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Characterization of the alternative excision repair pathway of UV-damaged DNA in *Schizosaccharomyces pombe*

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ABSTRACT

***Schizosaccharomyces pombe* cells deficient in nucleotide excision repair (NER) are still able to remove photoproducts from cellular DNA, showing that there is a second pathway for repair of UV damage in this organism. We have characterized this repair pathway by cloning and disruption of the genomic gene encoding UV damage endonuclease (UVDE). Although *uvde* gene disruptant cells are only mildly UV sensitive, a double disruptant of *uvde* and *rad13* (a *S.pombe* mutant defective in NER) was synergistically more sensitive than either single disruptant and was unable to remove any photoproducts from cellular DNA. Analysis of the kinetics of photoproduct removal in different mutants showed that the UVDE-mediated pathway operates much more rapidly than NER. In contrast to a previous report, our genetic analysis showed that *rad12* and *uvde* are not the same gene. Disruption of the *rad2* gene encoding a structure-specific flap endonuclease makes cells UV sensitive, but much of this sensitivity is not observed if the *uvde* gene is also disrupted. Further genetic and immunochemical analyses suggest that DNA incised by UVDE is processed by two separate mechanisms, one dependent and one independent of flap endonuclease.**

INTRODUCTION

Various repair mechanisms have evolved to counteract the deleterious effects of UV irradiation on cellular DNA. One of the most widely distributed repair enzymes is photolyase, which utilizes visible light to monomerize UV-induced DNA damage (1). In the bacterium *Micrococcus luteus* and T4 phage-infected *Escherichia coli*, specific excision repair enzymes for UV-induced cyclobutane pyrimidine dimers (CPDs) were found which introduce nicks at CPDs in DNA and have been designated UV endonucleases. These enzymes are, however, pyrimidine dimer DNA glycosylases with a DNA lyase activity which acts 3′ of the abasic site created by the glycosylase activity (for a review see 2). They do not produce nicks at (6–4) photoproducts (6–4 PPs).

The most widely distributed DNA repair process is nucleotide excision repair (NER), in which an oligonucleotide containing damaged DNA is removed and repair is completed by DNA synthesis and subsequent rejoining of the gaps. In NER, many proteins are necessary for the early steps of damage recognition and introduction of nicks flanking the UV-induced DNA damage (3). NER can repair not only UV-induced DNA damage, but also many other types of DNA damage, including chemically induced DNA monoadducts and DNA–DNA crosslinks. NER has been thought to be the only mechanism by which cells can excise CPDs as well as 6–4 PPs from their DNA. In *Saccharomyces cerevisiae*, for example, null mutants in NER are completely unable to remove such damage. In the fission yeast *Schizosaccharomyces pombe*, however, strains carrying deletion mutations in NER genes retain a substantial ability to remove both CPDs and 6–4 PPs from their DNA. This has provided evidence for the presence of another excision repair pathway in this organism (4,5). In previous work we used epistasis analysis to identify a series of candidate genes that were likely to be involved in this process (6,7), but the gene encoding the enzyme which could carry out the incision step was not identified and all the double mutants that we analyzed retained the ability to remove photoproducts.

An enzymatic activity has been found in extracts of *S.pombe* cells that introduces nicks immediately 5′ of both CPDs and 6–4 PPs (8). We recently isolated a cDNA from a *S.pombe* library which corrected the UV sensitivity of a totally repair deficient (*uvrA recA phr*) strain of *E.coli* (9). This gene encodes a protein of 69 kDa with significant sequence similarity to an UV endonuclease which we previously isolated from *Neurospora crassa* (10). Since the partially purified recombinant protein prepared from *E.coli* cells harboring the cloned *S.pombe* gene showed the same cleavage activity as the *Neurospora* protein and the reported extract of *S.pombe*, we concluded that the gene encodes a homolog of the *N.crassa* UV endonuclease, UV damage endonuclease (UVDE). To understand the mechanism of the putative alternative

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excision repair, possibly initiated by nicks introduced by UVDE at the damaged sites, we have constructed various *S.pombe* strains with single and double disruptions in different repair genes. We report here that UVDE initiates the alternative excision repair process, which is distinct from NER. UVDE-mediated excision repair removes UV damage much more rapidly than NER. Furthermore, we show that the nicked sites are further processed by two separate mechanisms, one dependent and one independent of Rad2, a structure-specific flap endonuclease.

MATERIALS AND METHODS

Cell strains

Escherichia coli strain SY2 (*uvrA recA phr*) (11) was used for screening of UV-resistant transformants. For gene disruptions in *S.pombe*, the *S.cerevisiae LEU2* marker gene was introduced into the cloned genomic *uvde* gene. Double and triple disruption mutants were constructed by crossing the *uvde* disruptant with existing repair-defective *rad* disruptants (or by disruption of the *uvde* gene in the *rad* disruptants). Table 1 lists the *S.pombe* strains used in this paper.

Cell survival

For UV irradiation, 254 nm UVC light was used. Overnight cultures of cells were washed and resuspended in water. Cells were irradiated at ~1×10^6 cells/ml, after which appropriate cell dilutions were plated on YPD (10 g/l yeast extract, 20 g/l polypeptone, 20 g/l glucose, 15 g/l agar) plates. Alternatively, cells were grown for 4 h in liquid medium, resuspended in H_2O and plated on YPD prior to UV irradiation.

Cloning of the *uvde* gene from a genomic library

A *S.pombe* genomic library (a generous gift of Dr M.Yanagida) was introduced into *E.coli* SY2 cells. Aliquots of 100 µl of an overnight culture of SY2 cells transformed with the genomic library were irradiated with a UV dose of 0.1 J/m^2 on a LB plate with four antibiotics (ampicillin, kanamycin, chloramphenicol and tetracycline) and incubated overnight. Surviving colonies were collected and cultured before the next round of UV irradiation. After three rounds of selection, a number of surviving colonies were examined to test UV resistance. One clone contained a 6.7 kb insert which conferred UV resistance to the recipient cells. By comparison of the restriction maps and partial sequence between the cloned genomic gene and the previously isolated *S.pombe* cDNA (9) encoding the *uvde* homolog of *N.crassa* (10), we found that the cloned genomic DNA contains the *uvde* gene of *S.pombe*.

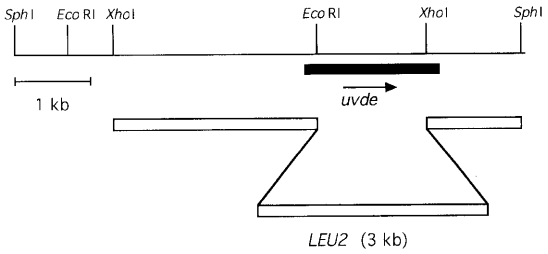

Figure 1. Restriction map of the 6.7 kb cloned genomic DNA fragment harboring the *uvde* gene. The coding region is indicated by the closed bar, with an arrow showing the direction of transcription. The open bar depicts the genomic DNA with insertion of the *S.cerevisiae LEU2* gene. This fragment was used for the disruption of the *uvde* gene.

Immunoassay for repair of photoproducts

Samples of 180 ml cells were irradiated with a dose of 100 J/m^2 UVC in water at a density of between 1 and 2×10^7 cells/ml. To these were added 18 ml 10× yeast extract medium and cells were incubated at 30°C. Aliquots of 30 ml were harvested at various times into an equal volume of ethanol to stop repair. DNA was extracted, using glass beads and prolonged vortex mixing of the disrupted cells, followed by phenol/chloroform extraction, ethanol precipitation and RNase treatment. The amounts of DNA in the samples were equalized by running aliquots on agarose gels, scanning the gels and readjusting as necessary. The dot blot immunoassay for CPDs and 6–4 PPs has been described in detail previously (5). The two classes of photoproducts are readily distinguished because of their differential sensitivities to hot alkali (6–4 PPs) and *E.coli* photolyase (CPDs). Lesions were detected using a polyclonal rabbit antiserum, a biotin/ExtrAvidin (Sigma) detection system and a BioRad model GS-670 imaging densitometer.

Table 1. Strains used in this study

Strain	Phenotype	Genotype	Source
sp011	Wild-type	*ade6-704 ura4-D18 leu1-32 h*$^-$	(12)
sp012	Wild-type	*ade6-704 ura4-D18 leu1-32 h*$^+$	(12)
ry001	*uvde*d	*uvde*::*LEU2*$^+$ *ade6-704 ura4-D18 leu1-32 h*$^-$	This study
sp217	*rad2*d	*rad2*::*ura4*$^+$ *ade6-704 ura4-D18 leu1-32 h*$^-$	(12)
ry003	*rad2*d *uvde*d	*rad2*::*ura4*$^+$ *uvde*::*LEU2*$^+$ *ade6-704 ura4-D18 leu1-32 h*$^-$	This study
spA4	*rad12*$^-$	*rad12–502 Ch16*(*ade6-M216*) *ade6-M210 ura4-D18*	(12)
ry004	*rad12*$^-$ *uvde*d	*rad12-502 Ch16*(*ade6-M216*) *uvde*::*LEU2*$^+$ *ade6-M210 ura4-D18*	This study
sp222	*rad13*d	*rad13*::*ura4*$^+$ *ade6-704 ura4-D18 leu1-32 h*$^-$	(12)
ry008	*rad13*d *uvde*d	*rad13*::*ura4*$^+$ *uvde*::*LEU2*$^+$ *ade6-704 ura4-D18 leu1-32 h*$^-$	This study
sp210	*rad2*d *rad13*d	*rad2*::*ura4*$^+$ *rad13*::*ura4*$^+$ *ade6-704 ura4-D18 leu1-32 h*$^+$	(12)
ry010	*rad2*d *rad13*d *uvde*d	*rad2*::*ura4*$^+$ *rad13*::*ura4*$^+$ *uvde*::*LEU2*$^+$ *ade6-704 ura4-D18 leu1-32 h*$^+$	This study

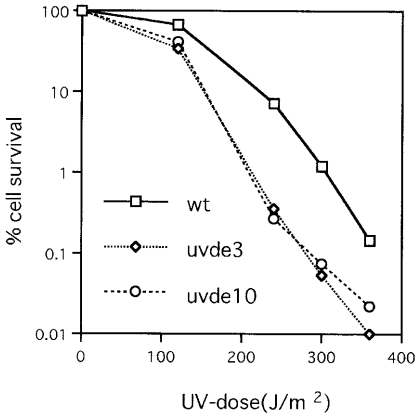

Figure 2. Influence of *uvde* disruption on UV sensitivities of wild-type cells. UV survival curves of a wild-type (wt) and two *uvde* disruptants (uvde3 and uvde10) are shown.

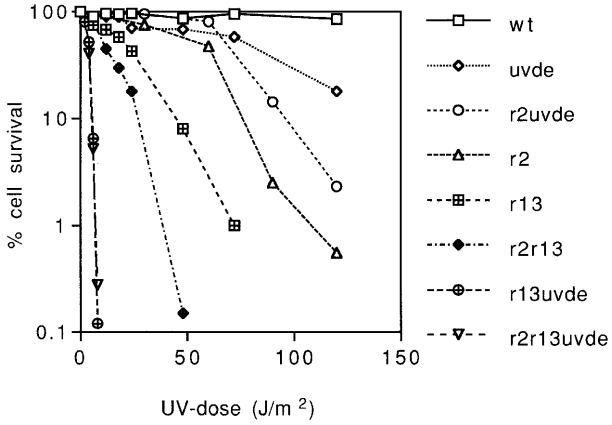

Figure 3. Influence of *uvde* disruption on UV sensitivities of various repair-deficient mutant cells. UV survival curves are compared among wild-type (wt), *uvde*d (uvde), *rad2*d (r2), *rad2*d *uvde*d (r2uvde), *rad13*d (r13), *rad2*d *rad13*d (r2r13), *rad13*d *uvde*d (r13uvde) and *rad2*d *rad13*d *uvde*d (r2r13uvde).

RESULTS

Cloning and disruption of the *uvde* gene in wild-type cells

We previously isolated a *uvde* cDNA clone of *S.pombe* that showed significant sequence similarity to the *uvde* gene from *N.crassa* and we designated the *S.pombe* gene *uvde* (9). In order to construct a deletion in the *uvde* gene we have isolated a *S.pombe* genomic DNA fragment containing the *uvde* gene by complementation of UV sensitivity in repair-deficient *E.coli* SY2 cells with a genomic library of *S.pombe* DNA (see Materials and Methods). By comparison of the restriction maps of the cloned genomic and the cDNA fragments, as well as by partial sequencing of the genomic DNA, we found that the cloned genomic DNA contains the *uvde* gene and that the gene does not contain any introns. A part of the cloned *uvde* gene was replaced with the *S.cerevisiae LEU2* gene (Fig. 1) and introduced into a wild-type *S.pombe* strain, resulting in a *uvde* gene disruptant (*uvde*d). This disruption was confirmed by analysis of the genomic DNA of the disruptant using gene-specific oligonucleotides and PCR (not shown).

UVDE acts in a rapid repair process distinct from NER

Figure 2 shows the UV sensitivities of two *uvde* disruptants (uvde3 and uvde10) independently constructed from a wild-type strain. The *uvde* gene disruption made cells only mildly sensitive to UV, even after high doses. A *uvde* gene disruption was also introduced into a *rad13*d NER-defective strain. *rad13* is the *S.pombe* homolog of the human XPG and *S.cerevisiae RAD2* genes, which encode an endonuclease which cleaves DNA 3′ of the damage during NER. Figure 3 shows that *rad13*d cells were more sensitive to UV than *uvde*d and the double disruption was much more sensitive than *rad13*d. These data indicate that the UVDE-mediated repair pathway is distinct from NER in this organism.

In order to obtain biochemical support for the above genetic studies, we have measured the repair of UV-induced CPDs and 6–4 PPs using antibodies raised against both substrates (Fig. 4). As previously reported (5), the rate of photoproduct removal in *rad13*d was only slightly lower than that in wild-type cells. In the *uvde* disruptant (in which only NER is operative) the rate of repair of both photoproducts was considerably slower. The *rad13*d *uvde*d double mutant cells were unable to remove either type of damage, even during 3 h post-UV incubation. Since the antibody used in this experiment can recognize both types of damage, even after nicks have been introduced by UVDE at the damaged sites (S.J.McCready, unpublished results), the above results indicate that UVDE-mediated repair not only introduced nicks at damaged sites but also removed the damage more rapidly than NER.

Post-incision steps

Schizosaccharomyces pombe rad2 encodes a homolog of mouse FEN-1, which is a ‘flap’ structure-specific endonuclease (12,13). A *rad2*d mutant is sensitive to UV and was shown to be deficient in a repair pathway distinct from NER (7). We found that the double mutant *uvde*d *rad2*d was more resistant than a *rad2*d single mutant (Fig. 3). This implies that the Rad2 protein is very important for processing nicks introduced by UVDE. If, however, UVDE is not present in the cell, the role of Rad2 in repair of UV damage is less important. The influence of *rad2* was further tested by measuring the UV sensitivities of other double and triple mutants. In contrast to the results in NER-proficient cells, in the absence of NER *uvde*d *rad13*d cells are much more UV sensitive than *rad2*d *rad13*d. Furthermore, a *rad13*d *uvde*d *rad2*d triple disruptant had the same UV sensitivity as the *rad13*d *uvde*d double disruptant. These results show that the Rad2 protein is involved only in the UVDE-mediated second pathway and that there are both *rad2*-dependent and *rad2*-independent components of the UVDE-mediated repair pathway.

Relation of Rad12 to UVDE

A UV-sensitive *S.pombe* mutant, *rad12*, has been reported to be severely deficient in UV endonuclease activity (14). Furthermore, a *rad13 rad12* double mutant was more sensitive than either single mutant. These results were interpreted as suggesting that *rad12* defined a second repair pathway and they raised the possibility that *rad12* might encode UVDE (14). However, in a cross between a *uvde*d and a *rad12-502* point mutant (*rad12* has not yet been cloned), the two genes segregated as unlinked loci. Furthermore, as shown in Figure 5, the *rad12-502 uvde*d double

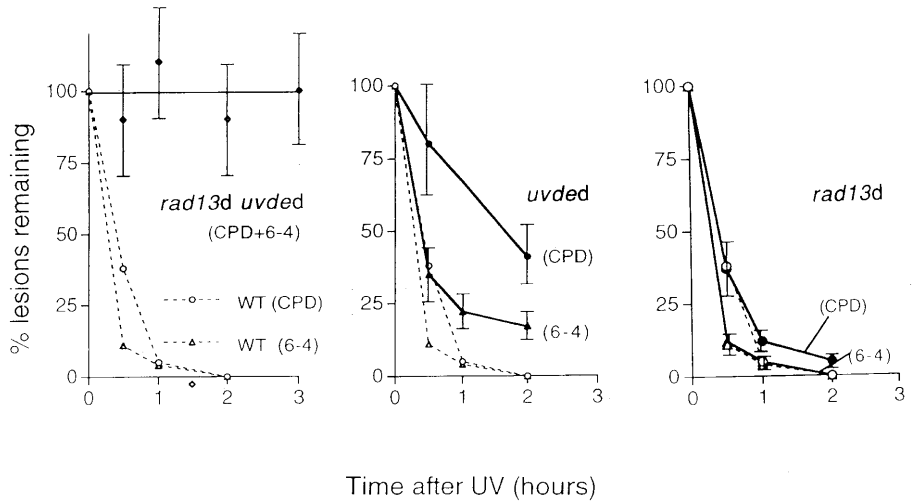

Figure 4. Removal of photoproducts from DNA in UV-irradiated *S.pombe* cells. The amounts of remaining CPDs as well as 6–4 PPs are shown for cells of *rad13*d, *uvde*d and *rad13*d *uvde*d cells and compared with those for wild-type cells (dashed lines).

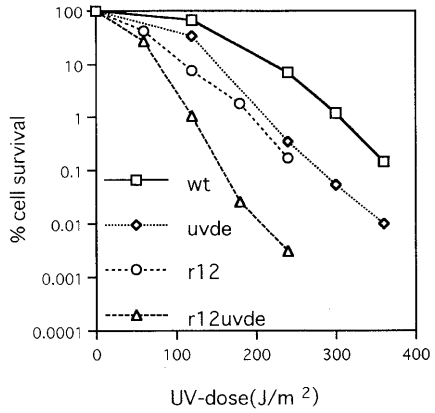

Figure 5. Influence of *uvde* disruption on UV sensitivity of *rad12* mutant cells.

mutant was more sensitive to UV than either single mutant. These results rule out the possibility that *rad12* encodes UVDE and demonstrate that the UV sensitivity of the *rad12* mutant is not caused by a defect in the *uvde* pathway.

Effects of the introduction of the *uvde* gene in a multi-copy plasmid into repair-deficient cells

To determine the influence of increased *uvde* expression on UV resistance in various repair-deficient strains, we introduced the genomic *uvde* gene with a 3.9 kb 5′ upstream sequence in a multi-copy plasmid harboring *ade6* as the selection marker into different mutants. The plasmid (pSPuvde-g) rendered *uvde*d (Fig. 6a), *uvde*d *rad2*d *rad13*d (Fig. 6b) and *uvde*d *rad12-502* (Fig. 6c) cells more resistant to UV than the corresponding *uvde*$^+$ strains, namely wild-type, *rad2*d *rad13*d and *rad12-502* respectively. Interestingly, introduction of the plasmid into *uvde*d *rad2*d cells had no influence on their UV response (Fig. 6d). This shows that in NER$^+$ cells high level expression of UVDE increases the biological effectiveness of photoproduct repair, but only if Rad2 is also present.

DISCUSSION

In an earlier report we showed that *S.pombe* has a second repair pathway, distinct from NER, for removing UV photoproducts (5). Further work implicated several genes in this second repair pathway (6), but we were not able to identify the protein which initiates the pathway. In the current work we have shown conclusively that UVDE is this key enzyme. The double disruptant of *uvde* and the NER *rad13* gene is synergistically more sensitive to UV than either single disruptant (Fig. 3), demonstrating that UVDE-mediated repair is distinct from NER. Furthermore, this double disruptant is completely unable to repair UV photoproducts (Fig. 4). Since partially purified UVDE of *S.pombe* has an endonuclease activity for CPDs and 6–4 PPs in *in vitro* analyses (9) and since both types of damage disappeared rapidly in *rad13*d cells, we infer that the second excision repair pathway of *S.pombe* is a rapid process initiated by the nicking activity of UVDE at both types of UV damage.

By using cells disrupted in *uvde* and other repair genes, we have carried out a genetic analysis of this pathway. The single *uvde* disruptant is mildly sensitive to UV, as was also found with *uvde*-deficient *mus-18* mutants of *N.crassa* (15,10). Several other genes have been proposed to be involved in the second repair pathway. We suggested that the *rad2*, *rad18* and *rhp51* genes, which belong to the same epistasis group, distinct from NER, were involved in the later stages of the second pathway (6). A *rad2* disruptant is more sensitive to UV than a *rad2 uvde* double disruptant (Fig. 3). This indicates that Rad2 has a crucial role in processing of UVDE-nicked sites, resulting from the action of UVDE. We infer that following cleavage of the damaged DNA by UVDE, strand displacement synthesis occurs from the nicked sites with the formation of a flap structure, known to be an excellent substrate for Rad2-like nucleases (12,13). In the absence of Rad2, incisions by UVDE at damaged sites results in the accumulation of potentially lethal intermediates, thereby conferring UV sensitivity upon *rad2* mutants. If UVDE is also absent, these intermediates are not produced and there is a reduced requirement for Rad2. Thus the *rad2 uvde* double mutant is less sensitive than the *rad2* single mutant.

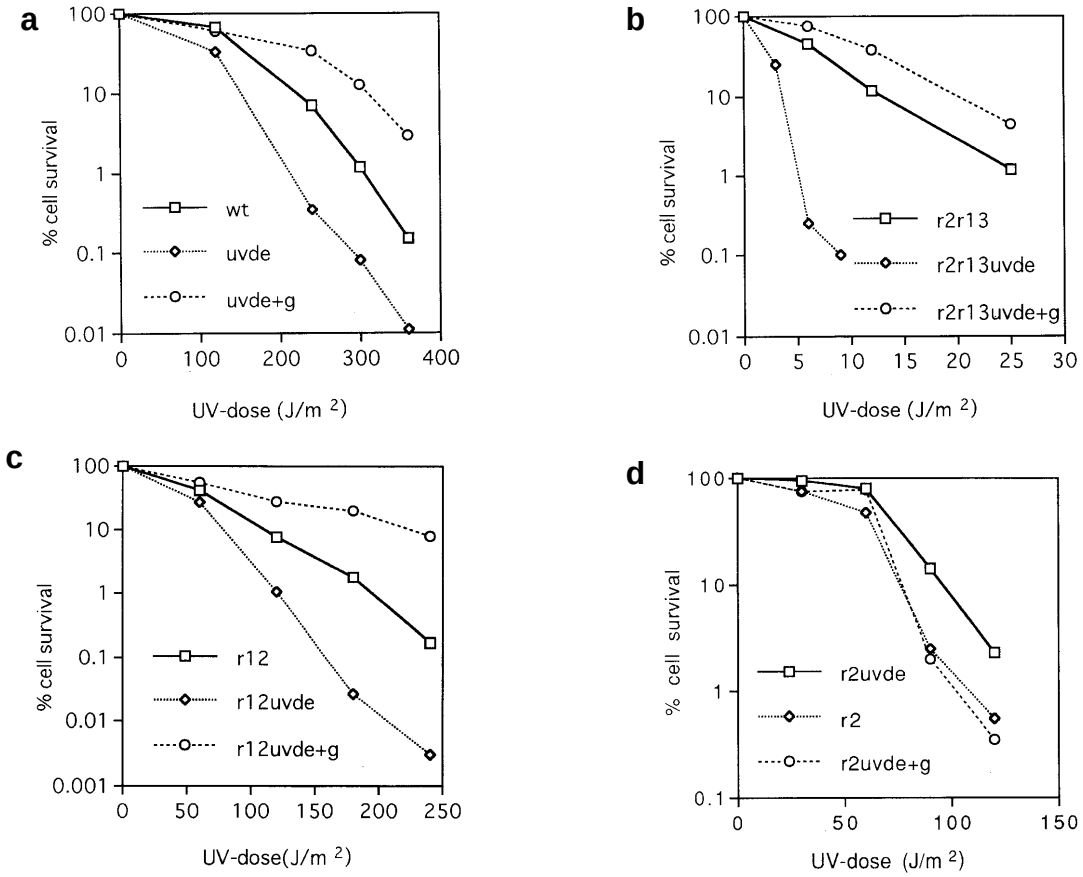

Figure 6. Influence of the introduced pSPuvde-g plasmid on UV survival of various *uvde* disruptants. Host cells are (**a**) *uvde*d (uvde); (**b**) *rad2*d *rad13*d *uvde*d (r2r13uvde); (**c**) *rad12* (r12); (**d**) *rad2*d (r2). UV survival of transformed cell is indicated by addition of +g to the names of host cells.

Expression experiments further showed the importance of Rad2 in the UVDE-dependent repair pathway in NER-proficient cells. Whereas introduction of a genomic *uvde* fragment made *uvde*d cells more UV resistant than wild-type cells, it had no influence on *rad2*d cells. This confirms that Rad2 is most important for processing UVDE-induced nicks in NER-proficient cells. As discussed below, UVDE-induced nicks are also substrates for a rapid Rad2-independent repair process, only a part of which, however, leads to complete repair of DNA damage and cell survival. Therefore, in *rad2*d cells, UV-induced damage processing is greater by the incomplete Rad2-independent repair than by the complete NER. Reasonably, high expression of UVDE in *rad2*d cells does not increase cell survival. In the absence of NER, the *rad2 rad13 uvde* triple disruptant was more sensitive than the *rad2 rad13* double disruptant, indicating that some nicks produced by UVDE can also be processed and repaired by a Rad2-independent pathway(s). Introduction of a high copy plasmid harboring a genomic *uvde* gene increased the resistance of the triple disruptant to a level slightly higher than that of the double *rad2 rad13* disruptants. These results are quite different from those for NER$^+$ cells, because the Rad2-independent repair process is the only repair mechanism available in the *rad13 rad2* double mutant.

Another gene, *rad12*, has been reported to be related to UVDE-mediated repair (14). Endonuclease activity against a synthetic oligonucleotide containing a thymine–thymine dimer was not detected in extracts of *rad12-502* cells. Genetic crosses showed, however, that *uvde* and *rad12* are unlinked loci and, as shown in Figure 5, *uvde rad12* double mutants were more UV sensitive than either single mutant, indicating that *rad12* is not the structural gene for UVDE and that the UV sensitivity of *rad12* is not related to a deficiency in UVDE-mediated repair. These data are consistent with the report that *rad12* and *rad2* belong to different epistasis groups for UV sensitivity (14). *rad12* may be involved in regulation of the expression of *uvde*, so that it can be expressed at the right moment in the cell cycle or in response to DNA damages. However, mutation in the *rad12* gene had no influence on the increase in UV resistance due to introduction of a plasmid containing the genomic *uvde* gene with 3.9 kb upstream sequence (Fig. 6c). Although UVDE activity in extracts of *S.pombe* appears to be UV inducible (14; J.M.Murray and A.M.Carr, in preparation), Northern analysis of the *uvde* gene did not show any UV (200 J/m^2 for wild-type and 20 J/m^2 for *rad13*d) inducibility at the transcription level in the first hour after UV (not shown).

Figure 7 depicts a model for the excision repair pathways of UV-induced damage in *S.pombe*. In the absence of Rad2 in a NER-proficient background, UVDE-mediated repair may produce abortive intermediates and may, therefore, interfere with NER, leading to cell death. The data in Figure 4 suggest that in *S.pombe* wild-type cells more UV-induced damage is likely to be processed by UVDE-mediated repair than by NER. However,

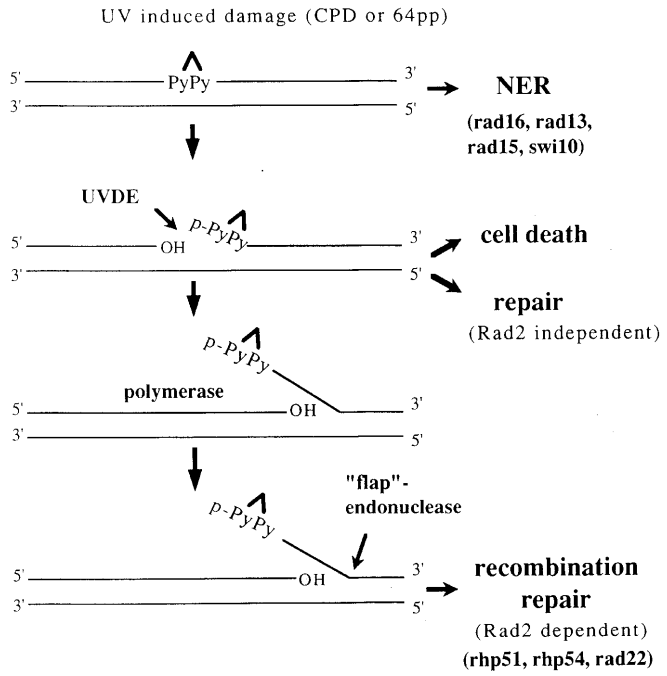

Figure 7. A model for the UVDE-dependent repair pathway.

since wild-type cells as well as wild-type cells expressing the *uvde* gene from a plasmid are hyper-resistant to UV, it is possible that there is a delicate balance between the two repair pathways and that their control might be linked. The presence of two excision repair pathways with different repair characteristics may be advantageous for cells, especially for those irradiated with high doses of UV, which produces a large number of CPD and 6–4 PP and additionally various other kinds of UV damage than CPD and 6–4 PP (2). Repair efficiency of such damage may influence survival of the deletion mutants shown in Figure 3.

We have shown here that the presence of two distinct excision repair pathways contributes to the hyper-resistance of *S.pombe* cells to UV. However, it is not clear why *S.pombe* possesses two excision repair pathways. Recently we isolated a UVDE homolog from the bacterium *Bacillus subtilis*, suggesting a wide distribution and an ancient origin of this excision repair mechanism for UV damage (9). As we pointed out before (10), neither *S.pombe* nor *B.subtilis* possesses photolyase activity for CPDs (16,17). UVDE in both organisms lacking photolyase might therefore provide an alternative method for the repair of CPDs. Another possible explanation for the presence of two excision repair pathways is that they might operate differentially in different cell cycle and growth phases. Rapid UVDE-mediated repair may be advantageous if cells are growing rapidly in a nutrient- and UV-rich environment.

Finally, we now have two excision repair systems and it is of interest to compare UVDE-mediated repair with the well-characterized NER. For the rapid removal of nicked damage from DNA, UVDE protein may be associated with other proteins for effective recognition, cleavage and processing of DNA damage. However, *in vitro* experiments using purified enzymes showed that damage recognition and cleavage of DNA can be performed by this single protein. This simple nicking mechanism rapidly creates strand breaks, which cannot be directly ligated, because of tension due to UV-induced damage. Instead of repair, the strand break may lead to DNA degradation or other lethal intermediates, if subsequent processing does not follow, for example in *rad2*d cells. In contrast, the NER system operates via a multi-protein complex containing a number of proteins besides endonucleases, including single-strand binding proteins, helicases within the TFIIH complex for unwinding of DNA and DNA polymerase (3). This complicated system, which ensures that all nicked sites are carefully guarded from other DNA catalyzing activities (including DNA degradation enzymes or recombination systems) and are coordinatedly processed, needs time for organization of the whole complex when compared with UVDE-mediated repair, but it may be safer than UVDE-mediated repair. Maybe, therefore, in spite of its complexity, the NER system is more widely distributed in life than UVDE-mediated repair. It is likely that an important factor in determining UV sensitivity is the balance between incision and the later steps in the repair process and that in this respect NER, though slower, is more controlled and needs many factors at once for incision on both sides of the damage. However, the UVDE-mediated repair system is present in a number of organisms and, because it is an effective, rapid and relatively simple procedure, it may be a powerful repair system in rapidly growing cells and might have been a dominant repair system against UV-induced damage in the early stages of evolution.

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