Alternative Excision Repair Pathway of UV-damaged DNA in Schizosaccharomyces pombe Operates Both in Nucleus and in Mitochondria*

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The fission yeast, Schizosaccharomyces pombe, possesses a UV-damaged DNA endonuclease-dependent excision repair (UVER) pathway in addition to nucleotide excision repair pathway for UV-induced DNA damage. We examined cyclobutane pyrimidine dimer removal from the myo2 locus on the nuclear genome and the colI locus on the mitochondrial genome by the two repair pathways. While nucleotide excision repair repairs damage only on the nuclear genome, UVER efficiently removes cyclobutane pyrimidine dimers on both nuclear and mitochondrial genomes. The ectopically expressed wild type UV-damaged DNA endonuclease was localized to both nucleus and mitochondria, while modifications of N-terminal methionine codons restricted its localization to either of two organelles, suggesting an alternative usage of multiple translation initiation sites for targeting the protein to different organelles. By introducing the same mutations into the chromosomal copy of the uvde* gene, we selectively inactivated UVER in either the nucleus or the mitochondria. The results of UV survival experiments indicate that although UVER efficiently removes damage on the mitochondrial genome, UVER in the mitochondria hardly contributes to UV resistance of S. pombe cells. We suggest a possible UVER function in mitochondria as a backup system for other UV damage tolerance mechanisms.

Eukaryotic cells generally possess multiple pathways as a defense against UV-induced DNA damage. Among them, nucleotide excision repair (NER) is distributed most widely and its core mechanism is considered to have been conserved throughout evolution (1). NER is driven by the coordinated action of more than a dozen proteins. These proteins are thought to form either a huge repairsome complex (2) or several subcomplexes, which act cooperatively on DNA damage (3). While NER is able to repair a wide variety of DNA damage in addition to UV-induced damage, most organisms also have simpler, UV damage-specific repair systems. Photoreactivation is one of those alternatives to NER, in which a single enzyme called photolyase catalyzes complete repair of UV-induced DNA damage without scissions of the phosphodiester bonds in DNA strands. Two types of photolyase that differ in substrate specificity have been described, one exclusively acting on cyclobutane pyrimidine dimers (CPDs) (4) and the other acting on (6–4) photoproducts (5, 6).

The fission yeast, Schizosaccharomyces pombe, and the filamentous mold Neurospora crassa have another type of UV damage-specific repair system called UV-damaged DNA endonuclease (UVDE)-dependent excision repair (UVER) (7–9). In this system the UVDE protein catalyzes a specific cleavage of a phosphodiester bond 5’ adjacent to a CPD or a (6–4) photoproduct. After nicking of damaged DNA, repair is thought to be completed by a base excision repair (BER)-like process (10). In contrast to photolyase, a single type of UVDE can recognize both CPDs and (6–4) photoproducts, which have been considered to have significantly different conformation.

The reason for the existence of multiple repair pathways for UV damage is not fully understood. One might think that UV-specific pathways such as photoreactivation or UVER are only a backup to NER and functionally redundant, or that they are just remnants of primitive repair systems that existed when UV irradiation was much stronger than today. However, it is difficult to imagine that such a completely redundant pathway would have been maintained for long during evolution. The most likely possibility is that these multiple pathways have partially overlapping but also differentiated functions.

Multiple organelles harboring their own genomes exist in most eukaryotes. These organelles have fundamental roles in respiration or photosynthesis, and the gene products encoded by their genomes are essential for these biochemical events. Therefore, these genomes should be protected by DNA repair systems, as are nuclear genomes, from harmful DNA damage. Photoreactivation has been reported to function in mitochondria or in chloroplasts in some species. In the green alga Euglena gracilis, UV irradiation induces the appearance of white color colonies consisting of cells lacking chloroplasts in addition to cell killing. This “bleached” colony induction is known to be photoreactivable (11). UV irradiation also causes induction of petite colonies lacking mitochondria with remarkably high efficiency in budding yeast Saccharomyces cerevisiae. Petite induction is also alleviated by photoreactivation of UV damage in mitochondrial DNA (12, 13). Our previous results showed that the N-terminal protruding sequence of S. cerevisiae photolyase is crucial for its mitochondrial function (14). In both cases, however, NER cannot repair UV damage in organelle genomes. Although cells lacking chloroplasts or mitochondria are viable under laboratory condition, they would be expected to suffer from great disadvantage compared with wild type cells in nature.

Interestingly, S. pombe totally lacks photolyase activity (15) and instead possesses UVDE. In the current study, we exam-
ined the possible function of UVER in mitochondria of *S. pombe*. We demonstrate that UVER works efficiently in mitochondria as well as in the nucleus by means of a repair kinetics assay and cytological observation of UVDE protein.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Transformation of *S. pombe***—Strains used in this study are listed in Table I. All strains were grown in either YES or MM at 30 °C (16). Transformation of *S. pombe* cells was performed by electroporation with a ECM600 Electro Cell Manipulator (BTX) following the manufacturer's protocol. Double mutants were made by crossing each of the single mutants.

**UV Survival Experiment**—Exponentially growing cells (1–2 × 10^7^ ml) in YES liquid medium were appropriately diluted with distilled water and 500 or 10,000 cells/plate were seeded on YES agar plates. 254-nm UV was administered using a set of germicidal lamps (GL-10, Toshiba, Japan) at a dose rate of 0.4–3.3 J/m^2^ s. After incubation at 30 °C for 3 days, colonies were counted.

**Strand-specific CPD Assay**—Exponentially growing cells in YES medium were washed twice with distilled water and resuspended in phosphate-buffered saline at 5 × 10^8^ cells/ml. Approximately 30 ml of cell suspension was poured into a 15-cm plastic dish (Falcon) and irradiated with 100 J/m^2^ 254-nm UV. During the washing and UV irradiation processes cells were kept on ice and all materials were pre-chilled. For each strain, a total of 300 ml of UV irradiated cell suspension was prepared and divided into 10 aliquots. Each aliquot was centrifuged, and the cells were resuspended in 30 ml of pre-warmed YES medium and incubated at 30 °C for 0, 20, 40, 60, or 120 min (two aliquots for each time point). The cells were pelleted again, frozen with liquid nitrogen, and stored at −80 °C until DNA preparation. Genomic DNA was isolated as described previously (16) with a slight modification. After digestion with HindIII, DNA was treated with T4 endonuclease and phosphorylated under alkaline condition (17). DNA was transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech). Blots were prepared in duplicate with the same DNA sample, and one was hybridized with a sense strand-specific myo2 or col probe the other with an antisense specific probe (see below) using ExpressHyb hybridization solution (CLONTECH) following the manufacturer's protocol. The blots were analyzed using a FLA-2000 fluorographic image analyzer (Fuji, Tokyo, Japan). The number of CPDs in the target fragment was calculated assuming Poisson distribution. After the first hybridization and analysis, the blots were incubated at 100 °C in 0.5% SDS for 5 min to remove probes and rehybridized reciprocally with the opposite strand-specific probes.

**Generation of myo2 and col Strand-specific Probes**—A part of the *myo2* gene (Ref. 18, nucleotides 1496–2302 of GenBank Accession U75357) was amplified by PCR with primers SY78 (5′-CCCCCGGATCCAAAAAGCTATGACCTTAAA-3′) and SY79 (5′-CCCCCGGATCCATTACGCTCATCTGACCTTAAA-3′) using Y4 strain genomic DNA as a template, and cloned into pBluescriptII SK− (Stratagene). Strand-specific probes were generated by asymmetric PCR using the cloned fragment as a template and either SY79 or SY78 as a primer (19). After the PCR reaction, the probe was purified by passing through G-50. Approximately 3 × 10^6^ cm^2^/m^2^ was used for hybridization. For *col* strand-specific probes, a part of the gene (nucleotides 4901–5405 of GenBank accession no. X54421) was PCR-amplified with primers SY61 (5′-CCCCGGATCCACTTATGTTAATAGATGGATATTCT-3′) and SY62 (5′-CCCCGGATCCAAAGCTACTTTATTGGTATTTT-3′) and similarly labeled.

**Computer Analysis of UVDE Sequence**—Subcellular localization of UVDE was predicted by PSORTII, which is available on the Internet (20).

**Site-directed In Vitro Mutagenesis of *uvde* Gene**—The *uvde*-M1 mutation was introduced into the cloned genomic fragment with a Mutan-Express Km kit (Takara, Tokyo, Japan) and SY96 (5′-CTCGTTGCATTATTTTTTTTAAAGGCTATGGATATTCT-3′). This mutation disrupts the first methionine codon of the *uvde* and creates a HindIII site. The sequences of the *uvde*-M6A4 mutation, which converts the second and the third methionine codons to alanine codons, was introduced by PCR-based method with SY95 (5′-TGCCCAACCCTCTCCTGGAGATTAGGTT-3′), SY101 (5′-GTTTACTTCCAGGAGCTCAAAACACACTCCTACCGTTACCGCAAGG-3′), SY102 (5′-CCGTTGCGATCTTTTTCACGACCTG-3′), and SY103 (5′-TGAGCTCGTGAGAATGAAACTTTAAGGTT-3′). Sequences were verified using a DSSQ-1000 DNA sequencer (Shimadzu, Kyoto, Japan).

**Expression of UVDE-GFP Fusion Protein**—The *uvde* gene from a pEGF-P-N1 (CLONTECH) vector together with its original multicloning site was isolated and inserted into the Xhol and *Sma* I sites of pREP4X (21). This plasmid was named pSY6. Wild type, 5′-truncated, or point mutated versions of *uvde* were cloned into the *Sac* II and *Bam* HI sites of pSY6 so that C-terminal GFP fusion protein was expressed from a nmt1 full-strength promoter (see Fig. 2b, pSY57-59, pSY74). When PCR was used in plasmid construction, sequences of the amplified region were verified. *S. pombe* transformants harboring either of these constructs were maintained in MM + thiamine (5 mg/ml) medium to minimize the toxicity due to overexpression of the fusion protein. Prior to microscopic observation, exponentially growing transformants were washed once with water, diluted 50–100-fold into thiamine-free MM and cultured at 30 °C for 16 h. The subcellular localization of UVDE-GFP fusion protein was observed by using Leica DM LB microscope (Leica, Germany) with an appropriate filter for GFP.

**Gene Replacement of *uvde* Gene**—Gene replacement was conducted by a pop-in/pop-out method (16) using *ura4* as the selectable marker. The mutated *uvde* gene and *ura4* marker are subcloned next to each other into a *ara-less* vector. The plasmid was integrated into the *uvde* locus and targeted integration was confirmed by Southern hybridization. This strain was cultured in YES medium for several generations and plated on MM + 5-FOA plates for selection against *ura4* marker. The pop-out event was checked by Southern hybridization and the nucleotide sequences of replaced region were confirmed by direct sequence of the PCR-amplified fragment.

**RESULTS**

**UVER in Mitochondria**—It has been reported that photolysis in *S. cerevisiae* functions in mitochondria as well as in the nucleus and that it actually contributes to the reduction of the petite induction rate by UV irradiation (12–14). While *S. cerevisiae* cells do not possess UVER, *S. pombe* lacks any detectable photoreactivation of UV damage. Each repair system depends on a single protein, photolyase or UVDE. This complementary relationship between two repair pathways in the two yeast species prompted us to examine whether UVER functions in the mitochondria of *S. pombe* cells.

We first looked at CPD removal from the *myo2* locus on the nuclear genome and the *col* locus on the mitochondrial genome by using a T4 endonuclease-based strand-specific damage as-

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**Table I**

| Strain | Genotype |
|--------|----------|
| Y4     | h* ade6-M216 ura3-D18 leu1-32 |
| Y21    | h* ade6-M216 ura3-D18 leu1-32 rad13Δ::ura4 |
| Y26    | h* ade6-M216 ura3-D18 leu1-32 rad13Δ::ura4 ura4Δ::LEU2 |
| Y53    | h* ade6-M216 ura3-D18 leu1-32 ura4Δ::LEU2 |
| Y61    | Y4/psY8 (see Fig. 2b) |
| X74    | Y4/psSY75 (see Fig. 2b) |
| Y72    | Y4/psSY55 (see Fig. 2b) |
| Y73    | Y4/psSY75 (see Fig. 2b) |
| Y79    | Y4/psSY74 (see Fig. 2b) |
| Y82    | h* ade6-M216 ura3-D18 leu1-32 ura4Δ::M6A4 |
| Y84    | h* ade6-M216 ura3-D18 ura4Δ::M6A4 |
| Y86    | h* ade6-M216 ura3-D18 leu1-32 ura4Δ::M6A4 rad13Δ::ura4 |
| Y87    | h* ade6-M216 ura3-D18 leu1-32 ura4Δ::M6A4 rad13Δ::ura4 |

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These loci encode a type II myosin heavy chain and a cytochrome oxidase subunit I, respectively. When digested with HindIII, these loci give rise to a 4.1- or a 4.3-kilobase pair signal in Southern hybridization experiments with the appropriate probes (see “Experimental Procedures”). Wild type cells were able to repair CPDs in both loci quite efficiently (Fig. 1, WT). The repair kinetics were not remarkably affected when functional NER was eliminated by the disruption of rad13, XPG/RAD2 homolog of S. pombe (22), suggesting that UVER operates both in the nucleus and mitochondria (Fig. 1, rad13Δ).

When UVER was eliminated, CPDs in the myo2 locus were removed with a clear strand bias (Fig. 1, rad13Δ, left column). Transcription-coupled repair by NER in S. pombe was described elsewhere (23). In contrast, NER did not repair damage at the coI locus during the 120 min incubation time (Fig. 1, rad13Δ, right column) indicating that NER does not function in mitochondria. Strains lacking both types of excision repair did not show any ability to repair damage at either locus (Fig. 1, rad13Δ, uvedeh).

Subcellular Localization of UVDE—UVER is initiated by the nicking of damaged DNA by UVDE (7, 8). In order for UVER to repair damage on the mitochondrial genome, UVDE itself should exist in mitochondria. Therefore, we next examined the subcellular localization of UVDE. The uvedeh nucleotide sequence possesses three putative initiation methionine codons, Met-1, Met-56, and Met-64 at its N terminus (Fig. 2a). Scanning of the full-length UVDE amino acid sequence (599 amino acids) with PSORTII program (20) picked up features of a mitochondrial protein between the first and the second methionines, as well as motifs for nuclear localization after the third methionine (Fig. 2a). The full-length protein was predicted to be mitochondrial with a score of 56.5%, while the N-terminal truncated version in which the sequence preceding the second methionine was removed (544 amino acids) was predicted to be nuclear with a score of 65.2%. These sequence features suggest that alternative usage of two or three methionine codons for translation initiation may target UVDE to two different organelles.

We then observed the localization of ectopically expressed UVDE-GFP fusion protein microscopically. Typical mitochondria in S. pombe appear as a stringlike structure, as has been shown (24, 25). Wild type UVDE-GFP was localized to both nucleus and mitochondria (Fig. 2b, pSY57), which is consistent with the repair kinetics result (Fig. 1, WT). In contrast, UVDE-GFP expressed from the second or the third methionine codons was observed exclusively in nucleus (pSY58 and pSY59) (Fig. 2b). We also expressed the mutant version of UVDE-GFP, where both the second and the third methionine codons were replaced with alanine codons. This mutation would repress the translation initiation from these two sites and consequently repress targeting to the nucleus. As expected, the mutant UVDE-GFP was localized only to mitochondria (pSY74). All of the wild type and mutant versions of UVDE-GFP fusion proteins were functional as UVDE in a repair-deficient Escherichia coli-based survival assay (7) (data not shown).

Effect of UVDE Mutation on Repair Kinetics—Cytological observation of ectopically expressed UVDE-GFP suggested an
alternative usage of two or three methionine initiation codons for targeting of this protein to multiple organelles. We next introduced the same mutations into the chromosomal copy of the *uvde* gene and studied its effect on repair kinetics of *myo2* and *col* loci in NER-deficient (*rad13Δ*) background. When the first methionine was disrupted, UVER repaired damage only at the *myo2* locus (Fig. 3, *rad13Δ uvde-M1*). When the second and the third methionine codons were replaced with alanine codons, UVER operated only on the *col* locus (Fig. 3, *rad13Δ uvde-M56AM64A*) as anticipated.

**Contribution of Nuclear and Mitochondrial UVER to UV Survival**—As shown above, UVER is the only excision repair system for UV damage in the mitochondrial genome of *S. pombe*. To evaluate its relative significance in damage repair in nucleus and mitochondria, we examined the UV survival of the strains whose nuclear or mitochondrial UVER was selectively inactivated.

When mitochondrial UVER was eliminated, UV survival was apparently unchanged (Fig. 4, a (*uvde-M1*) and b (*rad13Δ uvde-M1*)). On the other hand, inactivation of nuclear UVER (*uvde-M56AM64A*) rendered the cells sensitive to UV almost to the same degree as complete inactivation of UVER (Fig. 4, a (*uvde-M56AM64A*) and b (*rad13Δ uvde-M56AM64A*)). The result was the same whether or not NER was functional. The surviving colonies of the mitochondrial UVER-deficient strains did not show any retardation growth, and therefore were not considered to have respiration defect. Although UVER efficiently works on UV damage in the mitochondrial genome, its mitochondrial function may not contribute to UV resistance of *S. pombe* cells.

**DISCUSSION**

There are many examples of products of a single gene localized to several different intracellular compartments (26). A variety of mechanisms are responsible for this multiple localization, such as variable pre-mRNA processing (including alternative splicing), transcription initiation at multiple sites, translation initiation at multiple sites, or variable post-translational modification, depending on each case. The *S. pombe uvde* gene possesses three putative initiation methionine codons at its N terminus and the amino acid sequence preceding the third methionine is dispensable for its enzymatic activity (8). Although it remains to be confirmed by direct examination of N-terminal sequence of the protein, our repair kinetics and cytological data in the present study strongly suggest that multiple subcellular localization of UVDE is accomplished by translation initiation at multiple methionine start codons. According to Zhang and Marr (27), the most conserved nucleotide around translation initiation sites in *S. pombe* is A at −3 position. None of the three methionine codons at the N terminus of UVDE meet this criterion (T for the first and the third methionine codons, and C for the second methionine codon). The absence of any strongly preferred translation initiation sites may enable the usage of multiple sites.

In the present study, we demonstrated that UVER efficiently removes UV damage on the mitochondrial genome as well as on the nuclear genome. This means that, after nicking of damaged DNA by UVDE, two independent but analogous mechanisms may complete the repair reaction in the respective organelles. We previously showed that at least a part of UVER is dependent on Rad2p (10), a FEN-1 homolog of *S. pombe*, which has also been implicated in a proliferating cell nuclear antigen-dependent subpathway of BER (28). This suggests the possibility that UVER and BER share their later steps in nuclei. On the other hand, recent several works unequivocally showed the existence of BER in mitochondria probably involving DNA polymerase γ and mitochondrial DNA ligase (29–31). Although Rad2p itself is unlikely to be involved in mitochondrial BER (and UVDE) judging from its amino acid sequence, it is highly possible that UVER and BER share some steps also in mitochondria.

As mentioned above, the lack of mitochondrial DNA repair increases the rate of petite induction by UV in *S. cerevisiae* (12, 13). We expected that inactivation of mitochondrial DNA repair would lead to an increased sensitivity of *S. pombe* to UV, since this species has been long regarded as a petite-negative yeast and loss of the mitochondrial genome was thought to result in cell death (but also see Refs. 32 and 33). However, our results demonstrated that the defect in mitochondrial UVER does not affect the UV survival.

The first question is why apparent absence of DNA repair in mitochondria of the mutant *S. pombe* strains does not lead to an increased UV sensitivity. Two explanations for this will be possible. One is that any types of DNA damage tolerance systems in mitochondria are irrelevant to UV resistance of *S. pombe* cells. The other is that other type(s) of damage tolerance mechanisms in mitochondria (which should not be damage repair in a true sense, since we did not observe any damage removal in the absence of UVER) mask the effect of UVER. Since the size of the nuclear genome is much larger and the
copy number is much lower than those of the mitochondrial genome, one can easily imagine that the nuclear genome would be much more sensitive to the same number of damage per unit length of DNA than the mitochondrial genome. Under such conditions, cells will die before the effect of the damage tolerance system in the mitochondria is realized. This way of argument supports the former explanation. In our experimental condition, however, 100 J/m² UV induced about 10 CPDs in a single copy of S. pombe mitochondrial genome in average (data not shown). Taking into account that the copy number of mitochondrial genome is several hundred at most (34), this means that virtually any DNA molecules of mitochondrial genome bear at least a few CPDs. At this UV dose, about 50% of wild type and mitochondrial UVER-deficient cells are still survived (Fig. 4a). We also know from earlier work that CPDs are a strong barrier to DNA replication and transcription in the absence of any damage tolerance systems (35–38). This argues that the former explanation is very unlikely, and suggests the possible existence of other types of damage tolerance systems in the mitochondria of S. pombe. Ling et al. (39) described how, in S. cerevisiae mitochondrial DNA, recombination has an important role in damage tolerance. If a similar mechanism operates in the mitochondria of S. pombe, it would be a good candidate for major mitochondrial damage tolerance. This should be examined in a future study.

In the Introduction, we argued that completely redundant mechanisms cannot be maintained during evolution. Following this argument, mitochondrial UVER is expected to give an advantage at least under some condition. The second question is what conditions would these be? In contrast to UV, reactive oxygen species generated by respiration may cause the damage. Recent studies have suggested that the peroxisome proliferator-activated receptor-gamma (PPAR-γ) may have a greater significance than for UV damage. Recently we identified an apurinic/apyrimidinic endonuclease activity of UVDE (40). This may suggest a possible involvement of UVER in repair of oxidative damage on mitochondrial DNA and its contribution to cell survival. Although we have not found any increased sensitivity of ude disruptants cells against oxidative damage, this possibility remains to be elucidated.

The copy number of the mitochondrial genome is known to fluctuate depending on culture conditions. In S. cerevisiae, a well known phenomenon, glucose repression, also includes a decrease in mitochondrial DNA copy number (41). This is quite reasonable since, if a carbon source is abundant, energy generation does not have to depend solely on respiration. Additionally, earlier work on S. pombe showed that the mitochondrial DNA content in exponentially growing cell is much lower than that in stationary phase cells (34). As discussed above, when the mitochondrial DNA copy number is lower, mitochondrial DNA would have a greater significance. Mitochondrial UVER might be crucial under such conditions. At this moment, we do not have any positive evidence for this.

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