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Saccharomyces cerevisiae mms19 mutants are deficient in transcription-coupled and global nucleotide excision repair

Marcel Lombaerts, Marcel Tijsterman, Richard A. Verhage and Jaap Brouwer*

Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands

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ABSTRACT

The recently cloned *Saccharomyces cerevisiae MMS19* gene appears to be involved in both nucleotide excision repair (NER) and transcription, which is also the case for components of the NER/transcription complex TFIIH. Unlike TFIIH however, the Mms19 protein does not affect NER in a highly purified *in vitro* system. In order to investigate the role of Mms19 in NER, we have analysed the repair capacity of the *mms19* disruption mutant. We find that a cell-free extract of this mutant is deficient for NER *in vitro*. Since *mms19* mutants are only moderately sensitive to irradiation with ultraviolet (UV) light, it is possible that such mutants are specifically deficient in one of the two modes of NER, i.e. transcription-coupled or global genome repair. To investigate this possibility, we have analysed the removal of cyclobutane–pyrimidine dimers (CPDs) at the nucleotide level in an *mms19* mutant. Repair of CPDs was not detectable for both transcribed and non-transcribed sequences in this mutant, demonstrating a requirement for Mms19 in both transcription-coupled and global genome repair. Our data, combined with those obtained by others, suggest that Mms19 is required for NER in yeast, although it seems likely that the protein plays an indirect role in this process.

INTRODUCTION

Nucleotide excision repair (NER) is a multi-step process capable of removing a variety of lesions from the DNA (reviewed in 1). The characteristic step of the NER reaction comprises single stranded incisions on both sides of the lesion in the damaged strand. NER has been reconstituted in defined systems by using purified yeast or mammalian proteins (2–4). Besides the minimal set of proteins necessary for the damage-dependent dual incisions that is revealed by those experiments, other proteins may influence the proficiency of NER inside the cell. The kinetics of NER is not equal for different regions of the genome, and NER can be divided into two subpathways. Transcription-coupled repair is a mode of NER that accomplishes fast repair of transcribed strands, while non-transcribed DNA relies on the generally slower global genome repair subpathway. Specific proteins are implicated in each of these pathways. Such proteins are accessory to the NER reaction, not being required for the reconstituted *in vitro* NER reaction with highly purified components, but greatly influencing the NER reaction *in vivo*. Besides the phenomenon of transcription-coupled repair, in which NER enzymes are specifically attracted towards lesions on the transcribed strand that have stalled the transcription machinery (5), another intimate link between NER and transcription exists. The multisubunit complex TFIIH is required for transcription and for NER (reviewed in 6). TFIIH is required for repair of both transcribed and non-transcribed DNA (7,8), demonstrating that its role is not confined to the transcription-coupled repair subpathway of NER.

Saccharomyces cerevisiae mms19 mutants are moderately UV-sensitive (9,10), as compared to several NER-deficient *rad* mutants that are highly UV-sensitive. This suggests an accessory role for the Mms19 protein in the NER process. Recently the *MMS19* gene has been cloned, and the encoded protein appeared to be involved in both NER and in transcription, like TFIIH components (10). However, Mms19 is not a subunit of TFIIH, nor appears to be stably associated with this NER/transcription factor (10). Instead, Mms19 might influence TFIIH as an upstream factor, since a thermolabile transcription defect of *mms19* cell-free extracts could be complemented by addition of purified TFIIH and not by purified Mms19 protein (10). The pleiotropic effects of *mms19* mutations might be partly caused as a secondary consequence of a transcription defect in such mutants. Although it could be concluded that *mms19* mutants have an NER-defect based on a deficiency in removal of UV-induced lesions and UV-sensitivity (9,10), the purified Mms19 protein does not affect the damage-dependent incisions in a highly defined reconstituted NER system (10). It was, therefore, of considerable interest to determine the repair capacity of *mms19* mutants, compared to other mutants in which the proficiency of NER is affected, in order to gain insight into the role of Mms19 in NER. The moderate UV-sensitivity of *mms19* mutants could result from a deficiency in NER of a subset of lesions, as has been found for *rad7* and *rad16* mutants, which also display a moderate

*To whom correspondence should be addressed. Tel: +31 71 5274755; Fax +31 71 5274537; Email: brouwer@chem.leidenuniv.nl

UV-sensitivity. These latter mutants are specifically deficient in repair of lesions in non-transcribed DNA, whereas transcription-coupled repair is unaffected (11). On the other hand, Mms19 is known to affect transcription (10), and therefore, it is also possible that the *mms19* mutant is specifically deficient for transcription-coupled repair. Therefore, this mutant might be disturbed exclusively in either one of the subpathways of NER. Alternatively, NER of all lesions might be affected. Here we describe the characterization of the NER defect in the *mms19* disruption mutant. We demonstrate that such a mutation augments the UV-sensitivity of several partially NER deficient mutants disturbed in transcription-coupled repair, global genome repair or both. We reveal a NER deficiency in cell-extracts from the *mms19* mutant *in vitro*. Finally, we show that this mutant is defective in both transcription-coupled and global genome NER *in vivo*. These repair data extend the observations of Lauder *et al.* (10), and are in accordance with a role of the Mms19 protein upstream of TFIIH, as suggested by these authors.

MATERIALS AND METHODS

Yeast strains and media

The *S.cerevisiae* wild-type strain used for this study is W303-1B (MATα *ade2-1 trp1-1 can1-100 his3-11,15 leu2-3,112 ura3-1*). An isogenic *mms19*Δ mutant was constructed by a PCR based strategy (12). The following primers were used for PCR: 5′-CACAGTAACGGATTCTATTGTACACCGATCATCTTT-GACAGCTTATCATC-3′ and 5′-GTGTCCTTCAGAGTTTC-TAAAGCGGATACCCACTCGTGCACCC-3′. PCR was carried out for five cycles at 45°C, followed by 30 cycles at 72°C, using pYES2 as a target. The PCR fragment was transformed to W303-1B. The resulting *mms19*Δ strain MGSC217, in which almost the entire open reading frame of the *MMS19* gene was replaced by *URA3*, had characteristics consistent with *mms19* mutants described in literature (10). The PCR-fragment was also used to construct the *mms19*Δ mutation in the following isogenic yeast strains that were described before (11,13,14): MGSC126 (*rad16*Δ), MGSC102 (*rad26*Δ), MGSC107 (*rad16*Δ *rad26*Δ) and MGSC139 (*rad14*Δ) to obtain strains MGSC219 (*rad16*Δ *mms19*Δ), MGSC218 (*rad26*Δ *mms19*Δ), MGSC220 (*rad16*Δ *rad26*Δ *mms19*Δ) and MGSC221 (*rad14*Δ *mms19*Δ). All strains were kept on selective YNB (0.67% yeast nitrogen base, 2% glucose, 2% bacto agar) supplemented with the appropriate markers (15). Cells were grown in complete medium (YEPD: 1% yeast extract, 2% bacto peptone, 2% glucose) at 28°C (15).

Preparation of yeast cell-free extracts

Yeast cell-free extracts were prepared as described (16). Yeast cells were converted to spheroplasts with yeast lytic enzyme (ICN). Spheroplasts were washed thoroughly and stored as pellets at –80°C until further use. The pellet was resuspended at 4 ml/g spheroplast in a hypotonic buffer (10 mM Tris–HCl pH 8, 1 mM EDTA, 5 mM DTT) containing protease inhibitors (benzamidin 300 µg/ml, leupeptin 1 µg/ml, antipain 1 µg/ml, pepstatin 1 µg/ml, chymostatin 1 µg/ml, PMSF 1 mM) and stirred for 20 min on ice. A sucrose solution (50 mM Tris–HCl pH 8, 10 mM $MgCl_2$, 25% sucrose, 50% glycerol, 2mM DTT) (4 ml/g spheroplasts) was added dropwise to this suspension while stirring gently. After another 20 min of stirring, 290 µl/ml of a neutralized solution of 4M $(NH_4)_2SO_4$ was added dropwise, and stirring on ice was continued for another 30 min. The suspension was centrifuged at 170 000 *g* for 1 h at 4°C (Beckman L7 ultracentrifuge, 75Ti rotor). The supernatant was transferred to a beaker on ice, leaving some residual supernatant. Per ml of supernatant, 330 mg of powdered $(NH_4)_2SO_4$ was added over the course of 30–40 min, while gently stirring on ice. After neutralization of the solution with 1M NaOH [10 µl/g $(NH_4)_2SO_4$ added], stirring on ice was continued for 30 min. The suspension was centrifuged at 25 000 *g* for 15 min at 4°C and the pellet was resuspended in 1/40–1/30 vol. of the ultracentrifugation supernatant in dialysis buffer [20 mM HEPES–KOH pH 7.5, 20% (v/v) glycerol, 10 mM $MgSO_4$, 10 mM EGTA, 5 mM DTT] containing protease inhibitors. After dialysis overnight on ice against dialysis buffer containing 1 mM PMSF, precipitates were removed by centrifugation at 15 000 *g* for 15 min at 4°C. The resulting yeast cell-free extract was snap-frozen using liquid nitrogen and stored at –80°C. Under these conditions the extract was stable for at least several months and could be used for *in vitro* NER experiments, even upon repeated thawing and freezing of the extract.

In vitro NER

To measure *in vitro* NER activity (method as in ref. 16,17), supercoiled plasmid pNP81 (4.4 kb) was purified as an undamaged control plasmid, while purified pUC18 (2.7 kb) was treated with *N*-acetoxy-2-acetylaminofluorene to obtain pUC18-AAF. Standard *in vitro* NER reactions (50 µl) contained 300 ng each of pNP81 and pUC18-AAF DNA, NER buffer (45 mM HEPES–KOH pH 7.8, 40 mM KCl, 7 mM $MgCl_2$, 1 mM DTT, 0.4 mM EDTA, 20 µM dGTP, 20 µM dATP, 20 µM dTTP, 4 µM dCTP, 1 µCi [α-^{32}P]dCTP, 2 mM ATP, 40 mM creatine phosphate, 2.5 µg creatine phosphokinase, 4% glycerol, 18 µg BSA, 5% polyethylene glycol (PEG 6000) and 250 µg yeast cell-free extract. Repair reactions were incubated for 2 h at 28°C. Reactions were stopped by addition of 2 µl of 0.5 M EDTA and incubation for 2 min on ice. Samples were treated with RNaseA (2 µg) for 10 min at 37°C, and DNA was subsequently purified by treatment of the reaction mixtures with 0.5% SDS and 1 µg/µl proteinase K at 50°C for 30 min and precipitation of protein with ammonium acetate (final concentration 2.4 M). DNA was precipitated with ethanol, resuspended in TE (10 mM Tris–HCl pH 8, 1 mM EDTA) and digested with *Bam*HI to linearize the plasmids. The samples were electrophoresed in 1% agarose gels containing ethidium bromide (EtBr) and photographed. The gels were dried and autoradiographs were prepared.

UV-survival experiments

Yeast cells were grown until stationary phase in YEPD at 28°C. Serial dilutions of the cells were prepared in water and spread on YEPD plates. The plates were irradiated with increasing doses of UV-light and incubated in the dark at 28°C. After 4–6 days, colonies were counted and survival was calculated.

Analysis of CPD removal *in vivo*

Yeast cells diluted in chilled phosphate-buffered saline were irradiated with 254 nm UV light (Philips T UV 30W) with 70 J/m^2. Cells were collected by centrifugation, resuspended in complete medium and incubated for various times in the dark at 28°C prior to DNA isolation. DNA samples were purified on CsCl gradients (18). DNA samples (25 µg) were digested with appropriate

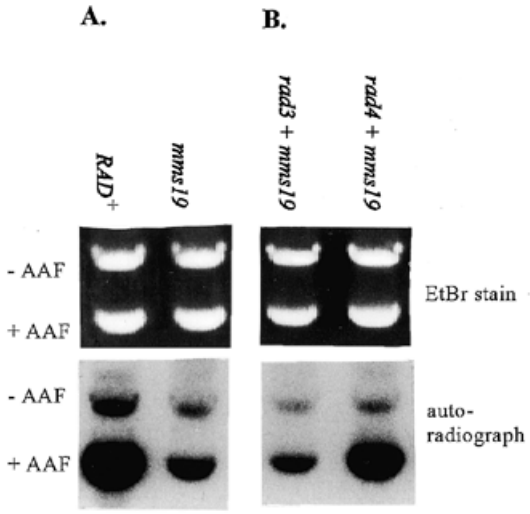

Figure 1. *In vitro* NER using yeast cell-free extracts. Yeast cell-free extracts from the indicated strains were prepared and 250 µg protein from these extracts was incubated for 2 h at 28°C with 300 ng each of undamaged pNP81 (4.4 kb) and pUC18 containing AAF-damage (pUC18-AAF, 2.7 kb) in the presence of [α-^{32}P]dCTP. DNA was purified, linearized and electrophoresed in 1% agarose. Top, shows an ethidium bromide (EtBr) stain of the gel, bottom, shows autoradiographs from the dried gel. (**A**) NER in W303-1B (*RAD*$^+$) and MGSC217 (*mms19*Δ) cell-free extracts. (**B**) *mms19*Δ cell-free extract was mixed (125 µg protein from each extract) with an extract prepared from either a *rad3-2* or a *rad4*Δ strain as indicated.

endonucleases, precipitated and *URA3* or *RPB2* fragments were isolated and end-labeled as described previously (19,20) using fragment-specific oligonucleotides (sequences available upon request). CPDs were identified using T4endoV. DNA samples were divided in two equal parts. One was incubated with T4endoV, the other was mock treated. Samples were subjected to spun column chromatography and lyophilized to small volumes. Approximately equal amounts of c.p.m. were loaded on 6% denaturing acrylamide gels. After drying, autoradiographs were prepared from the gels.

Quantification of repair rates

From each experiment, multiple autoradiographs were obtained with different exposure times to allow signal determination within the linear range of X-OMAT™ (AR) Scientific imaging films (Kodak) for each individual CPD. Autoradiographs were scanned using an LKB Ultrascan XL densitometer (Pharmacia) and analysed using ImageMaster™ software (Pharmacia). Background levels were subtracted and gel-band intensities were corrected for loading variations. OD-values were plotted against repair time for each CPD that gave sufficient signal to background ratio. Repair half-times ($t_{1/2}$), defined as the time at which 50% of the initial damage (signal at $t = 0$) was removed, were derived from these plots.

RESULTS

mms19 cell-free extracts are deficient for NER *in vitro*

The purified Mms19 protein does not influence the NER reaction when purified proteins are used to reconstitute this reaction (10).

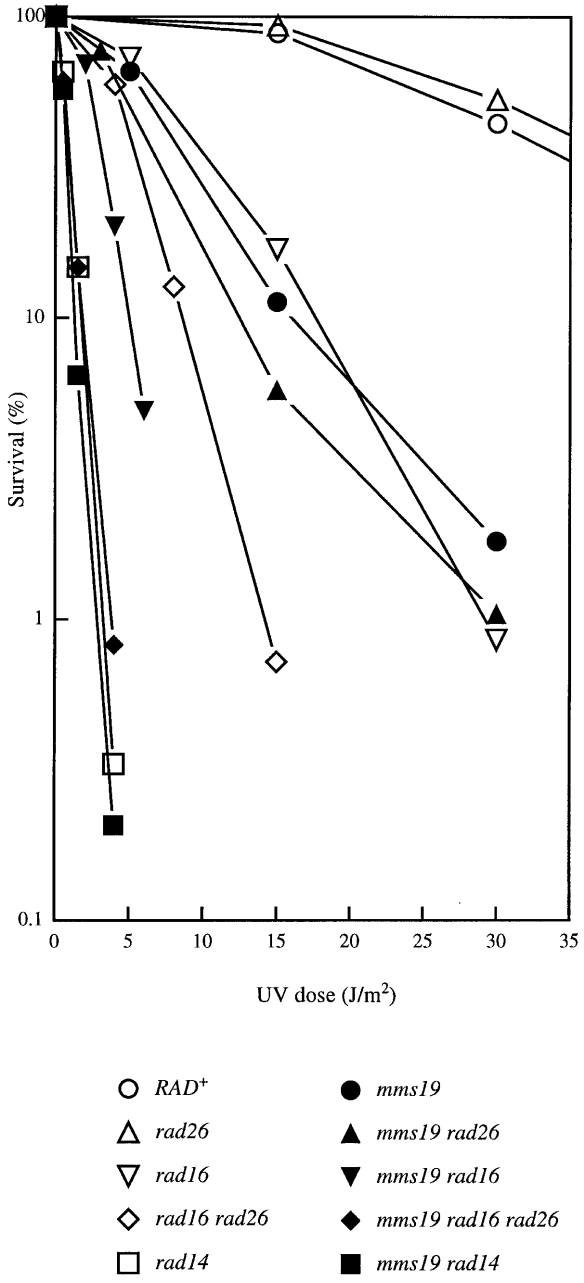

Figure 2. Effect of the *mms19*Δ mutation on survival of strains that already have a partial NER deficiency. Yeast cells on YEPD plates were irradiated with the indicated doses of UV-light, and after incubation at 28°C for 4–6 days in the dark, colonies were counted and survival was calculated.

Nevertheless, an *mms19* mutant is sensitive to UV and epistatic with NER deficient mutants, suggesting that Mms19 is involved in NER. We have determined the *in vitro* NER capacity of a cell-free extract of a yeast strain lacking Mms19. To this purpose an *mms19*Δ mutant was constructed by a PCR-based strategy (12). This mutant is a methionine auxotroph, grows slowly at 37°C and is sensitive towards MMS and UV-light, in agreement with the *mms19* mutants described before (10). The *in vitro* NER capacity of this mutant was investigated by determining the repair synthesis activity of a cell-free extract on a plasmid containing AAF damage, compared to a non-damaged plasmid (17).

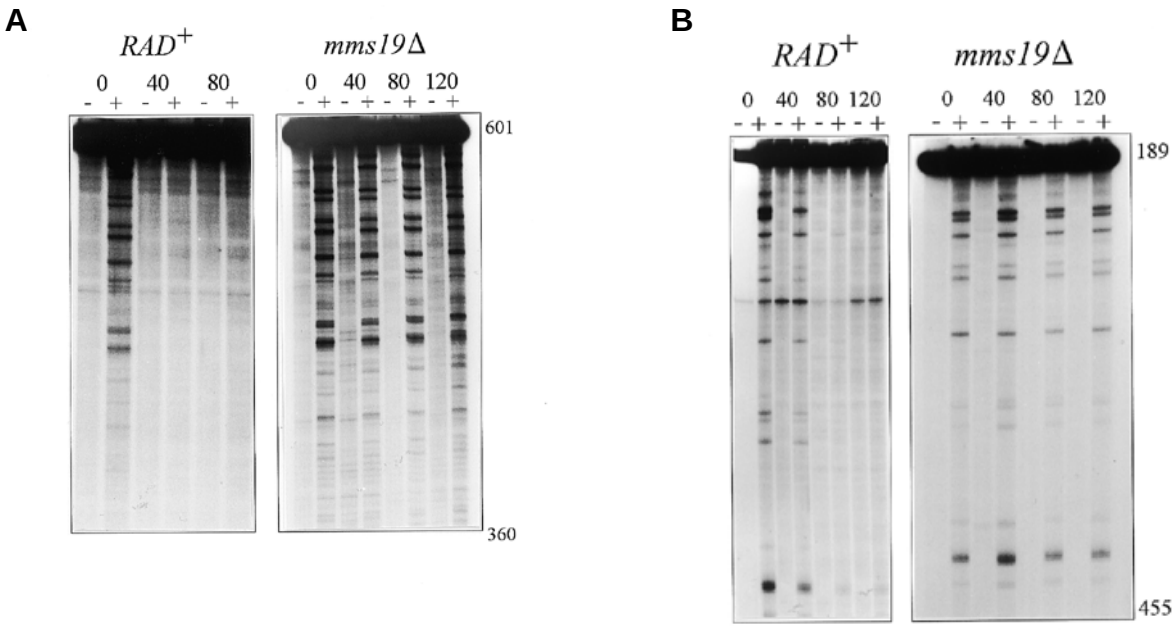

Figure 3. Repair of UV-induced CPDs at single nucleotide resolution in *RAD*$^+$ and *mms19*Δ cells. Sequences between nt +360 and nt +601 for the *URA3* transcribed strand (**A**) and between nt +189 and nt +455 for the non-transcribed strand (**B**) are shown. Cells were irradiated with 70 J/m^2 and repair was allowed for 0, 40, 80 and 120 min. Samples mock-treated or treated with the dimer specific enzyme T4endoV are denoted – and +, respectively.

Figure 1A shows the result of an *in vitro* NER experiment, in which repair in the NER proficient background (*RAD*$^+$) was compared to repair in the isogenic *mms19* strain. The ethidium bromide stain of the gel (Top) shows that the amount of undamaged plasmid (4.4 kb) is equal to the amount of AAF-damaged plasmid (2.7 kb). The autoradiograph of the dried gel (bottom), indicates the amount of incorporated radiolabeled dCTP as a result of the NER reaction. For a cell-free extract from the *RAD*$^+$ strain NER is visible as a high level of incorporation of radiolabel in the damaged plasmid, whereas for an extract of the *mms19* mutant the level of label incorporation is comparable to background levels in the undamaged plasmid. Therefore, a cell-free extract of an *mms19* mutant is deficient for NER *in vitro*.

Since the *mms19* mutation appears to affect the integrity of TFIIH in the cell (10), it is expected that the NER defect of the *mms19* cell-free extract cannot be complemented by extract that lacks functional TFIIH because of a mutation in one of the components of this complex (e.g. from a *rad3* mutant), whereas complementation by other NER-deficient extracts should be possible. This is indeed the case as Figure 1B shows that NER-deficient extracts of *mms19* and *rad3* mutants do not complement each other, whereas the NER defect of the *mms19* mutant extract is complemented by a NER deficient *rad4* whole cell extract. These data are in agreement with the hypothesis that the function of TFIIH is affected by the *mms19* mutation.

The *mms19* mutant is deficient in transcription-coupled and global genome NER *in vivo*

In vivo, the *mms19* mutant may be deficient in NER of all lesions, or in either transcription-coupled or global genome repair (see Introduction). By constructing the *mms19* mutation in genetic backgrounds that are already affected in one or both of the subpathways of NER, the influence of the *mms19* mutation on the remaining NER system can be studied by UV-survival experiments. When the *mms19* mutation leads to higher UV-sensitivity than found in the single mutant, Mms19 affects the remaining subpathway(s). As an example, *rad16 rad26* double mutants are more UV-sensitive than either single mutant, since *rad16* mutations lead to deficiencies in global genome repair while the *rad26* mutation affects the other, transcription-coupled, subpathway of NER (14). Therefore, we constructed the *mms19* mutation in strains that carry deletions of the *RAD16* gene (deficient for global genome repair, 11), the *RAD26* gene (partially deficient for transcription-coupled repair, 13,14), or both. To confirm epistasis with the *rad3* group of NER mutants (9,10), an *mms19* mutant was constructed in a completely NER deficient *rad14* mutant. Figure 2 shows the survival of these mutants after irradiation with UV light. The *mms19* mutation appears to increase the UV-sensitivity of the other partially NER deficient mutants in either subpathway, suggesting that Mms19 affects both transcription-coupled and global genome repair.

To test this directly, we performed repair analysis of UV-induced cyclobutane–pyrimidine dimers (CPDs) at the nucleotide level in the *mms19* mutant *in vivo*, using a method we previously described (19,20). Yeast cells were irradiated with UV and incubated at various times to allow repair, after which DNA was isolated. Lesions were detected by sizing end-labeled DNA, cleaved at the site of CPDs with the T4endoV enzyme, thus giving rise to a distinct band on a gel, reflecting the occurrence in the sequence of UV-sensitive pairs of adjacent pyrimidines (19). Disappearance of these bands from the lanes treated with T4endoV in the later time points is indicative for removal of CPDs. Figure 3A shows repair data for the transcribed strand of the *URA3* gene and Figure 3 shows repair of the non-transcribed strand of this locus. In the repair proficient strain, removal of CPDs after UV irradiation is evident from both the transcribed and the non-transcribed strand. Because of transcription-coupled

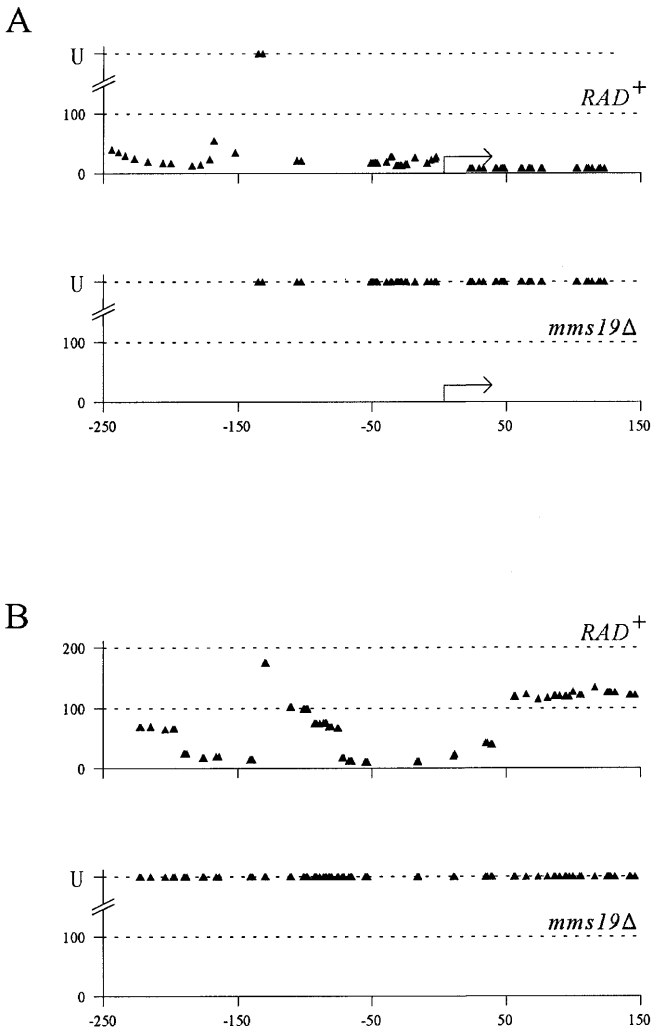

Figure 4. Graphic representation of quantified CPD repair rates along the transcription initiation site for (**A**) the template and (**B**) the non-template strand of the *RPB2* locus. The data obtained from wildtype *RAD*$^+$ cells (top) and from *mms19*Δ cells (bottom) is shown. The transcription initiation site is indicated by the arrow at position +1. Repair halftime values (in min), determined as the time at which 50% of the initial CPD signal was removed are depicted above their corresponding dipyrimidine position. repair $t_{1/2}$ = U indicates that repair was not observed after 2 h post-incubation.

repair, CPDs from the transcribed strand are removed faster than from the non-transcribed strand (compare Fig. 3A and B) in the *RAD*$^+$ strain (20). In contrast, CPDs are not removed after repair periods up to 2 h from either DNA strand of the *mms19* mutant (Fig. 3A and B). In addition, we analysed the removal of CPDs from the template and non-template strand around the transcription initiation site of the *RPB2* locus, allowing repair analysis of the transcribed strand and of non-transcribed DNA (promoter sequences and the non-transcribed strand of an active gene). In Figure 4A and B, the repair half-times of CPDs at specific dinucleotides in this gene are represented graphically, as calculated by quantification of the intensity of the corresponding bands on autoradiographs (19). Like the *URA3* gene, removal of CPDs from either strand of the *RPB2* gene is not detected in the *mms19* mutant, while lesions in these regions are repaired in the *RAD*$^+$ strain (Fig. 4A and B). From these data we conclude that the *mms19* mutant is deficient in transcription-coupled and global genome repair.

DISCUSSION

We have investigated the NER capacity of the yeast *mms19* mutant and find that it is deficient for NER *in vitro* and *in vivo*. Despite the moderate UV-sensitivity of this mutant, which might suggest a specific defect of either global genome or transcription-coupled repair, we demonstrate that this mutant is deficient in both subpathways of NER.

The Mms19 protein influences transcription (10), and probably acts upstream of TFIIH in the cell, giving a possible explanation for the pleiotropic phenotype of the *mms19* mutant. However, in contrast to the defect in transcription that is observed most clearly at 37°C, we observe a complete NER defect at 28°C, at which transcription still takes place in this mutant. Hence, it is not likely that the NER defect of an *mms19* mutant is caused by an indirect transcription defect. This idea is corroborated by the following observations: (i) the NER defect *in vivo* is not confined to the transcription-coupled repair subpathway and (ii) cell-free extracts of repair deficient strains that have functional TFIIH can be complemented by the *mms19* mutant, demonstrating that other NER proteins are still functional in the *mms19* mutant.

The reason for the only moderate UV-sensitivity of the *mms19* mutant is not known, as we failed to detect NER activity in this mutant. Probably, a residual NER capacity which leads to very slow removal of lesions exists in this mutant, but escapes detection in our *in vivo* repair analysis. A similar finding was reported for the *rad23* mutant (21, and discussion therein). Since complete absence of TFIIH is lethal, the *mms19* mutation probably affects the stability of TFIIH, without completely abrogating its function. This might lead to a slow turnover or a very low capacity of the NER reaction, rendering it difficult to measure repair activity in this mutant. The time for a survival experiment can suffice for residual repair. We cannot formally exclude the possibility that another class of UV-induced lesions, pyrimidine (6–4) pyrimidone photoproducts [(6–4)PPs], is repaired in the *mms19* mutant. However, we consider this very unlikely since the genetic requirements for NER of CPDs and (6–4)PPs are the same (22). The development of a method to analyse repair of (6–4)PPs at the nucleotide level in yeast is currently in progress in our laboratory, allowing to obtain experimental data on this presumption. The absence of detectable NER activity of cell-free *mms19* extracts *in vitro* is shared with the other moderate UV-sensitive *rad7*, *rad16* and *rad23* mutants (23,24, our unpublished results). From these, only *rad23* strains have a mutation in a protein that is required for damage-dependent incisions in a reconstituted NER reaction with purified components. This demonstrates that besides the minimal set of proteins required for NER in a purified system, other proteins are required for this complex process in less well defined systems *in vitro*, as is the case inside the living cell.

The experiments of Lauder *et al.* strongly suggested that Mms19 acts upstream of TFIIH in the cell (10). Our experiments are fully consistent with this hypothesis. Indeed, the NER defect of a cell-free extract from an *mms19* mutant cannot be complemented by an extract derived from a strain that has no functional TFIIH because one of the subunits of TFIIH (Rad3) is mutated, whereas complementation by NER-deficient extracts that contain intact TFIIH is possible. Furthermore, the *mms19* mutant is deficient for

both transcription-coupled and global genome repair, as are yeast strains with defects in TFIIH components (7,8). Despite the absence of a role for the purified Mms19 protein in NER with purified components as described by Lauder *et al.* (10), this report directly demonstrates a NER defect in the *mms19*Δ mutant. Therefore, Mms19 is involved in NER, albeit probably not in a direct manner but rather via upstream regulation of the integrity of TFIIH (10).

It has been suggested that mutations in a human homolog of *MMS19* might be responsible for certain diseases in which both transcription and NER are affected (10). Our data suggest that patients with such mutations, if they exist, will have a general NER deficiency rather than being specifically deficient for transcription-coupled or global genome repair.

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