Removal of cyclobutane pyrimidine dimers by the UV damage repair and nucleotide excision repair pathways of Schizosaccharomyces pombe at nucleotide resolution

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ABSTRACT

In Schizosaccharomyces pombe two different repair mechanisms remove UV-induced lesions from DNA, i.e. nucleotide excision repair (NER) and UV damage repair (UVDR). Here, the kinetics of removal of cyclobutane pyrimidine dimers (CPDs) by both pathways is determined at base resolution in the transcribed strand (TS) and the non-transcribed strand (NTS) of the sprpb2+ gene. UVDR does not remove lesions in a strand-specific manner, indicating that UVDR is neither stimulated nor inhibited by RNA polymerase II transcription. In contrast, in a UVDR-deficient strain the TS is repaired preferentially. This strong strand bias suggests that in S. pombe, as in other species, NER is coupled to transcription. In repair-proficient S. pombe the TS is repaired very rapidly, as a consequence of two efficiently operating pathways, while the NTS is repaired more slowly, mainly by UVDR. Furthermore, we demonstrate that UVDR is not always faster than NER.

INTRODUCTION

UV light induces lesions in DNA, mainly cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6–4) photoproducts that are potentially mutagenic and genotoxic. In order to maintain their genetic integrity, cells have evolved different mechanisms to remove these lesions from DNA (1). One repair mechanism, nucleotide excision repair (NER), is capable of removing a large variety of lesions from the DNA, including UV-induced lesions, and this pathway is evolutionarily strongly conserved from Saccharomyces cerevisiae to mammals (1). In vivo repair experiments revealed that DNA lesions are removed heterogeneously by NER; repair of transcriptionally active loci is much more efficient than repair of the bulk of the DNA (2) and the transcribed strand (TS) of active genes is repaired faster than the non-transcribed strand (NTS) (3). Furthermore, it was shown that polymerase II is efficiently blocked by thymine dimers in the TS (4). These observations ultimately led to the postulation of two subpathways of NER: transcription coupled repair (TCR) responsible for the repair of lesions from the TS of active genes; global genome repair (GGR) that repairs lesions from the genome overall (5). In this model a set of proteins referred to as core NER proteins is essential for all NER, while other factors are specifically devoted to one of the two subpathways. The relative efficiencies of both subpathways determine the difference in repair kinetics of the strands (reviewed in 6). In general TCR is fast while GGR is slower. The efficient repair of transcribed DNA was proposed to result from the direction of the NER machinery to sites of a stalled transcription unit (3,7).

In contrast to human and S. cerevisiae NER-deficient mutants, which are very sensitive to UV light, Schizosaccharomyces pombe NER-deficient mutants do not show a dramatic reduction in survival upon UV irradiation and these cells are still able to repair photolesions (8,9). Subsequently, it was demonstrated that S. pombe has an alternative repair system to remove UV-induced lesions from DNA, i.e. UV damage repair (UVDR) (8–11). The uvde+ gene, which codes for the enzyme performing the first step in the UVDR pathway (12), has recently been cloned (13). Homologues are found in the filamentous fungus Neurospora crassa (14) and in the Gram positive bacterium Bacillus subtilis (13), suggesting an ancient evolutionary origin. However, UVDR seems to be absent in other organisms. The mechanism of UVDR is less well understood than NER, although it is clear that the Uvde protein has a central role in recognising photolesions and incising the DNA 5’ of these damages (13). The study of NER and UVDR is complicated by the redundancy of the two repair pathways, both operating on UV-induced lesions in S. pombe. Here, rad13+ and uvde+ disruptants, which do not have NER or UVDR, respectively, are used to study the kinetics of either repair pathway separately.

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Based on lesion removal from genomic DNA using CPD-specific antibodies, Yonematsu and co-workers suggested that the UVDR pathway is fast, while NER is a much slower system in vivo (12). However, the antibody-based method that was applied measures the overall repair rate and no discrimination between transcribed and non-transcribed DNA can be made. We have recently developed a method to measure repair of CPDs at nucleotide resolution in vivo in S.cerevisiae (15). Here we use this method in the distantly related yeast S.pombe to demonstrate gene pathways in the TS and NTS of the RNA polymerase II transcribed gene sprpb2+ (DDBJ accession no. D13337).

MATERIALS AND METHODS

Yeast strains and media

Repair-proficient (h’ ade6-M216 leu1-32 ura4-D18) and isogenic rad13+ (rad13::ura4*) and rad13– (rad13::ura4+ wvdc::LEU2) strains were kindly provided by Drs Akira Yasui and Shinji Yasuhira (Tohoku University, Japan). Schizosaccharomyces pombe strains were maintained on complete medium (YES; 5 g l–1 yeast extract, 30 g l–1 glucose, 2% bacto agar supplemented with 75 mg l–1 adenine and 75 mg l–1 uracil). Cells were grown in liquid culture and with TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA). The target sequence to measure repair was a 532 bp RI fragment from the RNA polymerase II transcribed sprpb2+ gene (DDBJ accession no. D13337, positions +785 to +1317 relative to the start codon A+1). Dynal® magnetic particle concentrator, the immobilised streptavidin-coated magnetic beads were used to enrich for the desired end-labelling. To complete extension, 1 µl 10 mM dCTP was added followed by five additional cycles. After labelling, 4 µl 0.5 M EDTA was added to abolish sTAQ activity in the later stages.

Detection of CPD lesions in the sprpb2+ gene

The method is based on Tijsterman et al. (15) with modifications. Samples of 50 µg DNA, containing 2.5 × 109 copies of the yeast genome, were digested with EcoRI in OPA buffer (Pharmacia) and precipitated according to standard procedures. The target sequence to measure repair was a 532 bp EcoRI fragment from the RNA polymerase II transcribed sprpb2+ gene (DDBJ accession no. D13337, positions +785 to +1317 relative to the start codon ATG designated +1). Dynal M-280 streptavidin beads were used to enrich for the desired chromosomal DNA target. After 3 min incubation at 93ºC, 3 pmol of a biotinylated oligonucleotide complementary to the fragment of interest was annealed in 100 µl sTAQ buffer (50 mM KCl, 10 mM Tris–HCl pH 9.0, 1.5 mM MgCl2, 0.1% Triton X-100) for 20 min at 45ºC. Subsequently, 10 µl (1 mg µl–1) streptavidin-coated magnetic beads were added (prewashed with sTAQ buffer) and incubated for 20 min with occasional gentle agitation to avoid bead sedimentation. Using the Dynal® magnetic particle concentrator, the immobilised templates were washed with sTAQ buffer, BW solution (1.0 M NaCl, 10 mM Tris–HCl pH 7.5, 1.0 mM EDTA), sTAQ buffer and with TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA). The captured DNA fragments were eluted from the beads by incubating for 3 min at room temperature in 10 µl 0.1 M NaOH.

End-labelling conditions were as described (18). An oligomer complementary to the 3’-end of the desired DNA fragment contained a 6 nt non-complementary dGTP stretch. The primers were 5’-GGG GGG CTT GTC AGA CAA TTA GAG CAA CAC-3’ for isolation and labelling of the TS and 5’-GGG GGG CAC GAT TTG TTT CAA CGC ATT TC-3’ for the NTS (the sprpb2+ sequence is underlined). Both were biotinylated at the 5’-site. These primers were also synthesised without the six base non-complementary extension to generate PCR fragments for Maxam–Gilbert sequencing. The extended stretch was used as a template to extend the free 3’-hydroxyl end of the restriction fragment of interest with [α-32P]dCTP and sTAQ DNA polymerase (HT Biotechnology). The reaction mixture was generated by sequential addition of 10 µl 0.1 M NaOH containing the purified DNA fragment (see above), 1.0 µl 1 M HCl, 5.0 pmol oligonucleotide, 0.2 µl [α-32P]dCTP (3000 Ci mmol–1) and 0.2 U sTAQ polymerase in a final volume of 50 µl sTAQ buffer. Samples were denatured for 3 min at 93ºC and subjected to five consecutive rounds of denaturation (30 s at 93ºC), annealing (30 s at 45ºC) and extension (90 s at 72ºC) to optimise end-labelling. To complete extension, 1 µl 10 mM dCTP was added followed by five additional cycles. After labelling, 4 µl 0.5 M EDTA was added to abolish sTAQ activity in the later steps.

CPDs were identified using the T4 endonuclease V (T4EndoV) enzyme, which recognises CPDs and gives single-strand nicks in the DNA 5’ of these lesions. Since incision is most efficient on a double-stranded DNA substrate, the end-labelled fragments were subjected to a hybridisation protocol. An excess of complementary strand, synthesised by PCR amplification and bead isolation, was added followed by 3 min incubation at 93ºC and gradual cooling to room temperature. Native gel electrophoresis showed that all labelled DNA fragments were in the double-stranded configuration. The DNA was incubated with T4EndoV. After phenol/chloroform extraction to remove sTAQ and T4EndoV, samples were subjected to spin column chromatography and lyophilised to small volumes. Equal amounts of c.p.m. were loaded on 6% denaturing acrylamide gels alongside a Maxam–Gilbert sequencing ladder. After drying, autoradiograms were prepared from the gels.

Quantitation of repair rates

Autoradiograms were scanned at 300 d.p.i. and analysed using ImageMaster™ software (Pharmacia). Each experiment was carried out in duplicate or triplicate and audiographs were obtained with different exposure times to allow signal determination within the linear range of Kodak X-OMAT AR scientific imaging films. Background levels were subtracted and gel band intensities were corrected for loading variations. OD values were plotted against the repair time for each CPD that gave sufficient signal to background ratio. The repair half-times (t1/2) defined as the time at which 50% of the initial damage (signal at t = 0 min) was removed, were calculated from these plots.

Maxam–Gilbert sequencing reactions

Maxam–Gilbert sequencing ladders were obtained according to standard procedures (19) using PCR fragments identical to the chromosomal DNA fragment which was analysed. After the sequencing reactions the fragments were 32P-labelled using the (dGTP)2–tailed oligonucleotides (mentioned above). In this way, a 3’-end-labelled product identical to the chromosomal DNA fragment, used in the repair analysis, was obtained.
RESULTS

The rad13<sup>-</sup> uvde<sup>-</sup> double mutant does not remove CPDs

In S. pombe both NER and UVDR remove photolesions from DNA. Either pathway can be studied in uvde<sup>-</sup> or rad13<sup>-</sup> mutants, respectively, provided that no other mechanism repairs UV-induced lesions. Repair in NER- and UVDR-deficient cells was measured to detect possible removal of CPDs by other pathways. The absence of repair of CPDs in the TS in a rad13<sup>-</sup> uvde<sup>-</sup> double mutant is shown in Figure 1. A similar picture was obtained from the NTS (data not shown). A 532 bp fragment of the sprpb2<sup>+</sup> gene was isolated from S. pombe cells after various incubation times post-UV irradiation and subsequently treated with the T4EndoV enzyme that incises the DNA 5’ of CPDs. These incisions were visualised on sequencing gels and can be correlated to their cognate dipyrimidine sequences by comparing with a Maxam–Gilbert sequence ladder (data not shown). The initial lesion distribution is shown in Figure 1, lane 2 (t = 0 min).

The absence of repair of UV-induced CPDs along the TS of the rad13<sup>-</sup>– cells is caused by NER. Within both strands, CPDs are repaired heterogeneously. This is illustrated by the half-time values of lesions designated R (t½ = 11 min in the TS and 10 min in the NTS) and S (t½ = 27 min) repaired site are indicated, illustrating this heterogeneity.

The UVDR pathway is fast and not strand specific

To study whether the strand bias seen in wild-type S. pombe (Fig. 2) is caused by strand specificity of UVDR, we studied CPD removal in a rad13<sup>-</sup> mutant, which is NER-deficient. The results of the repair analysis of the TS are shown in Figure 3A. Here, individual lesions are removed rapidly, with t½ values ranging from 11 to 27 min. Repair of the NTS in rad13<sup>-</sup> cells is shown in Figure 3B. The half-times of different lesions in the NTS vary from 10 to 26 min. In general there is no difference between half-times of lesions in the TS and NTS, implying that UVDR is not strand specific. Apparently the strand bias seen in wild-type S. pombe (Fig. 2) is absent in a NER-deficient strain (Fig. 3), suggesting that strand specificity in repair-proficient cells is caused by NER. Within both strands, CPDs are repaired heterogeneously. This is illustrated by the half-time values of lesions designated R (t½ = 11 min in the TS and 10 min in the NTS) and S (t½ = 21 min in the TS and 25 min in the NTS) in Figure 3. Repair half-time plots for the TS and NTS of rad13<sup>-</sup> cells, given in Figure 5A and B, clearly demonstrate the heterogeneity in removal of differently positioned CPDs.

Figure 1. The absence of repair of UV-induced CPDs along the TS of the S. pombe sprpb2<sup>+</sup> locus in rad13<sup>-</sup> uvde<sup>-</sup> cells. Cells were irradiated with 40 J m<sup>-2</sup> UV light and allowed to repair DNA for the time periods indicated. DNA was isolated and the fragment of interest was labelled and treated with the CPD-specific enzyme T4EndoV. Lesion-specific bands in the TS (positions +864 to +1317) are shown in the autoradiogram. Lane 1 contains non-irradiated DNA which was also treated with T4EndoV (and was used to account for background levels). The initial lesion distribution is given in lane 2. Lanes 2–5 show that lesion-specific bands do not disappear with time. The positions are relative to the start codon A<sup>+</sup>G designated +1.
The NER pathway removes CPDs strand specifically

To substantiate the suggestion that NER causes strand specificity in repair-proficient cells, CPD removal in a uvede– mutant, only proficient for NER, was determined. Figure 4A shows repair of the TS in uvede– cells. The TS is repaired rapidly with half-times between 13 and 29 min. In contrast to the fast repair of the TS, lesions in the NTS are repaired very slowly, as can be seen in Figure 4B. The t₁/₂ values of lesions in the NTS range from 124 to >225 min. The strong preference for the TS in a UVDR-deficient strain shows that the strand bias in wild-type S. pombe cells (Fig. 2) can be attributed solely to NER. Repair of lesions in both the TS and NTS is not uniform. This is illustrated by some rapidly and slowly repaired sites, marked R and S in Figure 4. The t₁/₂ values of these lesions are 13 and 29 min in the TS and 124 and 308 min in the NTS (Fig. 4). Figure 5A and C, where repair half-times are plotted against dipyrimidine positions in the sprpb2+ locus, illustrates the heterogeneous repair of both strands and clearly shows the strand specificity in UVDR-deficient cells. Importantly, some sites in the TS are repaired faster by NER than by UVDR (Fig. 5A).
DISCUSSION

In *S. pombe* two distinct repair mechanisms are used to repair CPDs from DNA, UVDR and NER (reviewed in 20). To determine the rate of CPD removal by either pathway we used a method, developed in our laboratory (15), which allows us to study DNA repair in vivo at nucleotide resolution. Using rad13– or uvde– disruptants, deficient for NER and UVDR, respectively, we studied the repair kinetics of both systems operating on the TS and NTS of the RNA polymerase II transcribed gene *sprpb2*+. We show that: (i) in rad13– uvde– cells CPDs are not repaired; (ii) in repair-proficient cells there is very fast strand-specific repair; (iii) the UVDR mechanism has no strand bias; (iv) there is strong preferential repair of the TS by NER; (v) both pathways show profound heterogeneity of repair within the same strand; (vi) the UVDR mechanism is not faster than NER per se.

CPDs in the TS are removed heterogeneously by both the UVDR and NER pathways. Lesions that are close together show a considerable difference in t½ values, e.g. compare R with S in Figures 3A and 4A (see also Fig. 5A). Comparison of surrounding sequences of rapidly and slowly repaired CPDs

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**Figure 4.** Repair of UV-induced CPDs along the *S. pombe sprpb2*+ locus in *uvde*– cells. Cells were irradiated with 40 J m⁻² UV light and allowed to repair DNA for the time periods indicated. DNA was isolated and the fragment of interest was labelled and treated with the CPD-specific enzyme T4EndoV. Lesion-specific bands in (A) the TS (positions +864 to +1317) and (B) the NTS (positions +1258 to +785) are shown in the autoradiograms. Lanes 1 contain non-irradiated DNA which was also treated with T4EndoV (and was used to account for background levels). The initial lesion distribution is given in lanes 2. Lanes 2–5 show a time-dependent decrease in the intensity of the bands, indicating repair. R and S indicate rapidly and slowly repaired CPDs. In (A) R t½ = 13 min (position +938); S t½ = 29 min (position +930). In (B) R t½ = 124 min (position +1152); S t½ = 308 min (position +1103). The positions are relative to the start codon ATG designated +1. Note the different time periods in (A) and (B).

**Figure 5.** Quantified CPD repair rates in the *sprpb2*+ locus. Graphic representation showing half-times of CPDs in (A) the TS and (B and C) the NTS, depicted above the corresponding dipyrimidine position. All positions are relative to the start codon ATG designated +1. Closed circles represent t½ values of CPDs in the *sprpb2*+ gene in rad13– cells (A and B), open squares represent t½ values of CPDs in the *sprpb2*+ gene in *uvde*– cells (A and C). Note the different scales of the repair half-time axes between (A), (B) and (C).
did not hint at a correlation between primary sequence and the efficiency of repair, although the number of lesions studied is limited. Heterogeneity in repair in the TS is not unprecedented since others have found non-uniformity in repair rates as well. Lesions in the TS of the S. cerevisiae URA3 gene (21) and the human p53 gene (22) are repaired heterogeneously by NER. For the URA3 gene it was shown that this heterogeneity in the TS is not related to the chromatin structure (21). We previously reported that lesions in the TS of the S. cerevisiae RPB2 gene are removed with equal kinetics (15). Apparently, repair of the TS can be homogeneous or heterogeneous. A contribution of GGR to the heterogeneity in repair rates in the TS of the sprpb2Δ gene is unlikely since GGR seems to be very inefficient in S. pombe. This indicates that TCR itself can result in heterogeneous repair rates. Clearly, factors that determine the efficiency of NER and UVDR are different because some lesions are repaired faster by UVDR, while others are repaired faster by NER (Fig. 5A).

Lesions in the NTS are also repaired heterogeneously by NER and UVDR, e.g. compare R with S in Figures 3B and 4B (see also Fig. 5B and C). We (23) and others (21) found in S. cerevisiae that repair of the NTS of the URA3 gene is not uniform. Generally, slowly repaired lesions in the DNA are at positions occupied by proteins [e.g. nucleosomes (21) or TBP (24)]. Besides NER and UVDR, other repair systems, e.g. photocyst, also show heterogeneity in repair of lesions in the NTS (25). It is possible that the nucleosomal organisation of the sprpb2Δ gene causes heterogeneous repair of the NTS, however, this cannot be verified because there are no data available on the nucleosomal structure of this gene. Since the repair half-time plots for both NER- and UVDR-deficient cells show the same global pattern, identical factors might be involved in determining the efficiency of repair of the NTS (compare Fig. 5B and C).

The repair of CPDs by the UVDR pathway was studied in a NER-deficient strain. UV-induced CPDs are removed efficiently from both strands in rad13Δ cells (Figs 3 and 5A and B). The average t₀ values do not differ significantly for both strands and are between 11 and 27 min for the TS and 10 and 26 min for the NTS. This indicates that there is no strand preference, suggesting that, unlike for NER, there is no coupling between UVDR and transcription. Furthermore, it demonstrates that a stalled RNA polymerase is no block for the UVDR pathway, since in that case a preferential repair of the NTS would be expected (26). These results indicate that recognition by UVDR is fast compared to the transcription rate of this gene or that a stalled RNA polymerase is not a block for the Uvde protein.

In contrast to rad13Δ cells, which do not show strand specificity, repair analysis of both strands of the sprpb2Δ gene in a uvde-mutant does reveal a clear strand bias. The TS is repaired rapidly, while the NTS is repaired much more slowly (Figs 4 and 5A and C). As in S. cerevisiae and in human cells, the NER system preferentially repairs CPDs in transcribed strands (1,6), which is probably caused by TCR. The t₀ values of lesions in the TS repaired by NER are between 13 and 29 min, which are comparable to those found for the UVDR system (Fig. 5A). Repair of CPDs in the NTS by NER is much slower than repair by the UVDR pathway (Fig. 5B and C). The least slowly repaired lesions in UVDR-deficient cells have a half-time of 124 min (R in Fig. 4B), while for other lesions this is >225 min. This indicates that GGR in S. pombe is a slowly operating system.

In repair-proficient cells both UVDR and NER are functional. UV-induced CPDs in the TS are repaired very rapidly with half-times of <10 min. Lesions in the TS in wild-type S. pombe cells are repaired faster than in either of the mutants. Apparently both NER and UVDR operate on this strand, resulting in very fast repair (compare Figs 2A, 3A and 4A). Repair of CPDs in the NTS by the UVDR system is fast. In contrast, lesion removal from the NTS by the NER system is very slow and the role of GGR is negligible on a time scale of 80 min. Consequently, in repair-proficient cells CPDs in the NTS will be repaired mainly via the UVDR system. Indeed, in repair-proficient cells the NTS is repaired with the same kinetics as in a rad13Δ mutant (compare Figs 2B, 3B and 4B). Based on repair experiments using CPD-specific antibodies it has been suggested that repair via UVDR is faster than NER (12). We clearly show that repair via the UVDR system is not faster than repair via NER per se, in fact for some lesions NER is faster than UVDR (Fig. 5A). The overall slow repair of NER is the net result of the fast repair of the TS (TCR) and the very slow repair of non-transcribed DNA (GGR).

Previous work showed that the S. cerevisiae RAD7 and RAD16 genes are essential for GGR; null mutants in either of these genes are moderately UV sensitive and are unable to repair lesions from non-transcribed DNA (26). We recently cloned the S. pombe homologue of the S. cerevisiae RAD16 gene, named rhp16 (27). In contrast to S. cerevisiae rad16 mutants, S. pombe rhp16- disruptants are not sensitive to UV. We are currently studying the role of rhp16Δ in repair in S. pombe.

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