Quantitative characterization of pyrimidine dimer excision from UV-irradiated DNA (excision capacity) by cell-free extracts of the yeast *Saccharomyces cerevisiae*

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Cell-free extracts from wild-type yeast (RAD^+) and from rad mutants belonging to the RAD3 epistatic group (rad1-1, rad2-1, rad3-1, rad4-1) contain activities catalyzing the excision of pyrimidine dimers (PD) from purified ultraviolet-irradiated DNA which was not pre-treated with exogenous UV-endonuclease. The level of these activities in cell-free extracts from rad mutants did not differ from that in wild-type extract and was close to the in vivo excision capacity of the latter calculated from the LD_{37} (about 10^4 PD per haploid genome).

Pyrimidine dimer excision *Cell extract* *rad mutant* *Yeast*

1. INTRODUCTION

It is generally believed that UV-sensitive mutants belonging to the RAD3 epistatic group are defective with respect to in vivo incision of UV-damaged DNA. These data were obtained using the method of detection of UV-endonuclease-sensitive sites [1,2]; we confirmed this in experiments on the rad3-1 mutant using the same approach [3]. It was suggested that due to the excision defect mutants of this group are able to ligate the incisions in UV-irradiated DNA. However this suggestion does not seem to be valid in the light of data that were obtained with a double mutant, which carried both the rad mutation and the cdc9 mutation, that is also defective in DNA ligase [4]. Despite the absence of in vivo incision we showed that cell-free extracts of RAD3 group mutants contained a nuclease activity specific for UV-irradiated Col E1 DNA [5]. The level of this activity did not differ from that in wild-type extracts.

Simultaneously authors in [1] reported that they were unable to detect the incision of UV-irradiated DNA by yeast cell-free extracts. On the other hand, they reported that the extracts of both wild-type yeast and RAD3 group mutants catalyzed the excision of PD from UV-irradiated DNA, that had been pretreated with *Micrococcus luteus* UV-endonuclease activity [6]. They noted in [1] that their data disagree with ours [5]. This controversy prompted us to assay both qualitatively and quantitatively in vitro the PD excision activity in extracts of wild-type and rad mutant cells using UV-irradiated DNA that had not been pretreated with exogenous UV-endonuclease.

We describe here results showing that cell extracts from both wild-type yeast (RAD^+) and rad1, rad2, rad3 and rad4 mutants contain activities catalyzing the excision of PD from UV-irradiated purified DNA without pretreatment with exogenous UV-endonuclease activity. The levels of these activities were the same for all the strains studied and corresponded well to the in vivo excision capacity of wild-type cells. Our previous data [5] were confirmed in the special sets of experiments.

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2. EXPERIMENTAL

The haploid yeast strains used in this study, RAD^+, rad1-1, rad2-1, rad3-1, rad4-1 (from the collection of B.S. Cox [7,8]) were obtained from I.A. Zakharov (this laboratory). Cells were grown in YEP medium (1% yeast extract, 2% peptone, and 2% glucose) with constant agitation for 18 h at 28°C. Cells were collected by centrifugation, washed twice with buffer A [20 mM Tris–HCl (pH 7.8), 0.5 mM EDTA, 0.5 M KCl, 1 mM phenylmethylsulfonyl fluoride], resuspended in the same buffer and disrupted in a Hughes press at 1500 kg/cm^2. Extracts were centrifuged for 30 min at 10000 × g. All extracts were prepared from the same number of cells (1.5×10^9 cells/ml) and had the same protein concentration (about 15 mg/ml). The clarified extracts were diluted to 0.1 M KCl. All operations were performed at 4°C.

UV-endonuclease activity was determined by agarose gel electrophoresis, using ^{14}C-labeled Col E1 DNA as in [5]. UV-irradiated DNA as a substrate for this study was prepared by irradiation of Col E1 DNA at 254 nm at 60 J/m^2. All enzyme assays were done in duplicate with 3 independently prepared cell extracts.

Dimer excising activity in yeast extracts was determined by two independent methods: (i) chromatographic measurement of thymine dimer content; (ii) radioimmunoassay (RIA) for the detection of remaining PD in UV-irradiated DNA after incubation with extracts. [^{3}H]Thymidine-labeled *E. coli* DNA was used as a substrate for determination of PD excising activity. [^{3}H]DNA was purified from *E. coli* AB1157 thy$^-$ cell lysate by treatment with proteinase K (Sigma), extraction with buffered phenol–chloroform (1:1, v/v), and successive gel filtration on Sepharose CL-4B (Sigma). The final [^{3}H]DNA (3×10^5 cpm/μg) was irradiated at 254 nm with a dose sufficient to convert about 2% of the labeled thymine residues into thymine-containing PD. The standard reaction mixture (0.5–0.6 ml) for chromatographic measurement of PD excision contained 0.2–2 μg [^{3}H]DNA, 50 μg carrier DNA, 20 mM Tris–HCl (pH 7.8), 0.5 mM EDTA, 80 mM KCl and 0.5–5 mg extract protein. In control samples buffer A was added instead of cell extract. After incubation in the dark at 30°C for 90 min the reaction was terminated by the addition of SDS and NaCl (final concentrations 1% and 1 M, respectively). Following precipitation of the protein–SDS–NaCl complex at 0°C DNA was precipitated from the supernatant by addition of $HClO_4$ to 7%. Air-dried precipitates were hydrolyzed in 99% HCOOH at 175°C for 60 min. The pyrimidines and PD were separated by thin-layer chromatography on Silufol-254 nm plates (La Chema, CzSSR) in ethyl acetate–n-propanol–H_2O (4:1:1) as in [9]. The PD content was calculated as the percentage of radioactivity in thymine dimer containing spots to the total radioactivity in all thymine containing spots including the dimer region.

Radioimmunoassay of PD excision was performed as follows. UV-irradiated *E. coli* [^{3}H]DNA was incubated with or without cell extracts as in chromatographic PD determination. After precipitation of protein–SDS–NaCl complex as described above DNA was additionally deproteinized by a phenol–chloroform mixture (1:1, v/v) and dialyzed against buffer containing 10 mM Tris–HCl (pH 7.8) and 0.15 M NaCl. Filter RIA was carried out as in [10]. One hundred μl of dialyzed UV-irradiated *E. coli* [^{3}H]DNA was mixed with 10 μl of the antiserum against UV-irradiated DNA diluted 10-fold (large excess of antibodies). Antiserum was obtained from Dr L.A. Zamchuk (Institute of Chemical Physics, Moscow). After incubation for 20 min at 37°C the samples were diluted with the same buffer and passed through nitrocellulose filters (Synpor 6, CzSSR, 0.4 μm pore diameter). The filters were washed with the 10 ml of the same buffer, dried, and the radioactivity measured. PD excising activity was defined as activity catalyzing a reduction in UV-DNA retention on the filters in percent relative to appropriate controls, i.e., binding of unirradiated DNA by serum plus UV-DNA binding without yeast extract treatment.

3. RESULTS AND DISCUSSION

It was found that cell-free extracts of RAD^+ as well as rad1-1, rad2-1, rad3-1, and rad4-1 incised specifically UV-irradiated Col E1 DNA. The levels of this activity were equal to about $0.8–1 \times 10^{12}$ incisions per mg protein or 10^8 cells. Thus, good agreement was established between the level of PD-incising activity and in vivo excision capacity

of wild-type RAD$^+$ strain (about 10^4 PD per haploid cell) calculated according to LD_{37} [2,11,12].

The data for chromatographic determination of PD excision from UV-irradiated [^{3}H]DNA by cell-free extracts of yeast are shown in table 1. There are no significant differences in the PD-excision capacity between all strains used. The number of PD excised from UV-irradiated DNA was 1–1.4 × 10^{12} per mg protein or per 10^8 cells. About 40–60% of PD was released from UV-irradiated DNA under these conditions and about 6% of total labelled thymine was converted to an acid-soluble form (column 4, table 1). These data are in agreement with those in [6] from experiments with preincised DNA.

Similar results were obtained by determination of the PD-excising capacity of cell extracts from RAD$^+$ and rad3-1 strains of yeast by RIA (table 2). The good agreement between results obtained by two independent methods indicates that extracts from the excision-proficient RAD$^+$ strain as well as from UV-sensitive mutants of the RAD3 epistatic group were able to excise a significant fraction of PD from purified UV-irradiated DNA without its preincision by UV-endonuclease activities.

We do not know the reasons for the disagreement between the data of authors in [1] and ours, since these authors did not describe their methods of PD-incision determination. Possibly they used the same PD doses as for PD excision determination from preincised UV-irradiated DNA, in which case the total number of PD in the assay mixtures was significantly higher than the incision activity of the extracts. We have also been unsuccessful in demonstrating PD excision from UV-irradiated non-preincised DNA (see table 2, line 1) under reaction conditions similar to theirs (about 1.3 × 10^{14} PD per mg protein, i.e., the number of PD was more than 2 orders of magnitude higher than the excision capacity of yeast and UV-endonuclease activity of the cell extracts determined by us).

Our results suggest that the incision of UV-irradiated purified DNA by yeast cell extracts in vitro significantly differs from the incision process in vivo. Its successful completion probably requires coordinated action of several proteins and depends on organization of the genetic material in eukaryotic cells. An analogous situation was observed in fibroblasts of *Xeroderma pigmentosum* [14].

In the light of our data and other observations [1,2,6,13] it is clear that early events (incision or preincision) in PD excision are a limiting step.

Table 1

Percent removal of pyrimidine dimers from purified UV-irradiated *E. coli* DNA catalyzed by cell extracts of *S. cerevisiae* (chromatographic determination)

Strain	Protein in assay (mg)			
	0.5	1	2	4
RAD$^+$	12	24	41	44
rad1-1	14	23	28	46
rad2-1	10	30	29	40
rad3-1	8	23	36	48
rad4-1	14	27	32	61

One mg of protein corresponds to 10^8 cells. The number of PD in all assay samples was 5 × 10^{11}; 100% = 2% of the thymine base present in the DNA in the form of thymine-containing PD. The data show a difference in the percent of PD in UV-irradiated *E. coli* [^{3}H]DNA incubated with and without cell extracts of subsequent strains of yeast. All data are the means from duplicate determination with 3 independently prepared cell extracts; SD ± 10%

Table 2

Removal of pyrimidine dimers from purified UV-irradiated [^{3}H]DNA catalyzed by cell extracts of *S. cerevisiae* (%) (radioimmune determination)

	Contents in assay mixtures		PD excision (%)	
	Protein of cell extract (mg)	Number of PD (×10^{-11})	RAD$^+$	rad3-1
(1)	0.05	10	0	5
(2)	0.25	8	41	36
(3)	1.0	10	60	51
(4)	10.0	10	63	46

One mg of protein corresponds to 10^8 cells. The data are the means from 3 independent determinations

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