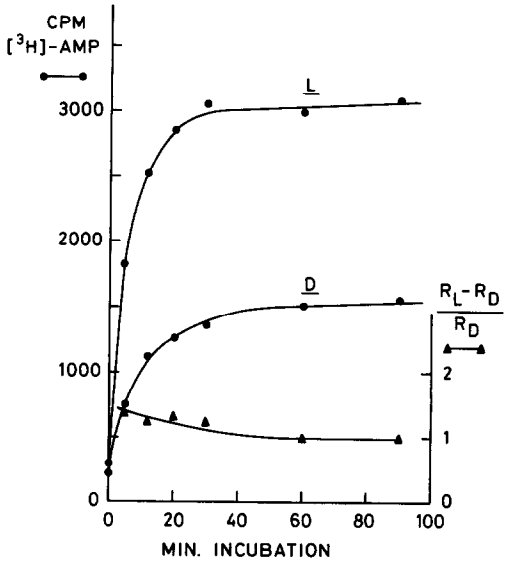

Fig. 4. Time course of the [³H]AMP incorporation with UV-DNA (33 000 erg/mm²) which had undergone dark (D) or photoreactivating (L) treatment. PRE was diluted 1:10.

photoproducts (C-T precursor of U-T adduct [14,15]) cannot be photoreactivated enzymatically.

The question arose as to whether the photoreactivability of template activity could be used as an assay for enzyme activity in PRE preparations. There is a need for a rapid test such as this since the transformation assay generally used [16] is fairly laborious and it takes at least 24 hr before results are known. This has led to the publication of alternative assays [17–19].

A linear correlation was found experimentally between PRE concentration and $(R_L-R_D)/R_D$ – see fig.3 – except for high PRE concentrations. R_D and R_L are the respective ribonucleotide incorporations after dark and photoreactivating treatment of the UV-DNA template. A similar correlation has been accepted for the transformation assay (see 2.4). However, from the slopes of the linear part of both curves in fig.3 and considering the experimental accuracies it can be estimated that the RNA-polymerase assay is less sensitive than the transformation assay.

The time course of [³H]AMP incorporation is shown in fig.4. Polymerisation ceased rather rapidly, suggesting that no reinitiation occurred. Furthermore $(R_L-R_D)/R_D$ does not vary much with incubation time.

The RNA-polymerase assay, yielding results within a few hours, was used routinely in PRE purification [6] for testing chromatographic column fractions. On the whole a good correspondence was obtained between the two assays (fig.5). However, the RNA-polymerase assay is sensitive to RNase and DNase and did not yield reliable results with crude extracts prepared from *S. griseus*.

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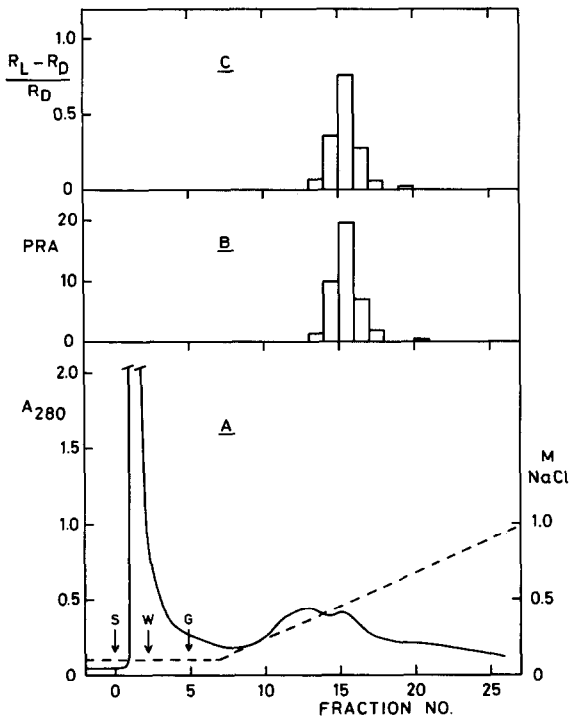

Fig. 5. Fractionation of partially purified PRE on a DNA-sepharose column (1 × 18 cm, flow rate 16 ml/hr, 15 min fractions). DNA was covalently linked by a cyanogenbromide treatment to Sepharose-4B [20]. A: absorbance at 280 nm measured in a 2.5 cm flow cell. B: photoreactivating activity measured with the transformation assay. C: the same with the RNA-polymerase assay. A sample of 140-fold purified PRE was applied (S) and after washing with enzyme dilution buffer minus BSA (W) elution was performed with a linear salt gradient (G).

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