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Molecular Mechanisms of Pyrimidine Dimer Excision in *Saccharomyces cerevisiae*: Incision of Ultraviolet-Irradiated Deoxyribonucleic Acid In Vivo

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A group of genetically related ultraviolet (UV)-sensitive mutants of *Saccharomyces cerevisiae* has been examined in terms of their survival after exposure to UV radiation, their ability to carry out excision repair of pyrimidine dimers as measured by the loss of sites (pyrimidine dimers) sensitive to a dimer-specific enzyme probe, and in terms of their ability to effect incision of their deoxyribonucleic acid (DNA) during post-UV incubation in vivo (as measured by the detection of single-strand breaks in nuclear DNA). In addition to a haploid *RAD*$^+$ strain (S288C), 11 different mutants representing six *RAD* loci (*RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD14*, and *RAD18*) were examined. Quantitative analysis of excision repair capacity, as determined by the loss of sites in DNA sensitive to an enzyme preparation from *M. luteus* which is specific for pyrimidine dimers, revealed a profound defect in this parameter in all but three of the strains examined. The *rad14-1* mutant showed reduced but significant residual capacity to remove enzyme-sensitive sites as did the *rad2-4* mutant. The latter was the only one of three different *rad2* alleles examined which was leaky in this respect. The UV-sensitive strain carrying the mutant allele *rad18-1* exhibited normal loss of enzyme-sensitive sites consistent with its assignment to the *RAD6* rather than the *RAD3* epistatic group. All strains having mutant alleles of the *RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD14* loci showed no detectable incubation-dependent strand breaks in nuclear DNA after exposure to UV radiation. These experiments suggest that the *RAD1*, *RAD2*, *RAD3*, *RAD4* (and probably *RAD14*) genes are all required for the incision of UV-irradiated DNA during pyrimidine dimer excision in vivo.

Current models of the molecular mechanism of pyrimidine dimer excision in ultraviolet (UV)-irradiated cells suggest that enzyme-catalyzed incision of deoxyribonucleic acid (DNA) is a very early biochemical event (see references 9 and 10 for recent review). The incision of DNA is thought to be followed by exonucleolytic removal of the damaged regions and by synthesis of new DNA using the opposite complementary strand as a template. The excision repair process is completed by the rejoining of newly synthesized to extant DNA by DNA ligase. It is obvious that such models predict the presence of single-strand breaks in the DNA of UV-irradiated cells. These single-strand breaks are expected to persist until strand rejoining by DNA ligase is completed; hence, their detection and subsequent disappearance constitutes a probe for studying the excision of pyrimidine dimers in vivo.

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The appearance of single-strand breaks in DNA after UV irradiation has been observed in a variety of biological systems including *Escherichia coli* (1, 15, 30, 36), bacteriophage λ lysogens (29, 32), bovine fibroblasts (5), and human fibroblasts (6, 7, 31). The relationship of these DNA strand breaks to the excision of pyrimidine dimers is perhaps best established in *E. coli* (1, 15, 30, 36). Significantly fewer single-strand breaks are evident in strains of *E. coli* known to be abnormally UV-sensitive and defective in the excision of pyrimidine dimers, suggesting a relationship between the DNA strand breaks and the excision repair of these photoproducts. Further evidence in support of such a relationship has been provided by Youngs and Smith (36), who demonstrated that the single-strand breaks detected in the DNA of excision-proficient strains of *E. coli* are photoreactivable, whereas the residual level detected in the DNA of excision deficient strains is not.

The excision repair of UV-irradiated DNA in the yeast *Saccharomyces cerevisiae* appears to be genetically more complex than in procaryotes. Relative to normal strains, defective excision repair after exposure to UV radiation has been reported in strains mutant at 10 different loci, including *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD7*, *RAD10*, *RAD14*, *RAD16*, *MMS19*, and *UVS12* (3, 19–21, 23, 25, 26, 34, 35). With the exception of *UVS12* (which has not been extensively studied as yet), all of these loci belong to a single epistatic group (generally referred to as the *RAD3* group) and are thus believed to code for functions involved in the same biochemical pathway. Two additional loci (*CDC8* [22] and *CDC9* [14]) may also be involved in the excision repair of UV-irradiated DNA; however, the effect of mutations at these loci on the excision of pyrimidine dimers from nuclear DNA (n-DNA) has not been reported. A mutant of the *RAD18* locus, a member of the *RAD6* epistatic group, is also abnormally sensitive to killing by UV radiation but also has not been directly studied in terms of its excision repair capacity. Mutations that have been studied at other loci in the *RAD6* epistatic group are without effect on excision of pyrimidine dimers (20).

The exhaustive genetic analysis of UV radiation sensitivity in *S. cerevisiae* has not been accompanied by biochemical information on the products of the various *RAD* loci referred to above. Neither has it been precisely established at which step in the process of excision repair the many mutants in the *RAD3* epistatic group are defective. As a prelude to undertaking experiments aimed at the isolation and characterization of DNA repair-specific enzymes from yeast, we have examined the capacity of a normal strain (*RAD*$^+$) and a number of mutant strains to carry out specific steps in the excision repair of pyrimidine dimers in vivo. In this paper we report the results of studies in which we have used a technique designed to directly measure small numbers of DNA single-strand breaks in vivo. We present evidence that during the post-UV incubation of normal *S. cerevisiae* (strain S288C), or of a UV-sensitive strain belonging to the *RAD6* epistatic group (*rad18-1*), single-strand breaks in n-DNA are readily detectable. All or most of these breaks are not observed in the DNA of 10 UV-sensitive mutants belonging to the *RAD3* epistatic group, suggesting that these strains are defective in the incision of their DNA after exposure to UV radiation. In addition, all except two of these mutants fail to show any detectable loss of sites from their DNA sensitive to a pyrimidine dimer-specific enzyme probe. To our knowledge this represents the first study in which such a large number of UV-sensitive mutants of *S. cerevisiae* have been examined under identical experimental conditions.

MATERIALS AND METHODS

Yeast strains. The UV-sensitive strains used in this study were originally isolated by Richard Snow (Department of Genetics, University of California at Davis) and are genetically closely related to the haploid *RAD*$^+$ strain S288C (33; R. Snow, personal communication) that was used as a normal control. The strain *rad1-19* was previously (27) incorrectly designated as *Rad1-11*. These strains were kindly provided by Dorma Gottlieb and Richard Snow.

Media and culturing conditions. A solidified medium (YPD agar) containing 1% (wt/vol) yeast extract (Difco), 2% (wt/vol) peptone (Difco), 2% (wt/vol) glucose, and 2% (wt/vol) agar (Difco) was used for maintenance of cultures and for cell viability determinations. Before UV irradiation, cells were routinely cultured in a synthetic liquid medium (YNB-D/0.5%) containing 0.67% (wt/vol) yeast nitrogen base without amino acids (Difco) and 0.5% (wt/vol) glucose.

For all experiments cultures were inoculated into YNB-D/0.5% from single colonies grown on YPD agar plates. After 12 to 15 h of growth at 29°C, cultures were diluted into fresh YNB-D/0.5% at a density of 5×10^6 cells/ml, and growth was continued at 29°C with vigorous aeration. Normal exponential growth was restored after a single population doubling, and cells to be examined for the loss of enzyme-sensitive sites (ESS) or for the appearance of single-strand breaks in n-DNA were isotopically labeled. To label DNA, [6-^{3}H]uracil (23 or 27.5 Ci/mmol) or [2-^{14}C]uracil (54.0 mCi/mmol) was added to the culture medium to final concentrations of 10 and 2.5 μCi/ml, respectively. Cells were grown in the labeled medium for approximately two generations (3 to 3.5 h) as suggested by Hatzfeld (12). At the end of the labeling period, cells were harvested by centrifugation (ca. $400 \times g$, for 5 min at room temperature) and suspended in ice-cold 67 mM potassium phosphate buffer (pH 7.0) at a concentration of 3×10^6 cells/ml.

UV irradiation and postirradiation treatments. Cells in 67 mM phosphate buffer (pH 7.0) were irradiated in suspension with stirring at 0°C. The source of UV radiation (predominantly at 254 nm) was a General Electric germicidal lamp, and the incident dose rate was determined with an IL 254 germicidal photometer (International Light, Newburyport, Mass.). The average UV dose rate to cells in suspension was determined from the incident dose rate by the method of Morowitz (16) but may have been slightly overcorrected due to light scattering (13). Irradiated cells (still in phosphate buffer) were returned to 29°C and incubated with vigorous aeration for various periods from 0 to 3 h. Upon completion of irradiation and any postirradiation incubations, cells in phosphate buffer were cooled on ice to 0°C and held at that temperature until conversion to spheroplasts.

Spheroplast formation. Cells were converted to spheroplasts as described previously (26) except that reduction with 2-mercaptoethanol was carried out at 0°C and cells were incubated with glusalase (Endo Laboratories, Garden City, N.Y.) for 30 min at 30°C.

Measurement of cell viability. Viable cell numbers were determined for unirradiated and for UV-irradiated cultures from the ability of cells to form colonies on YPD agar plates. Cultures at densities of approximately 5×10^7 cells/ml were diluted with 67 mM potassium phosphate buffer to the desired cell densities, and samples were spread in duplicate onto YPD agar plates. Cells on the YPD agar plates were irradiated at 254 nm and then incubated at room temperature for 5 days before direct determination of colony numbers.

Measurement of single-strand breaks in n-DNA. The determination of single-strand breaks in yeast n-DNA has been described in detail elsewhere (27). Briefly, log-phase cells grown in YNB-D/0.5% containing either [6-^{3}H]uracil or [2-^{14}C]uracil were washed free of radioactive medium and suspended in 67 mM potassium phosphate buffer (pH 7.0). The suspended cells were UV irradiated at 0°C with a dose of 100 J/m^2 unless otherwise indicated and then aerated at 29°C for varying periods of time. Samples were collected and held on ice until all incubations were completed, at which time they were converted to spheroplasts as described above. The spheroplasts were gently lysed as previously described, and the lysates were layered onto 5 to 20% (wt/vol) alkaline sucrose gradients. After velocity sedimentation, the gradients were fractionated, the amount of radioactivity in each fraction was measured by liquid scintillation spectrometry, and weight-average molecular weights of the DNA were calculated from the distribution of radioactivity through the gradient. The number of single-strand breaks in UV-irradiated DNA was calculated relative to the sedimentation profiles of n-DNA from unirradiated, unincubated samples that were included in each experiment.

Measurement of ESS in n-DNA. Sites sensitive to attack by a pyrimidine dimer-specific DNA-incising activity in extracts of *Micrococcus luteus* were detected and quantitated by a modification of the procedure of Reynolds (26). Cells were grown as described above and irradiated at 3 J/m^2 of UV radiation. Pelleted spheroplasts (2×10^7) were suspended in 100 μl of a solution containing 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid, and 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.6). The suspended spheroplasts were lysed immediately by the addition of 100 μl of 5% (wt/vol) sodium-*N*-lauroyl sarcosinate (ICN Pharmaceuticals, Inc., Cleveland, Ohio), 1.0 M NaCl, 10 mM ethylenediaminetetraacetic acid, and 50 mM tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.6). Lysates (200 μl) were deproteinized by extraction with 0.5 ml of fresh phenol [neutralized by shaking equal volumes of water-saturated phenol and 1.0 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0)]. Before separation of the organic and aqueous phases by centrifugation, an additional 100 μl of deionized water was added to each sample to reduce the density of the aqueous phase and thereby facilitate separation of the two phases. After low-speed centrifugation, the organic phase was removed by aspiration and the aqueous phase plus residual interface material were re-extracted with an additional 0.5 ml of neutralized phenol. Approximately 250 μl of the aqueous phase was recovered after the second extraction and dialyzed in nitrocellulose bags against 20 ml of 50 mM NaCl–1 mM ethylenediaminetetraacetic acid–10 mM tris-(hydroxymethyl)aminomethane-hydrochloride (pH 7.6), to eliminate residual phenol and to adjust salt and pH to conditions optimal for treatment with the *M. luteus* UV DNA-incising activity. Samples were dialyzed against three changes of buffer with dialysis times of approximately 1.5, 6, and 12 h. Preparation of the nitrocellulose dialysis bags has been described previously (26).

After dialysis, 100-μl samples were incubated with or without excess amounts of enzyme prepared from *M. luteus* as described previously (4, 26). It was recently demonstrated that the relevant enzymatic reaction is not the result of a simple endonuclease but rather the combined actions of a pyrimidine-dimer DNA glycosylase and apurinic/apyrimidine endonuclease(s) found in *M. luteus* preparations (11). We use the term "UV DNA-incising activity" to refer to these combined activities. Reactions were terminated by placing samples on ice until just before sedimentation, at which time an additional 100 μl of 20 mM ethylenediaminetetraacetic acid and 1.0 M NaOH were added to each. Velocity sedimentation of DNA samples through precalibrated alkaline sucrose gradients and calculation of numbers of ESS from the resulting distributions of radioactivity through the gradients have been described elsewhere (26, 27).

RESULTS

UV sensitivity of *rad* mutants. Survival curves for the strains used in this study (Fig. 1 and 2) demonstrate significant differences in their relative sensitivity to UV radiation. In general the *rad1*, *rad2*, *rad3*, and *rad4* mutants displayed similar extreme sensitivities to UV radiation; however, the *rad2-4* mutant was significantly more UV resistant than the *rad2-2* and *rad2-3* mutants. The *rad14-1* mutant was the least UV sensitive of any of the mutant strains examined.

Presence of single-strand breaks in n-DNA. The capacity of UV-irradiated *S. cerevisiae* to catalyze the formation of single-strand breaks in DNA was determined primarily by velocity sedimentation through alkaline sucrose density gradients of gently lysed spheroplasts. With this experimental approach the potential for shearing of DNA is minimized, and small numbers of single-strand breaks can be reproducibly demonstrated with careful technique. Typical sedimentation profiles for n-DNA from the excision-proficient (*RAD*$^+$) haploid strain S288C are depicted in Fig. 3A. Nuclear DNA released by lysis of spheroplasts of unirradiated cells sedimented with a weight-average molecular weight of 173×10^6. DNA released from cells irradiated at 0°C with 100 J of UV light per m^2 before spheroplast formation sedimented more slowly, with a weight-average molecular weight

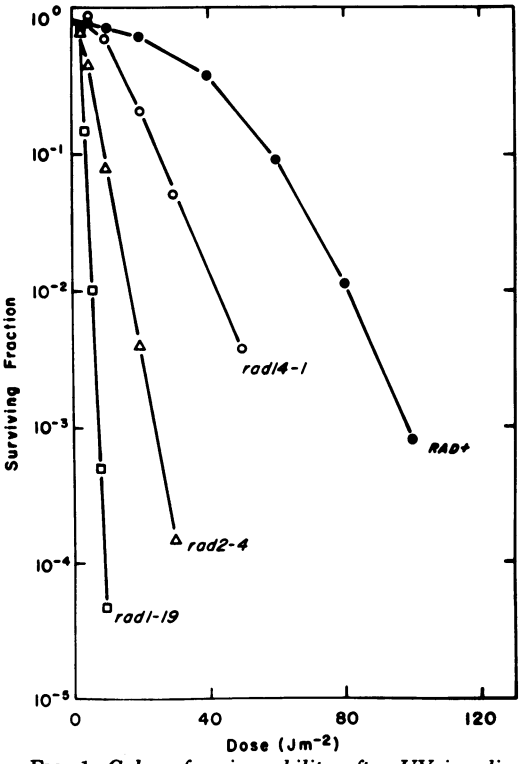

FIG. 1. *Colony-forming ability after UV irradiation. Symbols: rad1-19* (□), *rad2-4* (△), *rad14-1* (○), *and S288C (RAD⁺)* (●).

of 83 × 10^6. Incubation of cells at 29°C after irradiation but before conversion of the cells to spheroplasts resulted in a further time-dependent decrease in the sedimentation rate of n-DNA. As shown in Fig. 3A, n-DNA from normal cells incubated for 3 h after irradiation sedimented with an apparent weight-average molecular weight of 61 × 10^6. This time-dependent reduction in molecular weight in the excision-proficient strain was strictly dependent on incubation at temperatures above 0°C. No reduction in molecular weight beyond that observed in the DNA from cells converted to spheroplasts immediately after irradiation was evident when irradiated cells were held for up to 24 h at 0°C before being converted to spheroplasts (data not shown).

Results obtained with the UV-sensitive *rad1-2* mutant (Fig. 3B) are representative of many of the excision-defective strains examined. The sedimentation profile of the n-DNA released from spheroplasts of unirradiated *rad1-2* cells was indistinguishable from that obtained with unirradiated normal cells. Irradiation of *rad1-2* cells with 100 J of UV light per m^2 reduced the sedimentation rate of the n-DNA to a weight-average molecular weight of 84 × 10^6. In contrast to the results obtained with the normal *RAD⁺* strain S288C, however, no further reduction in the size of the n-DNA was evident when irradiated *rad1-2* cells were incubated at 29°C before conversion to spheroplasts.

Comparison of weight-average molecular weights calculated from sedimentation profiles similar to those depicted in Fig. 3 allowed us to determine the average number of single-strand breaks in n-DNA induced by a particular experimental protocol. The results of such comparisons for all of the strains examined are shown in Fig. 4 and suggest the presence of two classes of DNA strand breaks after exposure of *S. cerevisiae* to UV radiation. Relative to unirradiated controls, small numbers of single-strand breaks were consistently detected in the n-DNA of cells irradiated and maintained at 0°C until their conversion to spheroplasts. We refer to this class as "incubation-independent" DNA single-strand breaks. In repeated experiments, the average number of incubation-independent DNA single-strand breaks in the normal strain and in most UV-sensitive mutant strains was ~1.2 single-strand breaks per 10^8 molecular weight after irradiation at 100 J/m^2. This value represents about 1 break for every 400 pyrimidine dimers in n-DNA.

A second class of single-strand breaks appeared in the n-DNA of some strains during incubation at 29°C after exposure to UV radiation. We designate these as "incubation-dependent" single-strand breaks. In the normal strain, between 1 and 2 such single-strand breaks per 10^8 molecular weight were detected during the first 3 h of incubation after irradiation with 100 J of UV light per m^2. In previous studies (27) we demonstrated that the number of this class of strand breaks was significantly reduced if cells were incubated under conditions permissive for enzymatic photoreactivation. The photoreactivability of these lesions indicates that they are related to the presence of pyrimidine dimers in n-DNA. With the exception of *rad18-1*, none of the mutant strains presented in Fig. 4 exhibited any detectable incubation-dependent single-strand breaks in n-DNA in repeated experiments. The *rad18-1* mutant (Fig. 4D) differed from the *RAD⁺* strain S288C in that the incubation-dependent class of DNA single-strand breaks appeared with faster kinetics and was quantitatively greater over the 3-h incubation period examined. Preliminary experiments with the UV-sensitive mutant *rad7-1* yielded results indistinguishable from those observed with strain S288C (data not shown). However, the experimental conditions used with the *rad7-1* strain were slightly different from those generally employed in these experiments, and further

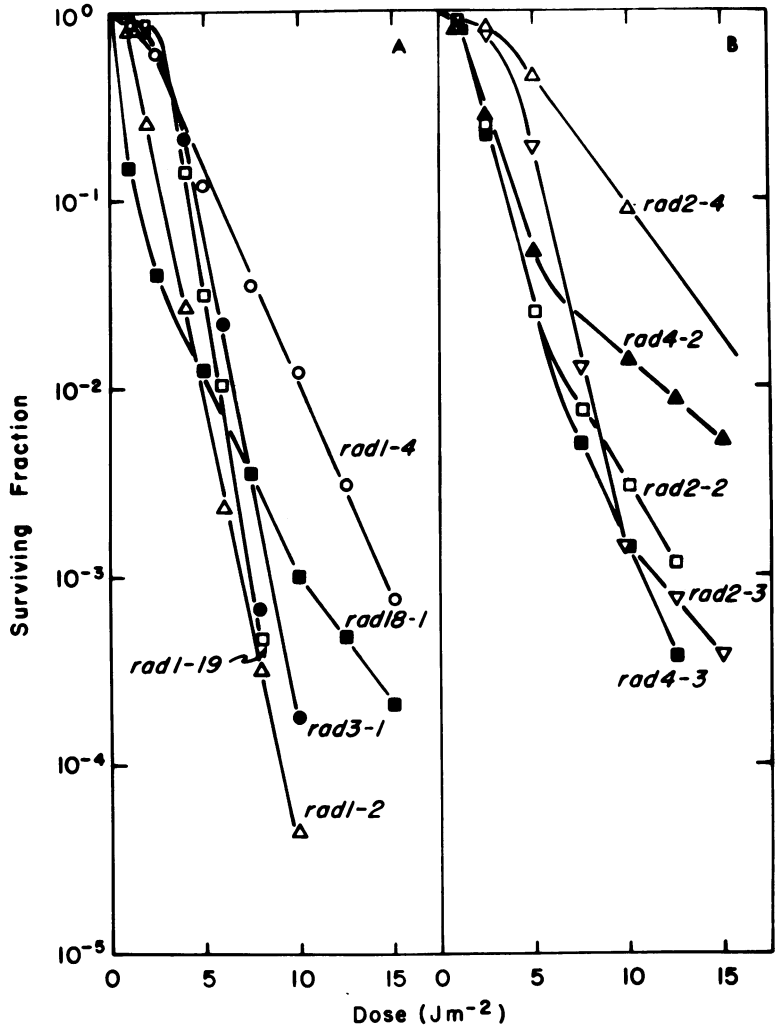

FIG. 2. *Colony-forming ability after UV irradiation. Symbols: A—rad1-2* (△), *rad1-4* (○), *rad1-19* (□), *rad3-1* (●) *and rad18-1* (■); *B—rad2-2* (□), *rad2-3* (▽), *rad2-4* (△), *rad4-2* (▲), *and rad4-3* (■).

studies are required to confirm this result.

An interesting and reproducible exception to the results obtained with the great majority of the mutants examined was observed with the *rad2-4* strain. This strain showed significantly increased numbers of incubation-independent single-strand breaks in n-DNA (~$2.5/10^8$ molecular weight) in the absence of incubation at 29°C after irradiation (Fig. 4B). The strict dependence of this result on UV irradiation as opposed to any other variable in the experimental protocol was demonstrated in an experiment in which a single culture of *rad2-4* cells was split into two equal fractions, one of which was grown in the presence of [2-^{14}C]uracil and the other of which was grown in the presence of [6-^{3}H]uracil. The ^{3}H-labeled culture was irradiated with 100 J/m^2, the two cultures were mixed, and all subsequent procedures were carried out on the mixed culture. It is evident from the data presented in Fig. 5 that an increase in the number of incubation-independent single-strand breaks relative to that typically observed (see Fig. 4) was detected only in the DNA of the irradiated *rad2-4* cells. This result was observed at all doses of UV radiation to which *rad2-4* cells were exposed, with relatively large numbers of single-strand breaks evident after exposure as low as 5 J/m^2 (Fig. 6). As regards incubation-dependent DNA single-strand breaks, strain *rad2-4* consistently demonstrated a time-dependent loss of the unusually large number of incubation-independent strain breaks during incubation at 29°C (Fig. 4B and 5).

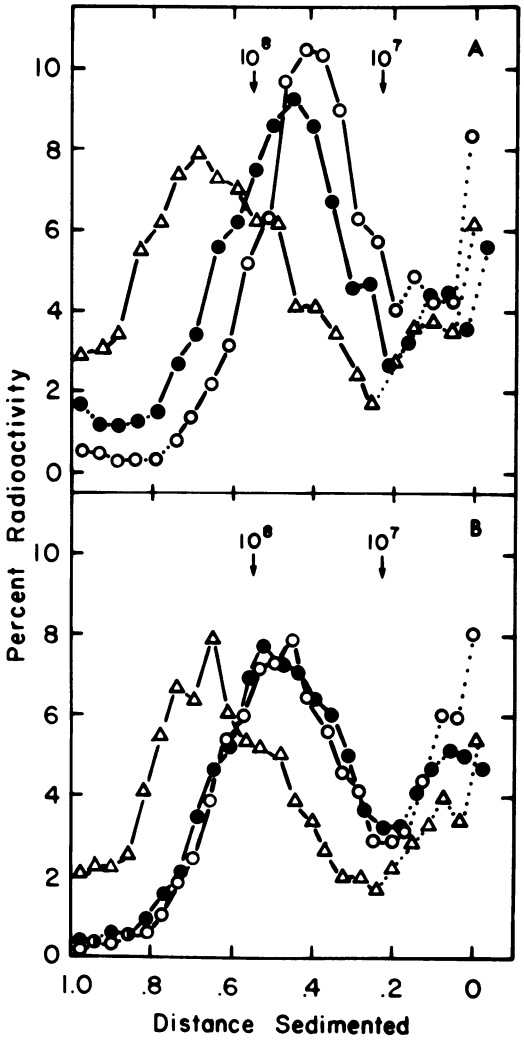

FIG. 3. *Effect of UV irradiation and subsequent aeration of cells at 29°C in potassium phosphate buffer on the sedimentation rate of yeast n-DNA through alkaline sucrose gradients. The positions of specific molecular weights as determined from the gradient calibrations are indicated by the arrows, and those portions of the distributions used in molecular weight calculations are indicated by the solid lines. Symbols: unirradiated cells (△); cells irradiated with 100 J/m² (●); and cells irradiated with 100 J/m² and aerated at 29°C in buffer for 3 h (○). (A) S288C (RAD⁺), excision proficient; (B) rad1-2, excision defective.*

Loss of sites sensitive to a pyrimidine dimer-specific enzyme probe (ESS). Duplex DNA containing pyrimidine dimers is sensitive to attack by the pyrimidine dimer-specific DNA glycosylase and associated AP endonuclease activities present in crude extracts of *M. luteus*. The specificity of similar *M. luteus* preparations for pyrimidine dimers in UV-irradiated DNAs has been demonstrated previously (17, 18, 26, 28). Thus, in the present context, ESS are considered indicative of pyrimidine dimers.

For the detection of ESS, DNA samples extracted from yeast nuclei were divided, and the two portions were incubated with or without the *M. luteus* enzyme preparation before sedimentation through alkaline sucrose gradients and determination of average molecular weights. Ideally, incision in DNA catalyzed at pyrimidine dimers in excision-proficient cells should register as strand breaks in the "minus-enzyme" controls. In practice, however, the anticipated reduction in the molecular weights of minus-enzyme controls from UV-irradiated cells is not usually observed. The inability of this assay to directly detect single-strand breaks induced in vivo results in part from the small numbers of breaks present at any given time after irradiation (see Fig. 3 and 4) and in part from a reduced sensitivity to their detection due to shear-induced breakage of the DNA during purification.

On the other hand, if dimer excision, repair synthesis, and DNA ligation are completed in vivo, the isolated DNA will be less sensitive to the enzyme treatment. Loss of ESS therefore provides an indication of completed dimer excision, including restoration of the covalent integrity of the DNA strands relative to the control DNA. By implication, a failure to detect the loss of any ESS as a function of post-UV incubation implies that pyrimidine dimers in the DNA are covalently intact and hence were not attacked by putative yeast DNA-incising activities during incubation in vivo. Thus this technique also provides an independent assessment of the capacity of a given yeast strain to catalyze the incision of its DNA at pyrimidine dimer sites specifically, after exposure to UV radiation.

To induce ESS in n-DNA, yeast cells were irradiated with doses of 3 J/m². After irradiation with this relatively low dose, no loss of viability could be detected in normal haploid cells and survival in the most sensitive of the mutant strains was 10% or greater (Fig. 1). This dose was, however, sufficient to introduce significant numbers of ESS into yeast n-DNA. In the absence of prior irradiation and/or enzyme treatment, weight-average molecular weights were typically between 50×10^6 and 70×10^6 (Table 1). Upon enzyme treatment of DNA extracted from cells irradiated with 3 J/m², weight-average molecular weights were reduced to approximately 11×10^6. This enzyme-dependent molecular weight reduction observed with DNA from UV-irradiated cells corresponds to approximately 0.14 ESS per 10^6 molecular weight.

Typical alkaline sucrose gradient profiles are

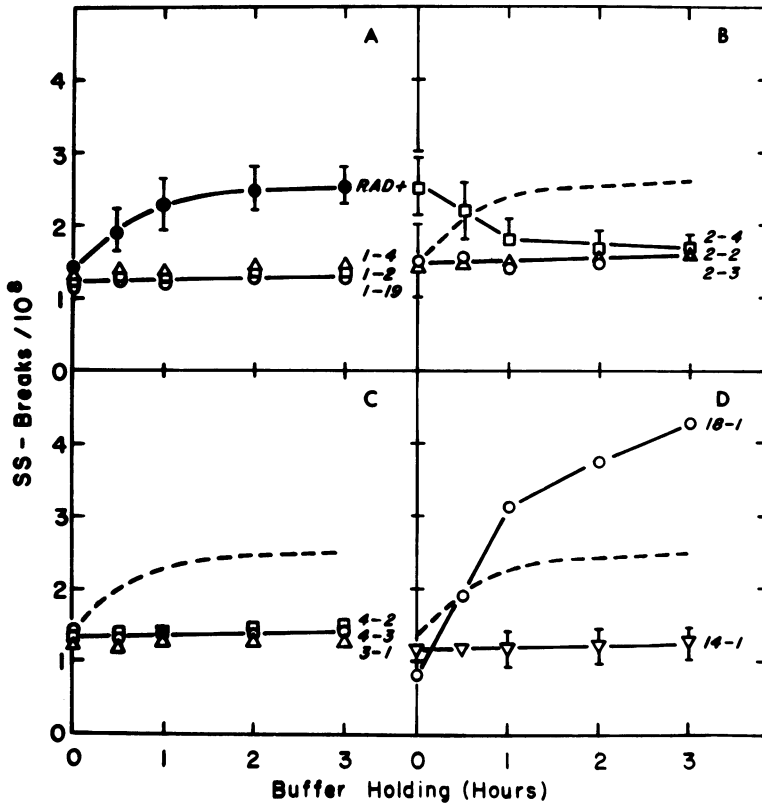

FIG. 4. *Appearance of single-strand breaks in yeast n-DNA after irradiation of cells with 100 J/m². Data points represent the means of two (rad1-4, rad2-2, rad18-1), three (rad1-2, rad2-3, rad3-1, rad14-1, and S288C), four (rad4-3), six (rad1-19), or seven (rad2-4, and rad4-2) determinations. Standard deviations are indicated when greater than 0.2 single-strand breaks per 10⁸. Dotted lines in panels B, C, and D indicate the response obtained with the normal strain S288C (A). Symbols: A—rad1-2 (□), rad1-4 (△), rad1-19 (○), and S288C (RAD⁺) (●); B—rad2-2 (○), rad2-3 (△), and rad2-4 (□); C—rad3-1 (△), rad4-2 (□), and rad4-3 (○); D—rad14-1 (▽) and rad18-1 (○).*

depicted in Fig. 7 for enzyme-treated samples from two different strains examined in the same experiment. Both strains are UV sensitive, but one strain carrying the *rad1-4* mutant allele is defective in a gene belonging to the *RAD3* epistatic group, whereas the other strain carrying the mutant allele *rad18-1* is defective in a gene belonging to the *RAD6* epistatic group (13). To enable direct comparisons, individual strains were selectively labeled with different radionuclides by growing the *rad1-4* and the *rad18-1* mutants in [2-^{14}C]uracil and [6-^{3}H]uracil, respectively. After 3 h of growth, the individual cultures were harvested from the labeled media and pooled. All subsequent procedures including UV irradiation, postirradiation incubation, spheroplast formation, and ESS determinations were carried out on the mixed cell suspension. After velocity sedimentation and gradient fractionation, results were obtained for each strain by measuring the amount of each radionuclide in each fraction with the aid of a two-channel liquid scintillation counter set to discriminate between ^{3}H and ^{14}C. Crossover corrections were made to adjust for the incomplete separation of the two radionuclides.

Relative to the DNA from unirradiated cells, a significant reduction in sedimentation rate was observed after enzyme treatment of DNA from UV-irradiated, unincubated cells; i.e., ESS were present. With the *rad18-1* mutant, DNA extracted from UV-irradiated cells incubated for 3 h at 29°C after irradiation sedimented much more rapidly after enzyme treatment than did enzyme-treated DNA extracted from irradiated, unincubated cells. This loss of sensitivity to the enzyme treatment is consistent with proficient excision (and hence incision) of pyrimidine dimers in these cells. In contrast, DNA extracted from UV-irradiated *rad1-4* cells after incubation at 29°C for 3 h after irradiation was indistinguishable in sensitivity to the *M. luteus* prepa-

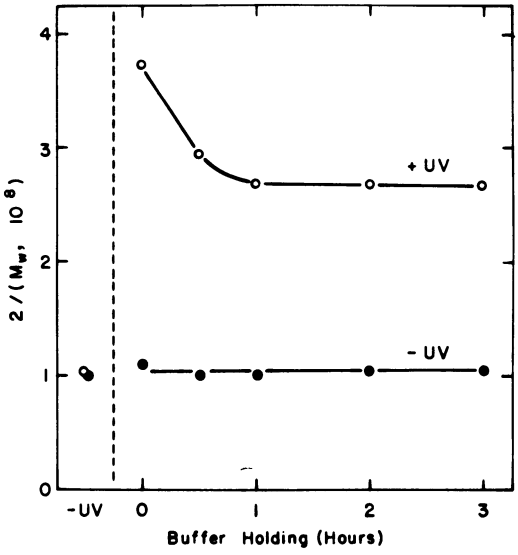

FIG. 5. *Dependence of single-strand breaks in rad2-4 upon prior UV irradiation. A single culture of exponentially growing cells was divided, and growth was continued in media containing either [6-^{3}H]uracil or [2-^{14}C]uracil for two generations. The ^{3}H-labeled cells (○) were either left unirradiated or irradiated with 100 J/m^2 and then mixed with the unirradiated ^{14}C-labeled cells (●). All subsequent procedures were carried out on the mixed culture.*

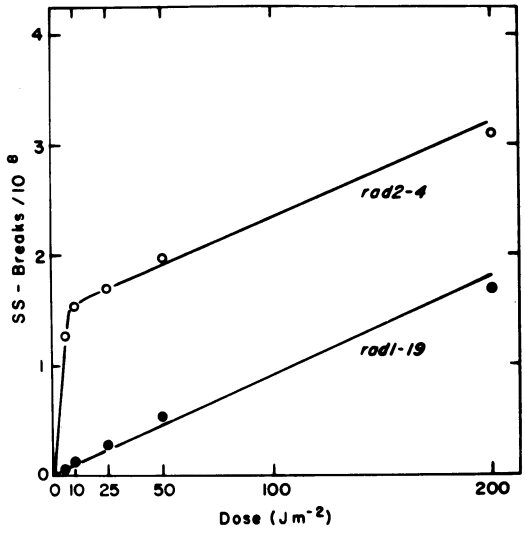

FIG. 6. *Dose response for the appearance of single strand breaks in the n-DNA of rad2-4 and rad1-19. Cultures of rad2-4 (○) and rad1-19 (●) were grown in the presence of [2-^{14}C]uracil and [6-^{3}H]uracil, respectively, and then pooled. Irradiation of the cells and all subsequent procedures were carried out on the pooled culture.*

TABLE 1. *ESS in the n-DNA of mutants rad18-1 and rad1-4*[a]

Strain	Dose (J/m^2)	Post-UV incubation (h)	10^6 Mol wt −Enz.	10^6 Mol wt +Enz.	ESS/10^6	% Total ESS (100% = 0.140)
rad18-1	0		58.2	45.3	0.010	
	3	0	53.2	13.1	0.115	82
	3	3	52.5	38.7	0.014	10
rad1-4	0		62.4	52.4	0.007	
	3	0	58.5	11.5	0.140	100
	3	3	57.8	12.0	0.132	94

[a] ESS were determined from a comparison of DNA average molecular weights after incubation of DNA samples with (+ Enz.) or without (− Enz.) *M. luteus* extracts as described in the text.

ration from DNA extracted from irradiated, unincubated cells. The absence of a time-dependent change in sensitivity to the enzyme treatment indicates a defect in the excision repair of pyrimidine dimers in the *rad1-4* mutant.

A quantitative analysis of the experiment depicted in Fig. 7 is presented in Table 1. Weight-average molecular weights for the minus-enzyme controls ranged from 52.5 × 10^6 to 64.3 × 10^6 in this experiment without significant deviation upon irradiation or postirradiation treatments of cells. Treatment of DNA from unirradiated cells with the *M. luteus* enzyme preparation revealed a small but reproducible amount of activity against both ^{14}C- and ^{3}H-labeled DNAs in the absence of prior cellular irradiation. This degradation was very limited with freshly prepared DNA and probably represents activity against DNA damage resulting from radionuclide decay (W. L. Carrier, personal communication). Similar activity was not detected in a previous study in which a more rapid DNA purification procedure was used (26). The numbers of sites attributable to this activity were never greater than 0.01 sites per 10^6 molecular weight, and all data presented are uncorrected for this activity.

The kinetics of the loss of ESS in a number of strains used in this study are presented in Fig. 8. Strains *rad1-2*, *rad1-4*, *rad1-19*, *rad2-2*, *rad2-3*, *rad3-1*, *rad4-2*, and *rad4-3* exhibited no significant loss of ESS during 3 h of post-UV incubation. The *rad2-4* (Fig. 8B), *rad14-1* (Fig. 8D), and *rad18-1* (Fig. 8D) strains did show significant loss of ESS as a function of the time of incubation. Both of the former two strains lost about 40% of total ESS during 3 h of incubation at 29°C, whereas the kinetics of loss of sites from the DNA of strain *rad18-1* were indistinguishable from those of the normal *RAD*$^+$ strain S288C. The loss of ESS from the DNA of the *rad14-1* mutant suggests that incision events

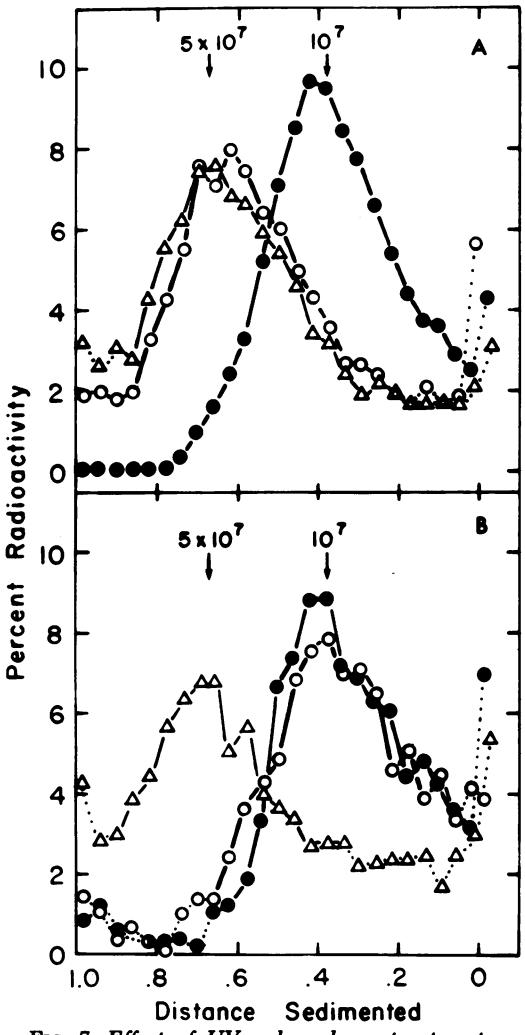

FIG. 7. *Effect of UV-endonuclease treatment on the alkaline sucrose gradient velocity sedimentation profiles of DNA purified from unirradiated cells (△), cells irradiated with 3 J/m² (●), and cells irradiated with 3 J/m² and aerated for 3 h at 29°C (○). To facilitate comparisons between the two strains, cultures were differentially labeled by growth in the presence of [6-³H]uracil (rad18-1) or [2-¹⁴C]uracil (rad1-4). The labeled cultures were mixed, and all subsequent procedures were carried out on the mixed cultures. Arrows denote the positions of specific molecular weights as determined from gradient calibrations, and solid lines indicate those portions of the distributions used in molecular weight calculations. (A) rad18-1, excision proficient; (B) rad1-4, excision defective.*

must have occurred during incubation in vivo. Since incubation-dependent single-strand breaks were not directly detected in the DNA of this mutant (Fig. 4D), the number of single-strand breaks present at any given time after irradiation is apparently reduced relative to the numbers present in the normal *RAD*⁺ strain S288C. Thus our data are consistent with reduced rates of incision but not with their complete absence in the *rad14-1* mutant. In preliminary experiments, the *rad7-1* mutant showed the time-dependent loss of a significant fraction of ESS (data not shown).

DISCUSSION

The yeast *S. cerevisiae* has been very extensively studied in terms of the genetics of the response of living cells to DNA damage by physical and chemical agents. As indicated by Haynes and Kunz in their recent comprehensive review (R. H. Haynes and B. A. Kunz, *in* J. Strathern, J. Broach, and E. W. Jones (ed.), *The Molecular Biology of the Yeast Saccharomyces*, in press), at least 85 genetic loci are known to affect sensitivity to radiation and to chemical mutagens. Many of these loci can be conveniently organized into three epistatic or non-overlapping groups based on the premise that mutations in different loci belonging to the same epistatic group do not result in additivity or synergism with respect to sensitivity to killing by the agents tested. Thus, epistasis implies that the gene products mediate steps in the same DNA repair pathway.

The *RAD3* epistatic group consists of a number of loci believed to be involved in the excision repair of certain types of DNA damage, notably pyrimidine dimers produced by UV radiation at ~254 nm. Included in this group are the following loci; *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD7*, *RAD10*, *RAD14*, *RAD16*, *MMS19*, and *CDC8*. Mutants at 9 of these loci (all of those just listed except *CDC8*) have been reported to be defective in the excision repair of pyrimidine dimers.

Three distinct experimental approaches have been used previously to monitor the excision repair capacity of wild-type strains and of mutants in the 10 loci identified above. Resnick and Setlow (25) used an assay based on the competition of pyrimidine dimers in yeast n-DNA for photoreactivating enzyme activity on transforming DNA. They reported that, during post-UV incubation, the *rad2-17* mutant failed to lose sites (pyrimidine dimers) which could compete for the photoreactivating enzyme. Unrau et al. (34) and Waters and Moustacchi (35) directly measured the loss of pyrimidine dimers from the acid-insoluble fraction of cells incubated for various periods of time after exposure to UV radiation and observed a defect in this parameter in *rad1-1* and *rad1-3* mutants, respectively.

The remaining studies in the literature have utilized the loss of ESS technique to monitor

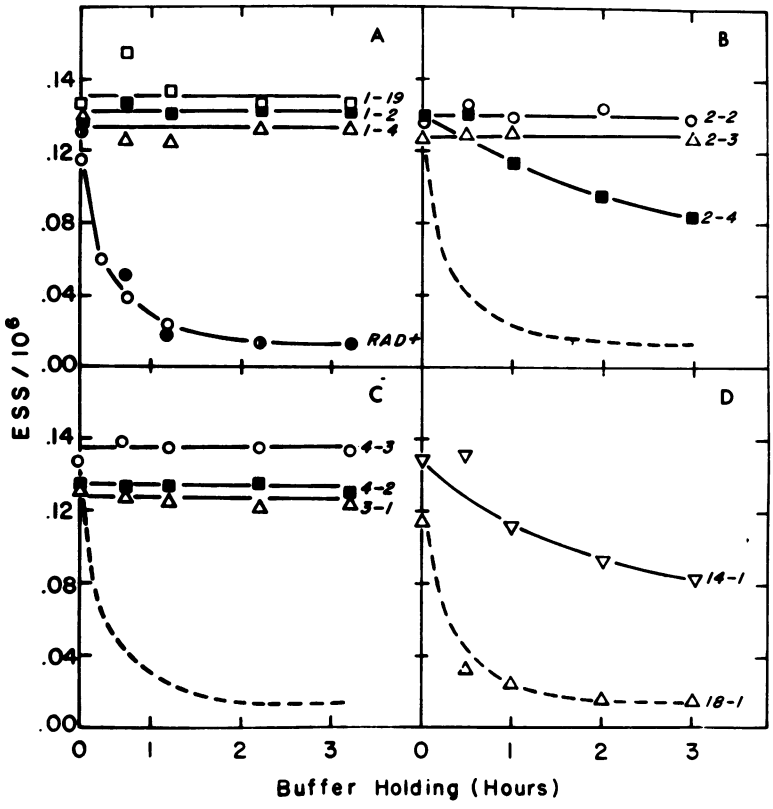

FIG. 8. *ESS removal in cells irradiated with 3 J/m² and aerated at 29°C in potassium phosphate buffer. Dotted lines in B, C, and D indicate the normal excision-proficient response as presented in A. Symbols: A—rad1-2 (■), rad1-4 (△), rad1-19 (□), and S288C (RAD⁺) [two independent determinations (○, ●)]; (B)—rad2-2 (○), rad2-3 (△), and rad2-4 (■); (C)—rad3-1 (△), rad4-2 (■), and rad4-3 (○); (D)—rad14-1 (▽) and rad18-1 (△).*

excision-repair proficiency of yeast strains. Using this procedure Prakash demonstrated a failure to lose ESS in the *rad1-2* mutant (19), and in independent studies similar results were observed in *rad3*, *rad4*, *rad10*, and *rad16* mutants (20, 21). Prakash and Prakash (23) also reported a failure to lose ESS in the *rad7*, *rad14*, and *mms19* mutants.

In the present studies we have investigated more than one mutant allele at a number of *RAD* loci and have also examined the UV-sensitive strain *rad18-1*, for which no data on excision repair capacity were previously available. In addition to the ESS assay, we have exploited a technique for the sedimentation of yeast n-DNA which is released from spheroplasts by gentle chemical lysis and immediately layered directly onto alkaline sucrose density gradients. Our earlier studies (27) demonstrated that this procedure allows the direct detection of DNA single-strand breaks and that those breaks, the appearance of which is dependent on incubation at 29°C with aeration of cells, are photoreactivable, strongly suggesting that they are related to the presence of pyrimidine dimers in DNA. In all cases we have compared our results on excision repair capacity with sensitivity to killing of cells by UV radiation.

The direct examination of the presence of DNA strand breaks in UV-irradiated cells either immediately after irradiation or during post-UV incubation at 29°C has provided evidence that a number of the UV-sensitive mutants examined are defective in the incision of their DNA. We have previously reported that single-strand breaks (or alkaline-labile sites) arise in the n-DNA of excision-proficient cells after UV irradiation (27). These DNA strand breaks appear to be of two classes which we have designated as incubation independent and incubation dependent. The former are evident in excision-proficient cells irradiated at 0°C and maintained at that temperature (except during conversion of cells to spheroplasts). The number of these single-strand breaks are independent of the time cells are held at 0°C for up to 24 h before

spheroplast formation and are independent of the duration of the steps involved in spheroplast formation. With the exception of the *rad2-4* mutant, similar numbers of incubation-independent single-strand breaks were observed in all strains examined. We believe it likely that these strand breaks are the result of direct photochemical nondimer damage to DNA (24). However, further studies are in progress to examine their origin.

The second class of DNA strand breaks observed in excision-proficient yeast is dependent on aeration of irradiated cells at 29°C. The number of these breaks is UV dose dependent; however, after longer times of incubation they decrease, consistent with the notion that they represent transient intermediates in the excision repair of pyrimidine dimers. This conclusion is further supported by the demonstration of their photoreactivability. The absence of this class of DNA strand breaks in mutants of five of the genetic loci examined suggests that all five loci (*RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD14*) are involved in the incision step of pyrimidine dimer excision. This conclusion is substantiated by the observation of incubation-dependent single-strand breaks in the DNA of the excision-proficient, UV-sensitive *rad18-1* mutant. In preliminary studies we have also observed incubation-dependent strand breaks in the DNA of the excision-defective *rad7-1* mutant. Whether or not this result represents leakiness of this particular strain, or is characteristic of all mutants at the *RAD7* locus, needs to be established by further study.

Strains that failed to demonstrate the presence of DNA strand breaks by this direct experimental approach would also be expected to retain all ESS in their DNA. Quantitative examination of this parameter revealed that most of the mutants defective in loci belonging to the *RAD3* epistatic group had extreme effects on excision repair capacity after irradiation of cells with low doses of UV. The severely affected mutants carried the mutant alleles *rad1-2*, *rad1-4*, *rad1-19*, *rad2-2*, *rad2-3*, *rad3-1*, *rad4-2*, and *rad4-3*. No significant loss of ESS could be detected in any of these strains during incubation for 3 h after irradiation at 3 J/m^2. The effects of these mutant alleles on excision repair of UV damage is consistent with their extreme UV sensitivities. Two strains carrying the mutant alleles *rad2-4* and *rad14-1* were able to remove a significant fraction of ESS from their DNA, although clearly less than the normal strain, despite the fact that neither strain showed clearly demonstrable incubation-dependent DNA strand breaks. Interestingly, these two strains were also found to have intermediate UV sensitivities relative to the extremely UV-sensitive strains and to the normal *RAD*$^+$ strain S288C. The results obtained with the *rad2-4* strain relative to the other *rad2* mutants examined suggest that incomplete molecular blocks in the pathway of pyrimidine dimer excision may be reflected in biological endpoints such as survival. The same may be true of the *rad14-1* mutant, although other alleles at the *RAD14* locus are not yet available for study. The possibility of such "leakiness" should be an important consideration in the selection of strains for biological and genetic studies.

A UV-sensitive strain carrying a mutant allele of the *RAD18* gene was also examined for ESS removal. Although the *rad18-1* mutant was extremely UV sensitive in our hands, the demonstration of normal kinetics for the loss of ESS is not unexpected since the *RAD18* locus is a member of the *RAD6* epistatic group, all members of which are thought to be excision repair proficient. Normal pyrimidine dimer excision has also been demonstrated in *rad6* and *rad9* mutants (20).

In conclusion, our studies indicate that a significant component of the genetic complexity associated with excision repair of pyrimidine dimers in *S. cerevisiae* is concentrated around the molecular events required for the incision of UV-irradiated DNA in vivo. This study provides clear evidence that at least four genetic loci (*RAD1*, *RAD2*, *RAD3*, and *RAD4*) are involved in this process. This conclusion is supported by the results of our earlier studies (27) in which we showed that mutants defective in the *RAD1*, *RAD2*, *RAD3*, and *RAD4* loci all contain normal levels of enzyme activity that in a cell-free system catalyzes the selective excision of thymine-containing pyrimidine dimers from UV-irradiated *E. coli* DNA previously incised by the dimer-specific UV DNA-incising activity of *M. luteus*. Further studies on the dimer-excising activity in cell-free systems will be reported elsewhere (R. J. Reynolds and E. C. Friedberg, manuscript in preparation). These conclusions are not in agreement with the recent report by Bekker et al. (2) who claim to have demonstrated normal levels of "UV endonuclease" activity in extracts of a number of *rad* mutants of *S. cerevisiae*. Our results with mutants at the *RAD7* and *RAD14* loci are not definitive, nor have we yet examined mutants at the *RAD10*, *RAD16*, or *MMS19* loci. Thus, potentially an additional five genetic loci may be involved in events associated with or preceding DNA incision.

The genetic complexity associated with the very early events in the excision repair of pyrimidine dimers in *S. cerevisiae* bears a striking

analogy to that demonstrated in human cells by the study of cell lines obtained from patients with xeroderma pigmentosum (see reference 8 for a recent review). At least seven complementation groups (exclusive of the xeroderma pigmentosum variant form) have been documented in this disease. Investigation of the loss of ESS from DNA extracted from one member of each complementation group (37) has demonstrated no significant loss of ESS in five of the seven groups. These results suggest that study of the molecular mechanisms of excision repair of DNA in *S. cerevisiae* may provide a very relevant model system for the understanding of this subject in higher eucaryotes, including humans.

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