DNA repair in a small yeast plasmid folded into chromatin

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ABSTRACT

The question of whether excision repair of yeast plasmids accurately reflects the repair of yeast genomic chromatin has yielded conflicting answers. These conflicts could have arisen from differences in the conformation of plasmid molecules used during these studies. We have examined excision repair of UV photoproducts in a small (2619 bp) autonomously replicating plasmid (YRp-TRURAP), known to be folded into chromatin with positioned nucleosomes *in vivo*, in the yeast *Saccharomyces cerevisiae*. A quantitative assay was used to measure the yield of cyclobutane pyrimidine dimers (PD) in plasmid DNA by measuring the fraction of Form I molecules resistant to T4 endonuclease V. After a UV dose of 100 J/m², which yields 1.2 PD/plasmid in irradiated cells, radiation insensitive (*wt*) cells repair ~70% of the PD in TRURAP chromatin in 2 hr (a rate comparable to that of genomic chromatin). On the other hand, no measurable repair occurs in TRURAP chromatin in radiation sensitive cells (*rad1*) during the same time period. Thus, this small plasmid contains sufficient chromatin structure *in vivo* to reflect the incompetent repair of genomic chromatin seen in a *rad* mutant, while maintaining the competent repair level in *wt* cells.

INTRODUCTION

The 1453 bp TRP1ARS1 circle is one of the smallest autonomously replicating yeast plasmids constructed to date and exists as extrachromosomal DNA in high copy numbers (1). This completely sequenced plasmid contains the *TRP1* gene, a segment required for autonomous replication (ARS1) and a segment of unknown function (UNF) (2, 3). The chromatin structure of TRP1ARS1 has been studied in detail (4–6). These studies indicate there are four 'unstable' nucleosomes covering most of the *TRP1* gene, three stable nucleosomes precisely positioned in the UNF region and two nonnucleosome 'gaps' between these regions.

More recently, Thoma and colleagues constructed a variety of plasmids containing insertions in TRP1ARS1 to investigate the influence of DNA sequence on nucleosome placement (6–8). One such construct is the plasmid YRp-TRURAP which contains the *URA3* gene (coding for orotidine-5'-phosphate carboxylase) on a 1166 bp *Hind* III fragment inserted into the *Hind* III site of

the *TRP1* gene (Figure 1). This plasmid contains seven additional nucleosomes, six in the coding sequence of *URA3* and one at the 3' end of the *Hind* III fragment (7). There are also two additional nonnucleosome gaps in TRURAP, a large one (~110 bp) just upstream from the *URA3* coding sequence and a smaller one just downstream from this sequence.

We have chosen the TRURAP plasmid as a simple, model substrate for studying DNA repair in chromatin. Studies on DNA repair in mammalian cells have revealed a number of distinctive features of the repair process in chromatin (9); however, the molecular details of such events will only be elucidated with a simple substrate of well-defined chromatin structure and the use of well-characterized repair mutants. Both the large number of repair mutants (*rad*) and the ease of plasmid manipulation, make yeast an ideal organism for examining repair at the molecular level (10). Indeed, numerous genetic studies have identified over 30 *RAD* (radiation sensitive) loci in yeast (10), and at least some of these genes may share homology with mammalian repair genes (11).

Only a limited number of reports have appeared on the repair of yeast plasmids (12–18). Most of these studies involved repair of exogenously added plasmids, which were damaged as isolated DNA prior to transformation or incubation with cell extracts (13–17). It was shown that *rad 1*, *2*, *3* and *4* mutants, which all belong to the same epistasis group and are incapable of repairing UV-induced PD in genomic DNA (19), are fully capable of 'reactivating' the exogenously added UV irradiated plasmid DNA (13, 14). Furthermore, cell-free extracts of these mutants contain activities catalyzing excision of PD from purified, UV-irradiated plasmid DNA (20). These authors suggested that the *RAD1*, *RAD2*, *RAD3* and *RAD4* genes may be required only for excision repair of nuclear DNA. In addition, the enhanced resistance of yeast to UV radiation acquired following pre-exposure to a low UV dose is not reflected by enhanced repair in exogenously added plasmid DNA (16). On the other hand, McCready and Cox (12) examined excision repair in the 2 μ m plasmid in intact *S. cerevisiae* cells. These authors reported that repair of the 2 μ m plasmid reflects the radiation sensitive phenotype of *rad* cells observed in repair of chromosomal DNA (12, 18). It is important to note that these latter results represent repair of plasmid *chromatin*, since the experiments were performed on intact cells which contained the 2 μ m plasmid prior to UV irradiation.

These different observations could reflect differences in the

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CHROMATIN STRUCTURE OF TRURAP

Figure 1. Chromatin structure and sequence construction of the plasmid YRp-TRURAP. The TRP1ARS1 circle was disrupted at the *Hind* III site (615 Map Units) in the *TRP1* gene by insertion of the *URA3-Hind* III fragment in the same orientation (7). The arrow heads and associated numbers indicate the approximate locations (± 20 bp) of the strong cutting sites for staphylococcal nuclease (7). Nucleosomes numbered 1–6 are tightly packed on the *URA3* gene (7) and nucleosomes I–III are positioned on the region of unknown function (UNF; 5–7).

response of repair enzymes operating on naked (or partially folded) DNA as compared to DNA already folded into chromatin. They also emphasize the need to establish the overall repair characteristics of a plasmid construction in both *wt* and *rad* cells if the plasmid is to be used as a model for genomic repair. Clearly, the results of McCready and co-workers (12, 18) indicate that the 2 μ m plasmid could be used as a model chromatin substrate. However, the structure of this plasmid is much more complex than YRp-TRURAP. For instance, it contains ~ 35 nucleosomes (21, 22) and at least 5 different genes (23). Thus, we have examined the overall UV damage and repair characteristics of YRp-TRURAP as chromatin in *S. cerevisiae* to determine if this simple, well-characterized plasmid can be used for detailed studies on chromatin repair.

MATERIALS AND METHODS

Yeast strains

Saccharomyces cerevisiae haploid strain FTY23 (MATa, *ura3-52*, *his3-1*, *gal2*, *gal10*, *trp1*, *cir*^o), containing the autonomously replicating plasmid YRp-TRURAP, was used as the radiation insensitive (*wt*) strain for all of these studies (7). Radiation sensitive strains LP2649-1A (MAT α , *leu2-3*, *leu2-112*, *ura3-52*, *can1*, *rad3-2*) and PRY43 (MATa, *his3- Δ 1*, *trp1-289*, *ura3-52*, *rad1 Δ*) were provided by Dr. L. Prakash (University of Rochester, Rochester, New York). The *rad1* strain was transformed with YRp-TRURAP using the lithium acetate procedure of Ito et al. (24). The presence of YRp-TRURAP in transformants was confirmed by Southern analysis using a labeled probe specific for a fragment of the TRURAP plasmid (Smerdon and Thoma, submitted). The resulting transformant was designated JMY43

Culture and UV irradiation of yeast cells

All liquid cultures of FTY23 and JMY43 cells were grown at 30°C in minimal media (25), supplemented with the necessary amino acids, to optical densities of 1.0 to 2.5 (at 600 nm) prior to irradiation. Cells were collected by centrifugation, resuspended in water to a concentration of $5-100 \times 10^6$ cells/ml and incubated at 30°C, with shaking, for 1 hr to insure arrest of cell division. At concentrations $\geq 5 \times 10^7$ cells/ml the PD yield was reduced (e.g., see Figure 5B) and, therefore, all dose-response experiments were carried out at concentrations below this value.] In most experiments, 50 to 100 ml of cell suspension were transferred to a 12 $\times$ 21 cm plastic tray to produce a thin (2–4 mm) layer of liquid (for uniform exposure). The cells were irradiated with a low pressure Hg lamp (predominantly 254 nm) at a flux of 2.6 W/m². The flux was measured with a black body-thermal radiometer. In other experiments, cells were irradiated in a 18 $\times$ 28 cm pyrex tray with a single (low pressure) Hg lamp at a flux of 4.8 W/m². In this case, the flux was measured by an Ultraviolet Products 'Black-Ray' UV meter. For 'no repair' samples, the cells were chilled to 0°C, irradiated on ice and kept on ice (in the dark) until harvest. For all other samples, cells were irradiated at 30°C and incubated in the dark at 30°C prior to harvest. During all post-irradiation steps, care was taken to not expose cells to photoreactivating light to insure that any repair occurred via the excision repair pathway (10).

Cell survival

Liquid cultures of each strain were grown in complete media (25) to an optical density at 600 nm of ~ 3 . After serial dilution in water, small aliquots (10 μ l) were spread on agar plates containing complete media. After ~ 5 min, the plates were exposed to varying doses of UV light, as described above, and incubated in the dark at 30°C for 2 days prior to determination of colonies/plate.

Preparation of supercoiled TRURAP plasmid

Cells were chilled on ice, collected by centrifugation at 4°C and resuspended in ice-cold zymolyase reaction buffer (250 mM EDTA, pH 8, 1 M sorbitol, 20 mM β -mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride). The high EDTA concentration was used to prevent excision repair during preparation of spheroplasts (see text). Spheroplasts were obtained by incubation with 0.1–0.2 mg/ml Zymolyase (100T; Miles Lab.) for 30–60 min at 30°C in the dark. Samples were then immediately chilled on ice, collected by centrifugation and washed in 1 M sorbitol, 10 mM EDTA (pH 8). Spheroplasts were lysed in alkaline-SDS solution (~ 0.13 N NaOH, 0.67% SDS), as described in Maniatis et al. (26), and the majority of genomic DNA precipitated in 1 M sodium acetate ($\sim$ pH 4.8). After 15 min on ice, samples were centrifuged at $16,000 \times g$ for 10 min, and the resulting supernatants extracted 2–3 times with phenol/chloroform prior to ethanol precipitation. Pellets were resuspended in 0.3 M sodium acetate and digested with 250 μ g/ml RNase A (Boehringer) for 30 min at 37°C. The digests were then clarified by brief centrifugation, extracted with phenol/chloroform and ethanol precipitated. The pellets were resuspended in TE buffer (10 mM Tris, pH 8, 1 mM EDTA) and, in some experiments, this 'crude preparation' of TRURAP was used (see Figures 5 and 6). For other experiments, the TRURAP molecules were further purified by preparative electrophoresis on 1% agarose gels containing TBE buffer (Tris-borate EDTA) (26) and 0.5 μ g/ml ethidium bromide. The supercoiled (Form I) TRURAP band was excised and electroeluted as described previously (27).

UV irradiation of isolated plasmid DNA

Plasmid DNA (15–30 $\mu\text{g/ml}$) was irradiated in T4 endo reaction buffer (see below) in 25 to 180 μl aliquots placed on parafilm or in tops of 1.6 ml microcentrifuge tubes. Samples were irradiated at 2.6 W/m^2 as described earlier.

T4 endonuclease V digestion

T4 endonuclease V (T4 endo) was prepared from *E. coli* strain AB2480 containing the plasmid ptcaden V. This clone was obtained from Drs. K. Valerie and J. de Riel (Temple University). Details on the preparation procedure will appear elsewhere (A. Murad and M. Smerdon, in preparation). Stock solutions of enzyme were stored at 4°C in 50 mM Tris (pH 7.5), 10% ethylene glycol, 10 mM EDTA and had an initial activity of 1.4×10^{12} PD cuts/min/ μl (A. Murad, unpublished results). Nonspecific endonuclease activity was not detected in this enzyme preparation.

Purified plasmid DNA was digested with T4 endonuclease V (1:100 or 1:10 dilution of stock solution) in T4 endo reaction buffer (10 mM Tris, pH 8, 10 mM EDTA, 80 mM NaCl, 1 mg/ml bovine serum albumin) at 37°C for 20 min. [Virtually all Form I DNA containing PD was relaxed to Form II DNA by 5 min and only a small decrease (~5%) in Form I DNA was observed during an additional 30 min incubation (unpublished results).] After digestion, Form I and Form II DNA molecules were separated on 1% agarose gels containing TBE buffer and 0.5 $\mu\text{g/ml}$ ethidium bromide. Gels were photographed through a red filter with Polaroid positive/negative film (types 665 and 55).

Analysis of the fraction of Form I plasmid

Photographic negatives were scanned on a laser densitometer (LKB model 2222). The scans were analyzed on an IBM PS/2 (model 60) computer equipped with a digitizing tablet (Jandel Scientific), using a computer program written by M. Snoddy, Technical Services, Washington State University. The fraction of Form I DNA was calculated from the expression (28, 29).

$$\text{Fraction Form I} = \frac{\text{EF} \int (10^{A_1(x)/\gamma} - 1) dx}{\text{EF} \int (10^{A_1(x)/\gamma} - 1) dx + \int (10^{A_2(x)/\gamma} - 1) dx}$$

where, EF is the efficiency factor for ethidium bromide binding by Form II DNA over that of Form I DNA, $A_i(x)$ is the absorbance of peak i above background at position x , and γ is the film contrast. [A_1 and A_2 correspond to Form I and Form II DNA, respectively.] All scans were in the log-linear portion of the characteristic curve for each film type (0.45–1.2 O.D. at 600 nm) and, therefore, values for γ of 0.75 and 0.65 were used for type 665 and 55, respectively (taken from Polaroid specifications). The value of 1.42 reported for the efficiency factor by Lloyd et al. (30) was taken as the value for EF.

RESULTS

UV sensitivity of FTY23

Since strain FTY23 had not been examined previously for repair competence, we compared the UV survival of this strain to two radiation-sensitive mutants (*rad1* and *rad3*). Actively growing FTY23 cells yielded a D_0 of 67 J/m^2 (i.e., the UV dose at which 37% of the irradiated cells survive). The two mutant strains yielded D_0 values of <2 J/m^2 . These values agree with past reports on *wt*, *rad1* and *rad3* (31,32) and indicate that FTY23 has normal UV sensitivity (i.e., is RAD^+).

Quantitation of Form I DNA

We have used a method similar to that reported by McCready and Cox (12) for monitoring DNA repair in the TRURAP plasmid, with some important modifications. This method makes use of the fact that supercoiled plasmids containing PD will relax to Form II molecules when treated with a UV specific endonuclease (T4 endo). A necessary feature of this analysis is the ability to accurately quantitate the fraction of Form I molecules that are resistant to T4 endo digestion. We have utilized a digitizer and computer program to analyze laser densitometer scans of photographic negatives taken of ethidium bromide stained gels (Materials and Methods). Figure 2 shows an example of a gel photograph (positive) and the resulting scans of the negative. Due to the nonlinear film response, simple area determination of the peaks in the scan will not yield accurate values for the DNA concentrations in each band (28, 29). Furthermore, the fact that Form I DNA binds less ethidium bromide than Form II (30), must also be accounted for in the analysis.

The method we have employed for this study was tested using scans of photographic negatives of restriction digests of sequenced DNAs. Thus, we could test the measured fractions of each band (or set of bands) in the digest against the value determined from the sequence. As shown in Figure 3, our analysis yielded a near one-to-one correlation for all of the fragments (or sets of fragments) analyzed. This was the case even when the concentration of total DNA per set of bands varied by >80 fold (Figure 3, solid squares).

Dose response of TRURAP in intact cells

The UV dose response of TRURAP in intact yeast cells was examined by irradiating FTY23 cells on ice. To prevent repair during the preparation of spheroplasts, the zymolyase digestion was carried out in high EDTA concentration (0.25 M). Inhibition of repair during this step was verified by comparing PD levels in *wt* and *rad* cells (see below). Furthermore, purified Form I DNA was isolated (by preparative electrophoresis) for these studies to eliminate genomic DNA and TRURAP molecules nicked during preparation. This was essential for accurate dose-response curves for naked DNA due to the high absorbance of the 'crude preparation' solution (unpublished observations).

Figure 4A shows the decrease with UV dose in the fraction of Form I DNA from irradiated *wt* cells that is resistant to T4 endo over the range of 0–300 J/m^2 . To determine the average PD yield/plasmid, it was assumed that the distribution of plasmids containing PD followed Poisson statistics. Therefore, the average PD yield at a given dose was calculated from $-\ln P_0$, where P_0 is the fraction of Form I molecules resistant to T4 endo cutting. This fraction was determined from the ratio R_D/R_0 , where R_D is the fraction of Form I DNA present at dose D after T4 endo digestion divided by the fraction of Form I DNA present before T4 endo digestion at the same dose D [i.e., $F(+\text{T4 endo})/F(-\text{T4 endo})$] and R_0 is this ratio of fractions for unirradiated cells. [We note that differences in absolute DNA concentrations between lanes (due to loading errors) are corrected for by this method of calculating the fraction of Form I DNA.] As shown in Figure 4B, this analysis yields a near linear response of PD/plasmid up to 150 J/m^2 . At higher doses, the fraction of Form I molecules resistant to T4 endo was extremely low (Figure 4A) and small amounts of Form III DNA were observed (data not shown). Thus, the error in this analysis increases dramatically at photoproduct levels > 2 PD/plasmid. Linear regression analysis of the data for UV doses 150 J/m^2 gave a yield of 5.42×10^{-6} PD/bp $\cdot \text{Jm}^{-2}$ or about 1.4 PD/plasmid at 100 J/m^2 .

Figure 2. Agarose gel (upper panel) and scans of the photographic negative (lower panel) for isolated Form I molecules of TRURAP exposed to different treatments with UV light and T4 endo. (Upper Panel) DNA samples were exposed (or not exposed) to 75 J/m² UV light, treated (or not treated) with T4 endo and electrophoresed on a 1% agarose gel containing ethidium bromide. The gel was photographed with Polaroid (type 665) positive/negative film. The far left lane contains a *Hind* III digest of λ DNA. (Lower Panel) The photographic negative was scanned using a laser densitometer (Materials and Methods). Scans A-D correspond to lanes 2-5 (starting at left), respectively.

Figure 3. Comparison of the fraction of different restriction fragments calculated from scans of the photographic negatives of ethidium bromide stained agarose gels (fraction measured) to that calculated from the sequence (fraction from sequence). One set of data ($\square$) are for a *Ps*I digest of plasmid pTUB (pBR322 + YRp-TRURAP), yielding fragments of 4.24, 1.57 and 1.17 kb, and photographed with type 665 Polaroid film. The other two sets of data are for a *H*indIII digest of λ DNA photographed with type 665 ($\blacksquare$) or type 55 ($\blacklozenge$) Polaroid film. One of these ($\blacksquare$) represents values determined for the following sets of fragments: 23.13, 9.42 and 6.56 kb (set 1); 2.32 and 2.03 kb (set 2); and 0.56 kb. Each set represents one point.

Because of the initial 'lag' in the data (Figure 4B), presumably due to increased light scattering in the intact cell suspension, this value is actually closer to 1.2 PD/plasmid at this dose. This value is significantly lower than the value we obtained for *isolated* TRURAP DNA (1.27×10^{-5} PD/bp $\cdot$ Jm⁻²) irradiated *in vitro* (Figure 4B). Thus, the shielding produced in intact cells appears to reduce the effective photon flux to less than half the incident flux. Of course, at least some of this 'shielding' must reflect the increased light scattering by the intact cell solutions. It is noted that our value for PD yield in irradiated, naked plasmid DNA (1.27×10^{-5} PD/bp $\cdot$ Jm⁻²) is significantly lower than some values reported previously in the literature for other plasmids. For example, from the dose response data reported for the 5020 bp plasmid pSV2cat (33), we calculate a yield of 2×10^{-5} PD/bp $\cdot$ Jm⁻². This discrepancy could arise from technical differences (e.g., differences in UV meters), the use of different methods of quantitating gel bands, or differences in the DNA sequences of the plasmids studied (i.e., in the number of PD forming sites present).

Repair of TRURAP in intact cells

Replication of TRURAP molecules during the repair period will artifactually increase the fraction of Form I molecules that are resistant to T4 endo cutting, and yield a higher rate of repair than actually occurs. Therefore, cells were incubated in water at 30°C for 1 hr prior to irradiation (10). As shown in Table I, during an 80 min incubation period after the time of irradiation, the number of yeast cells remained essentially constant, whether or not they were irradiated at time 0. [The small decrease observed between irradiated and unirradiated cells is due to our inability to retrieve all of the cells from the plastic tray used for the irradiation step (Materials and Methods).] A similar result was obtained with the *rad* mutant cells. Since replication of the TRP1ARS1 plasmid is directly coupled to replication of genomic

Figure 4. UV dose response of TRURAP. (A) Fraction of Form I molecules before (□) and after (▲) treatment with T4 endo following irradiation of intact yeast cells with different UV doses. Each set of data points (i.e., one -T4 endo and one +T4 endo) represents a different preparation of TRURAP plasmid. (B) Average PD yield at different UV doses, assuming a Poisson distribution, for irradiated TRURAP DNA (□) and TRURAP irradiated in intact cells (▲). Where applicable, the data represent the mean $\pm$ 1 S.D. of the values calculated from panel A.

TABLE I. Number of yeast cells before and after repair incubation

Repair Time (min)	Cells/ml $\times 10^7$ ^a	
	Before Irradiation ^b	After Repair
0	8.2 $\pm$ 1.4	7.2 $\pm$ 1.7
20	8.8 $\pm$ 1.6	7.5 $\pm$ 1.5
40	8.4 $\pm$ 1.4	7.9 $\pm$ 1.9
80	7.6 $\pm$ 2.0	6.4 $\pm$ 0.9

^a Numbers represent mean $\pm$ 1 SD of 4 determinations on 2 separate loadings of a standard hemocytometer using a 1:100 dilution of each sample. ^b Cells were incubated 60 min in water prior to the first determination.

Figure 5. Time course of the removal of PD from TRURAP chromatin in intact cells. (A) Form I molecules were prepared from FTY23 cells (*wt*) irradiated with 100 J/m² UV light and incubated in the dark for the times shown prior to harvest. The DNA was treated (◇, ▲) or mock treated (◆, △) with T4 endo, electrophoresed on agarose gels, photographed and the negatives analysed as described in Figure 3. The different sets of symbols represent data for both a crude preparation of TRURAP DNA (Materials and Methods) (◇, ◆) and Form I TRURAP molecules isolated by preparative electrophoresis (△, ▲). (B) Same as in panel A except JMY43 cells (*rad1*) were used. Data are for crude preparations of TRURAP, and 1×10^8 cells/ml (◇, ◆) or 0.5×10^8 cells/ml (△, ▲) during the radiation step. Note the decrease in PD yield at the higher cell concentration.

DNA (34), it was assumed that replication of TRURAP would also cease in stationary phase cells. This assumption was supported by quantitation of total plasmid molecules in cells after different repair incubations (not shown).

Using this protocol, we monitored the fraction of Form I DNA remaining after T4 endo digestion for different incubation times in *wt* cells. The data in Figure 5A show a clear increase in this fraction over a 2 hr post-irradiation period, indicating that PD are removed from the plasmid DNA. The data also indicate that, for repair studies, one can use the 'crude preparation' of TRURAP rather than purified Form I DNA (Materials and Methods). However, as expected, a larger fraction of nicked plasmid molecules (Form II) are obtained in the crude preparation (Figure 5A, -T4 Endo samples).

Repair of TRURAP chromatin was also examined in *rad* cells. As shown in Figure 5B, no measurable repair of plasmid DNA occurs in *rad1* cells during a 2 hr incubation. This was the case

for PD yields that were equal to, or about half, the initial yield shown in Figure 5A for *wt* cells. These results provide strong evidence that the removal of PD from TRURAP chromatin observed in Figure 5A is the result of nucleotide excision repair in FTY23 cells.

Once it was established that *rad1* cells do not repair TRURAP chromatin, the amount of (residual) repair during the spheroplasting step (Materials and Methods) was determined. This was done by comparing the fraction of Form I DNA resistant to T4 endo in *wt* and *rad1* cells following the same UV dose. Care was taken to adjust each strain to the same cell concentration prior to irradiation to insure the same PD yield/plasmid in each strain (i.e., for the same degree of shielding). The fraction of Form I DNA before and after T4 endo treatment was 0.91 and 0.57, respectively, for *wt* cells and 0.93 and 0.56, respectively, for *rad1* cells. This result indicates that little (if any) repair occurs during the 30–60 min zymolyase digestion step in high EDTA.

To obtain the rate and extent of repair of TRURAP chromatin in *wt* cells, the average number of PD/plasmid was calculated (as done for Figure 4B) for the different repair times. As shown in Figure 6, ~70% of the PD were repaired by 2 hr after a UV dose of 100 J/m². As mentioned earlier, this UV dose results in an initial (average) yield of 1.2 PD/plasmid or about one PD for every 2 kb. This level of PD kills ~87% of rapidly growing FTY23 cells (J. Mueller and M. Smerdon, unpublished results). Therefore, another advantage of arresting growth in water prior to irradiation is that many more cells remain viable and can carry out excision repair(10). This is particularly important in our study where we are examining repair in a very small plasmid.

Finally, the data in Figure 6 also indicate that the repair efficiency is greater after a dose of 50 J/m². In this case, only about 50% of the plasmids contain PD immediately after irradiation (Figure 4). This result indicates that the repair system

is not saturated in these cells by the level of PD following 50 J/m².

DISCUSSION

We have examined the repair of UV-induced PD in the 2619 bp autonomously replicating plasmid YRp-TRURAP in the yeast *S. cerevisiae*. By measuring the fraction of Form I molecules resistant to a UV-specific endonuclease, both the PD yield and the repair time course were measured. The general features of this method were first reported by McCready and Cox (12). In the present study, some improvements in this method are noted. First, isolation of Form I DNA (by preparative electrophoresis) eliminated the high background staining due to genomic DNA, which can be significant when this DNA migrates near the plasmid band (12), and many of the nicked plasmid molecules obtained in the crude preparation (Figure 2). Second, the use of a purified UV specific endonuclease (from the cloned T4 *denV* gene) to cleave Form I molecules may be more representative of repair of a single class of lesions (i.e., PD), since crude extracts can contain activities for cleaving at sites other than PD (35). Third, the laser scanning and computer digitizing methods allowed accurate determination of DNA concentrations in ethidium bromide stained bands from the photographic negatives and incorporated a correction for the differential binding of ethidium bromide to Form I and Form II DNA (Materials and Methods). Finally, use of growth-arrested cells improved the assay in two ways: (1) Plasmid replication, which yields an artifactual increase in repair by this assay, is (presumably) greatly reduced; and (2) The cells are much less sensitive to the doses of UV radiation required to form easily measurable levels of PD in the small TRURAP plasmid. However, even with these changes in the method, the *rate of repair* we observe in TRURAP chromatin is comparable to the rate of repair of the 2 μ m plasmid reported by McCready and Cox (12).

As mentioned in the Introduction, it has been reported that yeast *rad 1*, 2, 3 and 4 mutants, all belonging to the same epistasis group, are fully capable of repairing PD in exogenously added plasmids which are irradiated with UV light prior to transformation (13, 14). These authors suggested that the normal repair capacity elicited by the mutants in this assay may reflect defects in these mutants for repairing DNA folded into chromatin, but not the (naked) plasmid DNA added during transformation. This hypothesis is similar to that proposed by others, working with human cell extracts, who find near normal removal of PD from naked DNA in extracts of xeroderma pigmentosum cells (36, 37). On the other hand, McCready et al. (18) observed defective repair of UV damage in the 2 μ m plasmid in intact *rad 1*, 2, 3, 4 and 16 mutant cells. Our results confirm and extend these findings to a small plasmid, constructed in the laboratory, which contains positioned nucleosomes and nucleosome gaps (Figure 1).

Finally, the repair system we describe here has a number of advantages for studying the molecular details of excision repair in chromatin. As mentioned earlier, the detailed nucleosome mapping studies have yielded precise locations of nucleosomes in the TRURAP circle (Figure 1). Therefore, since this plasmid is small and sequenced, it is now possible to examine repair of DNA lesions at specific sites within these different structural domains to determine if correlations exist between repair efficiency and nucleosome placement.

Figure 6. Fraction of total PD removed from TRURAP in intact FTY23 cells following a UV dose of 50 J/m² (□) or 100 J/m² (◆, ▲, ■). The data were calculated from the equation

$$1 - \frac{\ln P_0(t)}{\ln P_0(0)},$$

where $P_0(t)$ is the fraction of Form I molecules resistant to T4 endo cutting after time t . The symbols (◆, ▲) correspond to the fraction of PD repaired, calculated from the data in Figure 5A. The symbol (■) corresponds to a set of values obtained for another crude preparation of TRURAP from FTY23 cells.

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