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# An Alternative Eukaryotic DNA Excision Repair Pathway

GREG A. FREYER,[1]* SCOTT DAVEY,[2] JASMINE V. FERRER,[1] AMY M. MARTIN,[3] DAVID BEACH,[2] AND PAUL W. DOETSCH[3]

*Center for Radiological Research, Columbia University College of Physicians and Surgeons, New York, New York 10032[1]; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724[2]; and Departments of Biochemistry and Radiation Oncology, Emory University School of Medicine, Atlanta, Georgia 30322[3]*

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**DNA lesions induced by UV light, cyclobutane pyrimidine dimers, and (6-4)pyrimidine pyrimidones are known to be repaired by the process of nucleotide excision repair (NER). However, in the fission yeast *Schizosaccharomyces pombe*, studies have demonstrated that at least two mechanisms for excising UV photoproducts exist; NER and a second, previously unidentified process. Recently we reported that *S. pombe* contains a DNA endonuclease, SPDE, which recognizes and cleaves at a position immediately adjacent to cyclobutane pyrimidine dimers and (6-4)pyrimidine pyrimidones. Here we report that the UV-sensitive *S. pombe rad12-502* mutant lacks SPDE activity. In addition, extracts prepared from the *rad12-502* mutant are deficient in DNA excision repair, as demonstrated in an in vitro excision repair assay. DNA repair activity was restored to wild-type levels in extracts prepared from *rad12-502* cells by the addition of partially purified SPDE to in vitro repair reaction mixtures. When the *rad12-502* mutant was crossed with the NER *rad13-A* mutant, the resulting double mutant was much more sensitive to UV radiation than either single mutant, demonstrating that the *rad12* gene product functions in a DNA repair pathway distinct from NER. These data directly link SPDE to this alternative excision repair process. We propose that the SPDE-dependent DNA repair pathway is the second DNA excision repair process present in *S. pombe*.**

The major UV light-induced DNA lesions, cyclobutane pyrimidine dimers (CPDs), and (6-4)pyrimidine pyrimidones (6-4 PPs), are known to be repaired by the mechanism of nucleotide excision repair (NER; reviewed in references 13, 28, and 34). NER is carried out by a multiprotein complex which recognizes DNA damage, cleaving at a distance both 5′ and 3′ to the lesion and removing the damaged nucleotides as an oligomer (15, 27). Human NER has been studied largely with cells of individuals suffering from the DNA repair deficiency syndrome xeroderma pigmentosum. These patients have reduced ability to repair the damage induced by UV light and are highly susceptible to certain cancers (7, 8, 16). Cell fusion studies established the existence of seven genetically distinct complementation groups (XP-A through XP-G) (36). Most of the genes which complement the XP mutant cell lines have been isolated (1, 34).

A large number of mutant *Schizosaccharomyces pombe* strains have been shown to be sensitive to either UV irradiation, gamma irradiation, or both (2, 19, 26, 30). Several of the genes which complement these radiation-sensitive mutants have been isolated, and the function in specific repair processes has been determined (3, 9, 18). The *rad13* and *rad15* genes have been shown to have homology with the *Saccharomyces cerevisiae RAD2* and *RAD3* genes and the human XP-G and XP-D genes, respectively (6, 22). The *rad13-A* and *rad15-P* mutations cause the cells to be sensitive to UV radiation, establishing a role for NER in repair of UV photoproducts in *S. pombe*. The *rad22* gene of *S. pombe* has homology to the *S. cerevisiae RAD52* gene, which is involved in recombinational repair (25).

Since NER is recognized as the process whereby CPDs and 6-4 PPs are repaired, it follows that *rad13* or *rad15* mutant strains should be deficient in UV photoproduct excision. However, several years ago it was demonstrated that both *rad13-A* and *rad15-P* mutant strains were still proficient in removing CPDs in vivo (4). More recently, results of an immunoassay using antibodies directed against either 6-4 photoproducts or CPDs demonstrated that in *S. pombe rad13-A*, *rad13*Δ, *rad15-P*, *rad16-N*, or *rad16*Δ NER mutants both CPDs and 6-4 photoproducts are efficiently removed (21). These data clearly demonstrate that *S. pombe* has an alternative process for the excision of UV photoproducts. No direct data demonstrating an alternative excision repair process in *S. pombe* exist. However, the *S. pombe rad2* gene product has been implicated as a possible component of a second excision repair process (23). Recent studies in our laboratories revealed an endonuclease which recognizes and cleaves at a position immediately adjacent to both CPDs and 6-4 PPs (5). Here we provide evidence that this endonuclease, SPDE (*S. pombe* DNA endonuclease), mediates an excision repair process. We propose that the SPDE-dependent DNA repair pathway is the second excision repair process in *S. pombe*.

## MATERIALS AND METHODS

***S. pombe* culture and genetic manipulations.** *S. pombe* was cultured by standard techniques (17). A summary of the strains used in this study is presented in Table 1. GF101 ($h^{+N}$ *rad12-502*) was backcrossed three times with wild-type *S. pombe*, twice with strain 972 ($h^{-S}$) (17), and once with GF110 ($h^{+N}$ *leu1-32*), producing an $h^{+N}$ *rad12-502 leu1-32* mutant (GF105). This strain was crossed with GF102 ($h^{-S}$ *rad13-A*), GF118 ($h^{-S}$ *rad22-67*), and GF150 ($h^{-S}$ *rad2-44*). The $h^{-S}$ *rad12-502 rad13-A* (GF106), $h^{-S}$ *rad12-502 rad2-44* (GF152), and $h^{-S}$ *rad12-502 rad22-67* double mutants were selected from nonparental ditype tetrads, and their genotypes were confirmed by outcrossing.

**Preparation of *S. pombe* whole-cell extracts.** Approximately $10^9$ *S. pombe* cells were cultured to late log phase in YEA (5 g of yeast extract, 30 g of dextrose, and 75 mg of adenine per liter), washed in water, and resuspended in an equal volume of extraction buffer (20 mM Tris-HCl [pH 7.9], 10% glycerol, 1 mM EDTA, 10 mM $MgCl_2$, 0.3 M $(NH_4)_2SO_4$, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). Cells were then frozen at −80°C. Frozen cells were thawed

* Corresponding author. Mailing address: Center for Radiological Research, Columbia University, Rm. VC 11-212, 630 W. 168th St., New York, NY 10032.

TABLE 1. Strains used in this study

| Strain | Genotype | Reference |
|---|---|---|
| 972 | $h^{-S}$ | 17 |
| GF101 | $h^{+N}$ *rad12-502* | 24 |
| GF102 | $h^{-S}$ *rad13-A* | 24 |
| GF103 | $h^{-S}$ *rad15-P* | 24 |
| GF105 | $h^{+N}$ *rad12-502 leu1-32* | This study |
| GF106 | $h^{-S}$ *rad12-502 rad13-A* | This study |
| GF110 | $h^{-S}$ *leu1-32* | This study |
| GF118 | $h^{-S}$ *rad22-67* | 24 |
| GF122 | $h^{-S}$ *rad12-502 rad22-67* | This study |
| GF150 | $h^{-S}$ *rad2-44* | 24 |
| GF152 | $h^{+N}$ *rad2-44 rad12-502* | This study |

and lysed by using a 50-ml bead beater (BioSpec Products Inc.). After the beads were separated, the cellular debris was removed by centrifugation for 1 h at 100,000 × *g*. The supernatant was dialyzed for 5 h to overnight against 100 volumes of dialysis buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-KOH [pH 7.6], 10 mM $MgSO_4$, 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 5 mM dithiothreitol, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride). Protein concentrations of the extracts, determined by the Bradford assay (Bio-Rad), were between 30 and 60 mg/ml.

**Preparation of the damaged oligonucleotide substrate.** The 6-4 PP 49-mer and CPD 49-mer were synthesized as described elsewhere (32). 3'-end labeling was carried out by incubating 1 pmol of the double-stranded oligonucleotide (Fig. 1a) with [α-$^{32}$P]dGTP (50 μCi, 3,000 Ci/mmol), 0.2 mM dATP, and 5 U of T4 DNA polymerase for 40 min at 14°C. This created a 3'-end-labeled 6-4 PP 51-mer or CPD 51-mer.

**SPDE assays.** Whole-cell extract (50 μg) was incubated with 0.02 pmol of the 3'-end-labeled 6-4 PP 51-mer at 37°C for 15 min in 45 mM HEPES-KOH (pH 7.8)–70 mM KCl–7 mM $MgCl_2$ in a 20-μl reaction mixture. The DNAs were isolated by proteinase K treatment followed by phenol-chloroform extraction. The DNAs were separated on a 15% polyacrylamide–urea gel. The gel was dried and exposed to X-ray film, and the results were quantitated with an Image Analysis system (Fuji).

**In vitro repair assay.** Extract preparation and reaction conditions were as described elsewhere (31). Following incubation at 30°C for 2 h plasmid DNA was repurified (31). The DNAs were separated on a 0.8% agarose gel. The gel was dried and exposed to X-ray film, and the results were quantitated with an Image Analysis system. Approximately 4 μg of partially purified SPDE (2 μg/μl) was added to some reaction mixtures prior to incubation.

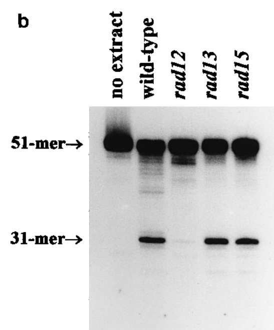
**SPDE purification.** *S. pombe* cells were grown in 1.5× YEA and a whole-cell extract was prepared as described above. Extract prepared for the in vitro repair reaction, the S-100 fraction, was bound to S/P Sepharose, and the SPDE activity was eluted by a linear 50 mM to 0.5 M NaCl gradient in buffer A (20 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 10% glycerol, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The pooled SPDE activity was bound to a mono-Q Sepharose column, and the SPDE activity was eluted by a linear 50 mM to 1.0 M NaCl gradient in buffer A. The eluted SPDE activity was then concentrated by ultrafiltration (Centricon) and size fractionated on tandem Superose 6 and Superose 12 columns. The peak activity fractions were pooled, concentrated as above, and used as the source of SPDE in these experiments. The concentration of protein was 2 mg/ml. This partially purified SPDE preparation contained very low levels of contaminating nucleases and was not active in the in vitro repair reaction (Fig. 2, lane 9).

**UV irradiations.** Irradiations were performed with 254-nm light at a dose rate of 2.68 J/m$^2$/s. Approximately 1,000 cells were plated on each plate. Following irradiation, plates were incubated at 30°C for 3 to 4 days and the number of viable colonies was determined. The results are based on three independent experiments. UV-damaged plasmid DNA was prepared by spotting 10-μl drop-

a

5'AGCTACCATGCACGAATTAAGCAATTCGTAATCATGGTCATAGCT 3'
3' GATGGTACGTGCTTAATTCGTTAAGCATTAGTACCAGTATCGACT 5'

b




c

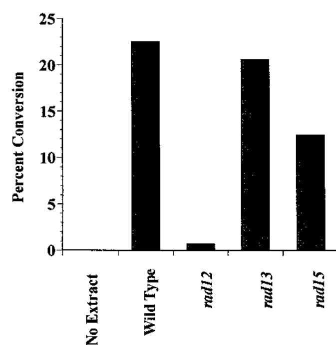
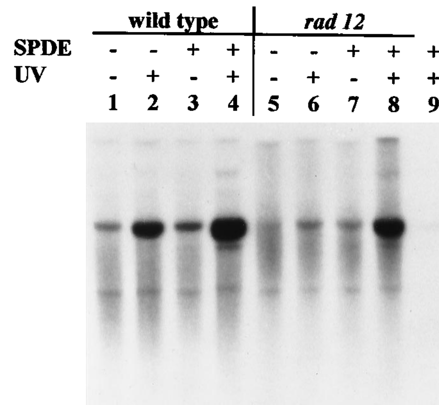



FIG. 1. The *rad12-502* mutant lacks SPDE activity. (a) A synthetic oligonucleotide (49-mer) containing a single 6-4 PP or CPD at the overlined site was used as the substrate for SPDE activity. The 49-mer was converted to a 3'-end-labeled 51-mer by a fill-in reaction containing T4 DNA polymerase with [α-$^{32}$P]dGTP and excess unlabeled dATP. (b) SPDE cleaves the phosphodiester bond immediately 5' to the (6-4) PP, generating a $^{32}$P-labeled 31-mer (5). Whole-cell extracts were prepared from *S. pombe*, as described elsewhere (29). Extracts from wild-type *S. pombe* and the *rad1* through *rad23* mutants were tested for SPDE activity. A representative result is shown for wild-type (strain 972), *rad12-502* (GF101), *rad13-A* (GF102), and *rad15-P* (GF103) cells. The labeled oligonucleotide was incubated in the presence of whole-cell extracts prepared from the strains indicated above each lane, and the products of the reaction were subjected to electrophoresis on a 15% denaturing polyacrylamide gel. (c) SPDE activity in each extract was quantitated by using a Fuji Image Analysis system. The data are percent conversion of the 51-mer to a 31-mer in the corresponding lanes of panel b.

a




b

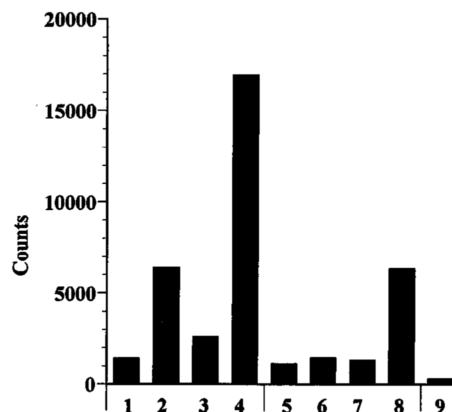



FIG. 2. SPDE complements the in vitro excision repair defect of extracts prepared from *rad12-502* cells. (a) The $^{32}$P 3'-end-labeled 6-4 PP 51-mer was incubated with a whole-cell extract prepared from a wild-type (strain 972) (lanes 1 to 4) or *rad12-502* (GF101) (lanes 5 to 8) strain. The extracts were incubated with damaged (lanes 2, 4, 6, 8, and 9) or undamaged (lanes 1, 3, 5, and 7) plasmid DNA (UV + and −, respectively). Exogenous SPDE was added as indicated (lanes 3, 4, and 7 to 9). Following incubation at 30°C for 2 h the plasmid DNA was isolated and the products were separated by electrophoresis on 0.8% agarose gels. The gels were dried and subjected to autoradiography. (b) The extent of repair DNA synthesis was quantitated by using a Fuji Image Analysis system. The data are relative extents of DNA synthesis in the corresponding lanes of panel a.

lets of supercoiled pUC18 DNA in Tris-EDTA at 0.1 μg/μl onto a sheet of Parafilm. The DNA was exposed to 100 J of 254-nm UV light per $m^2$.

## RESULTS

***rad12-502* mutants lack SPDE activity.** We previously demonstrated that extracts prepared from *S. pombe* had an activity which cleaves immediately 5′ to CPDs and 6-4 PPs on a double-stranded CPD 49-mer or 6-4 PP 49-mer oligonucleotide, respectively (5). We used this assay to screen for SPDE activity in 23 radiosensitive mutants of *S. pombe*, (*rad1* to *rad23* mutants) (19, 26). The substrate in these assays was a double-stranded $^{32}$P 3′-end-labeled CPD 51-mer or 6-4 PP 51-mer (Fig. 1a). SPDE activity was found in all of the strains except for the *rad12-502* mutant. While the other *rad* mutant strains varied in SPDE activity from 50 to 200% of that for the wild type, only *rad12-502* extracts consistently exhibited little to no detectable SPDE activity, ranging between 0 and 5% of that for the wild type (Fig. 1b and c). The *rad12-502* mutant has been shown to be UV sensitive but not sensitive to gamma rays (26). This suggests that the UV sensitivity of the *rad12-502* mutant is due to its lack of SPDE activity.

**Partially purified SPDE complements the in vitro excision repair deficiency of *rad12-502* extracts.** Whole-cell extracts prepared from wild-type *S. pombe* are active for DNA repair synthesis in vitro on an exogenous plasmid template, as shown by the incorporation of α-$^{32}$P-deoxynucleoside monophosphates (31). DNA repair synthesis in this assay is dependent on the presence of damage in the DNA (Fig. 2, lanes 1 and 2). Addition of partially purified SPDE to wild-type extracts increases the amount of DNA repair synthesis (Fig. 2; compare lanes 2 and 4), suggesting that SPDE is limiting in these reactions.

In contrast, whole-cell extracts prepared from *rad12-502* cells are deficient in DNA repair synthesis on a UV-damaged plasmid (Fig. 2, lane 6). Addition of partially purified SPDE to *rad12-502* extracts complements this defect and promotes DNA repair synthesis in the presence of a UV-damaged DNA substrate (Fig. 2, lanes 8). This directly demonstrates that SPDE is necessary for repair synthesis in these extracts and shows that the deficiency in DNA repair synthesis in *rad12-502* extracts is due to the lack of SPDE activity. The partially purified SPDE used in these reactions has no activity by itself in this in vitro excision repair assay (Fig. 2, lane 9).

***rad12* defines a repair pathway distinct from NER.** The *rad12* gene has not been previously identified to be genetically linked to any epistasis group in *S. pombe*. To further define the relationship of the SPDE-dependent excision repair pathway with other repair pathways previously described for *S. pombe*, epistasis studies were carried out. First, a *rad12-502 rad13-A* double mutant was selected from a cross of GF105 with GF102. The *rad13* gene is a functional homolog of *S. cerevisiae RAD2* (6, 27), the NER endonuclease responsible for excision 3′ to the UV photoproduct (24). The sensitivity of the *rad12-502 rad13-A* double mutant to UV light was compared with that of each single mutant and with that of wild type cells. The *rad12-502 rad13-A* double mutant is much more sensitive to UV light than either single mutant (Fig. 3). The double mutant showed a more than multiplicative sensitivity to UV light. For example at 50 J/$m^2$, *rad12-502* cells show 30% survival, *rad13-A* cells show 0.3% survival, and *rad12-502 rad13-A* cells show 0.0004% survival. These data indicate that the SPDE-dependent DNA repair pathway defined by *rad12* is distinct from the NER pathway. This conclusion is further supported by the fact that extracts prepared from *rad13-A* cells contain SPDE activity and are proficient in the repair of UV damage in vitro (data not shown).

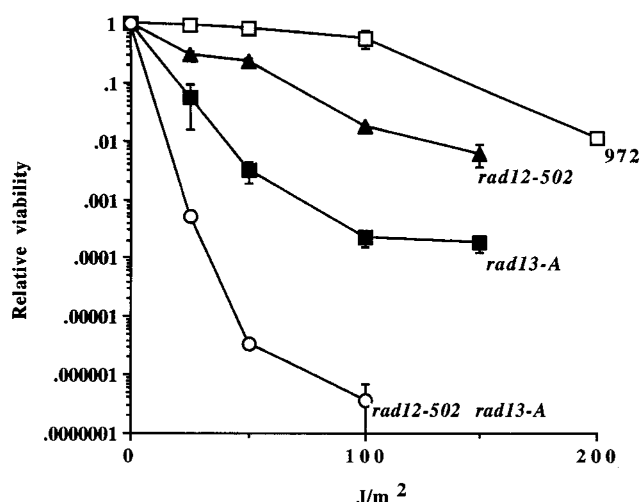



FIG. 3. *rad12* defines an excision repair pathway distinct from NER. The sensitivity to 254-nm UV light was determined for *rad12-502* and *rad13-A* single and double mutants. Cells were cultured to mid-log phase, transferred to plates, and irradiated with the indicated doses. Bars, standard deviations for three independent experiments. The *rad12-502 rad13-A* double mutant (GF106) shows a radiation sensitivity much higher than that of either the *rad12-502* (GF105) or the *rad13-A* (GF102) single mutant, indicating that the *rad12-502* and *rad13-A* mutations are in different repair pathways.

**The repair pathway defined by *rad12* may play a role in *rad22*-dependent recombinational repair.** While the data in Fig. 2 demonstrate that SPDE is part of an excision repair process, the possibility that it could also initiate a recombinational repair process exists. To test this possibility, a strain containing both the *rad12-502* and the *rad22-67* mutations was generated. The *rad22* gene is a functional homolog of the *S. cerevisiae* recombinational repair gene *RAD52* (25). The *rad22-67* mutant shows only slight UV sensitivity, suggesting that it plays a minor role in resistance to UV damage to DNA. The *rad12-502 rad22-67* double mutant shows sensitivity to UV light which is only slightly greater than that of the *rad12-502* mutant alone (Fig. 4). These data suggest that *rad12-502* and *rad22-67* define partially overlapping pathways.

**The *rad12-502* mutant does not show epistasis with *rad2*.** Since the *rad2* gene product has been implicated as a component of a repair process distinct from NER (23), it follows that it could be part of the same DNA repair pathway defined by *rad12-502*. A cross between the $h^{+N}$ *rad12-502 leu1-32* (GF105) and $h^{+S}$ *rad2-44* (GF118) strains was made. The UV sensitivity of the double mutant was the product of the sensitivities of the individual mutants, showing that these two mutants are in separate epistasis groups (Fig. 5).

## DISCUSSION

We have shown that the *rad12-502* mutant of *S. pombe* is deficient in both SPDE activity and UV damage-dependent excision repair synthesis in vitro. In whole-cell extracts prepared from *rad12-502* cells, DNA repair synthesis was reconstituted by the addition of partially purified SPDE. This demonstrates that the excision repair deficiency in *rad12-502* extracts is due to the lack of SPDE. UV survival studies clearly demonstrate that the *rad12* gene product is involved in a DNA repair pathway distinct from NER, as double mutants deficient in both the *rad12* and the *rad13* gene products are hypersen-

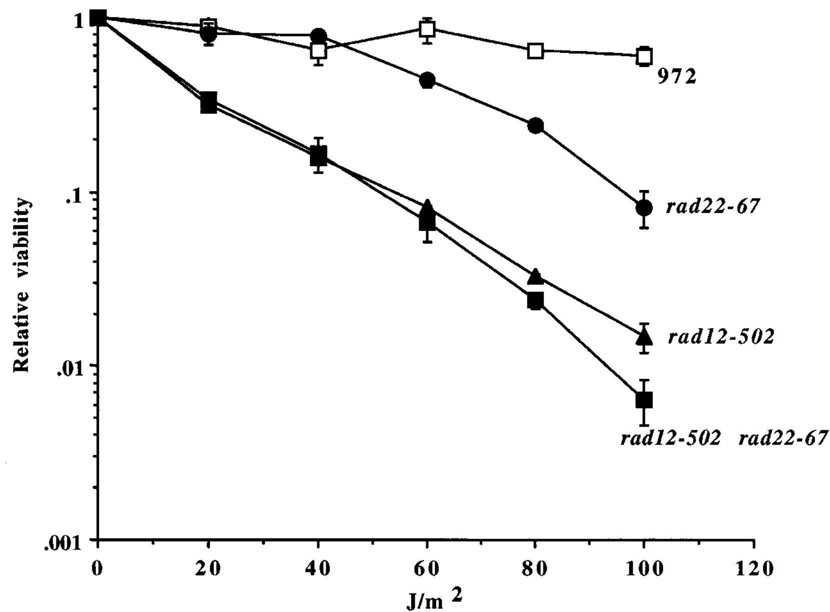



FIG. 4. The repair pathway defined by *rad12* may play a role in recombination. To test for the potential role of the *rad12* gene product in a recombinational repair process, a strain containing both the *rad12-502* and the *rad22-67* mutations was generated. Cells were grown to mid-log phase and plated at the appropriate dilutions. The cells were exposed to the indicated dose of 254-nm UV light. Cells were incubated for 3 to 4 days at 30°C and counted. Bars, standard deviations for three independent experiments.

sitive to UV radiation. Taken together, these data implicate SPDE in a novel form of excision repair. To date, we have not identified a substrate for SPDE other than CPDs and 6-4 PPs.

Generation of single-strand breaks in DNA has been shown to stimulate recombination-inducing processes (33). Since a single-strand break is the product of the SPDE incision, we wanted to test whether SPDE might be involved in recombinational repair. The *rad22* gene product is a homolog of the *S. cerevisiae RAD52* gene, which is involved in recombinational repair. A double mutant containing both the *rad12-502* and the *rad22-67* mutations was constructed and tested for sensitivity to UV radiation. UV survival studies showed that the *rad12-502 rad22-44* double mutant is slightly more sensitive than either of the single mutants but not multiplicatively, demon-

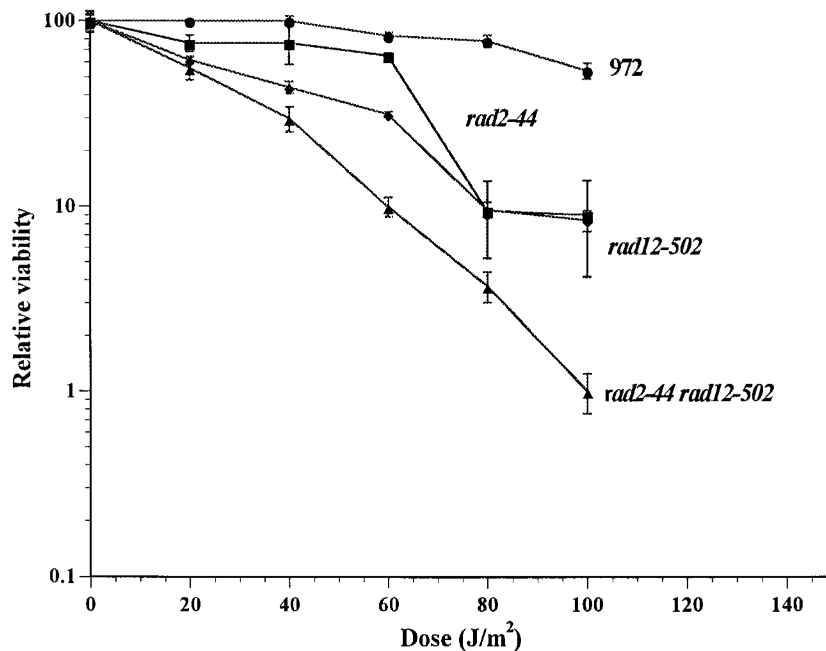



FIG. 5. The *rad12-502* mutant does not show epistasis with *rad2*. The sensitivity to 254-nm UV light was determined for *rad12-502* and *rad2-44* single and double mutants. Cells were cultured to mid-log phase, transferred to plates, and irradiated with the indicated doses. Bars, standard deviations for three independent experiments.

strating that the *rad12* gene product may play a role in recombinational repair. This does not diminish a role for SPDE in DNA excision repair, as shown in the in vitro excision repair assay. In contrast, *rad12* does not appear to be in the same epistasis group as *rad13* or the undefined pathway represented by *rad2-44.*

The existence of multiple excision repair pathways in *S. pombe* was first suggested by Birnboim and Nassim (4). They demonstrated that CPDs were efficiently removed from all nine of the *S. pombe* UV-sensitive strains that they tested. Recently, McCready and coworkers used an immunoassay to determine that in five NER-deficient strains of *S. pombe*—the *rad13-A*, *rad13*Δ, *rad15-P*, *rad16-U*, and *rad16*Δ strains—both CPDs and 6-4 PPs were efficiently removed (21). In their study the use of deletion mutants, *rad13*Δ and *rad16*Δ, ruled out the possibility that the original strains were leaky. On the basis of their results, these investigators concluded that *S. pombe* did indeed possess an additional excision repair pathway distinct from NER. We propose that the SDR pathway is initiated by incision immediately 5′ to CPDs and 6-4 PPs by SPDE followed by removal of the damaged nucleotides and resynthesis.

There are a variety of processes by which cells repair DNA damage. In addition to NER, DNA damage can be repaired by recombination (14), mismatch repair (11), or a host of enzymes involved in base excision repair, including uracil DNA glycosylase, which removes uracil generated by the deamination of cytosine (10). This process involves removing the uracil base, creating an abasic site, followed by cleavage by an apyrimidinic endonuclease (AP endonuclease) in a β-elimination reaction (20). The action of SPDE demonstrates a very different repair process. SPDE is not an *N*-glycosylase and cleaves immediately 5′ to the UV photoproduct. Cleavage by SPDE results in a 3′ hydroxyl terminus, the priming site for repair synthesis. The steps following excision by SPDE, including removal of the damaged nucleotides, remain to be deduced. The damaged bases could be removed by an exonuclease or incision 3′ to the site of damage by an endonuclease. We have previously demonstrated that DNA repair synthesis is completely dependent on the presence of ATP and an ATP-regenerating system. This could mean that ATP is required in the removal of the damaged bases. It is also consistent with DNA polymerase δ or ε, both of which require ATP-dependent PCNA (proliferating cell nuclear antigen) for activity (35).

While SPDE-dependent repair differs from NER, it is not necessarily independent of it. It is entirely possible that SPDE-dependent repair requires various components of NER. NER-deficient cells from people with xeroderma pigmentosum show a wide range of repair synthesis activity and capacity to excise UV photoproducts (12). If SPDE exists in human cells, a partial dependence on NER could explain this variability in cells from people with xeroderma pigmentosum. Recently it was reported that an activity identical to SPDE exists in *Neurospora crassa* (37). We have also found an SPDE-like activity in *Schizosaccharomyces japonicus* (unpublished results). It will be important to demonstrate the range of organisms having SPDE and SPDE-dependent repair.

For many years following its discovery, NER was believed to occur by incision adjacent to the damage site by a UV endonuclease followed by removal of the damaged bases by a 5′→3′ exonuclease. However, while its existence was supported by several studies (see reference 28 for a review), the required UV endonuclease was never identified. Meanwhile, it was determined that, in *Escherichia coli*, UV photoproducts are repaired by a protein complex which cleaves at a distance 3′ and 5′ to the site of damage, removing the adducts on an oligomer (NER). Resolving the mechanism of NER ended a search for the UV endonuclease. Our results support the existence of an alternative DNA excision repair pathway, which, interestingly, is reminiscent of the mechanism originally proposed for the process of NER. Although it is not known at present whether the later steps and components of this alternative excision repair pathway differ from NER, the initial DNA damage recognition and cleavage events are clearly distinct from those of NER. It will be important to establish the relative contributions of these alternative repair processes in other eukaryotes.

## ACKNOWLEDGMENTS

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