Repair of rDNA in *Saccharomyces cerevisiae*: *RAD4*-independent strand-specific nucleotide excision repair of RNA polymerase I transcribed genes

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ABSTRACT

Removal of UV-induced pyrimidine dimers from the individual strands of the rDNA locus in *Saccharomyces cerevisiae* was studied. Yeast rDNA, that is transcribed by RNA polymerase I (RNA pol I), is repaired efficiently, slightly strand-specific and independently of *RAD26*, which has been implicated in transcription-coupled repair of the RNA pol II transcribed *RPB2* gene. No repair of rDNA is observed in *rad1, 2, 3* and *14* mutants, demonstrating that dimer removal from this highly repetitive DNA is accomplished by nucleotide excision repair (NER). In *rad7* and *rad16* mutants, which are specifically deficient in repair of non-transcribed DNA, there is a clear preferential repair of the transcribed strand of rDNA, indicating that strand-specific and therefore probably transcription-coupled repair of RNA pol I transcribed genes does exist in yeast. Unexpectedly, the transcribed but not the non-transcribed strand of rDNA can be repaired in *rad4* mutants, which seem otherwise completely NER-deficient.

INTRODUCTION

Cyclobutane pyrimidine dimers induced in DNA by irradiation with UV-light can be removed by the nucleotide excision repair (NER) system to maintain the genetic integrity (reviewed in 1–3). Removal of dimers from DNA is heterogeneous throughout the genome (4,5) because dimers can be a substrate for either of two subpathways of NER: transcription-coupled and global genome repair (6). Transcription-coupled repair is a very efficient process in which lesion-stalled RNA polymerase II (RNA pol II) molecules may act as a condensation site for the assembly of repair complexes (7–9). Specific gene products might enhance the efficiency of this process. In *Escherichia coli*, a protein called TRCF (transcription repair coupling factor) couples the NER enzymes to a lesion-stalled RNA polymerase (10). Based on *in vitro* studies, the following model for transcription-coupled repair in *E.coli* has been proposed (10): TRCF releases the stalled polymerase together with the transcript, binds the NER protein UvrA, thereby recruiting the NER proteins to lesions that interfere with transcription. Subsequently these lesions are removed by the action of the Uvr enzymes. In mammalian cells the genes complementing the hereditary recessive disorder Cockayne syndrome groups A and B are involved in transcription-coupled repair (11–13), while in *S.cerevisiae* the homolog of the Cockayne syndrome B gene, *RAD26*, is implicated in this process (14). It is still unknown whether these genes encode coupling factors analogous to TRCF in *E.coli*, or are involved in transcription-coupled repair in a different way. Non-transcribed DNA obviously can not be a substrate for transcription-coupled repair. Nevertheless this DNA is repaired by NER enzymes, although slower than transcribed strands (4), in a process referred to as global genome repair. Specific genes have been shown to be essential for global genome repair. Notably, in human xeroderma pigmentosum group C (XP-C) cells, non-transcribed DNA is not repaired while transcribed strands of active DNA are repaired efficiently (15,16). In yeast the *RAD7* and *RAD16* genes are essential for repair of non-transcribed DNA (17,18). In *rad7* and *rad16* mutants the transcribed strand of active genes is repaired as efficiently as in RAD^+ cells, showing that transcription-coupled repair is not hampered in these mutants (18). The actual repair process is conducted by a complex of enzymes called repairosome (19), which contains most proteins that are essential for NER known so far. Most likely this multiprotein complex performs the incisions and subsequent steps in the same manner for both DNA strands. Possibly the repairosome is unable to remove dimers in DNA that is condensed into chromatin, and therefore is dependent on either global genome repair factors or transcription to be able to operate *in vivo* (6). Transcription-coupled repair has been demonstrated in eukaryotes for genes transcribed by RNA polymerase II (RNA pol II) (20–23), but not for genes transcribed by RNA pol I (24,25). Here we investigate the repair of ribosomal DNA (rDNA) in yeast, to find out whether RNA pol I transcribed DNA is repaired in a similar way as the genes transcribed by RNA pol II that have been studied so far. rDNA genes are highly repetitive in all organisms, with yeast having 100–200 copies (reviewed in 26,27). Two structurally and transcriptionally different subclasses of rDNA exist: some of the copies are inactive and packed in nucleosomal arrays which are not accessible for psoralen crosslinking while the other copies are transcriptionally active and in an open non-nucleosomal chromatin

conformation that can be crosslinked by psoralen (28,29). Removal of dimers from rDNA was virtually absent in hamster cells and inefficient in human cells (24,25). It was speculated that removal of dimers from the highly repetitive rDNA cluster could be due to recombination instead of NER (24), but subsequently it was shown that in XP-C and CS-B cells which are impaired in NER, repair of rDNA was inhibited (30). Repair of mammalian rDNA appeared to be not strand-specific (not transcription-coupled) and less efficient than repair of the genome overall (24,25). We have studied removal of dimers from the rDNA cluster of yeast in repair proficient (*RAD*+) cells and in various *rad* mutants that are disturbed in specific subpathways of NER. Our results reveal marked differences between repair of rDNA in yeast compared to results described for mammalian cells, as well as differences in repair of rDNA and genes that are transcribed by RNA pol II. The data also have implications for the function of Rad4p in NER, and possibly for its presumed human homolog, XPC.

MATERIALS AND METHODS

General procedures

All general procedures including DNA purification, restriction enzyme digestion, cloning and gel electrophoresis were performed according to standard procedures (31). Plasmids were propagated in *E.coli* strain JM101 under appropriate antibiotic selection.

Yeast strains and media

The yeast strains used for this study are listed in Table 1. All strains were kept on selective YNB (0.67% yeast nitrogen base, 2% glucose, 2% bacto agar) supplemented with the appropriate markers. Cells were grown in complete medium (YEPD: 1% yeast extract, 2% bacto peptone, 2% glucose) at 28°C under vigorous shaking conditions.

Construction of disruption mutants

Yeast cells were transformed by electroporation (2250 V/cm, 250 μF, 200 Ω). Cells were plated on YNB with the necessary amino acids and incubated at 28°C for 2–5 days. Disruption of the *RAD4* gene was accomplished by transformation of *Xba*I-digested pDG38 (gift of D. Gietz). Disruptions of the *RAD14* gene were obtained by transformation of *Sac*I/*Nco*I-digested pBM190 (gift of L. Prakash; 32). Disruption of the *RAD7*, *RAD16* and *RAD26* genes has been described earlier (18,14).

UV irradiation and DNA isolation

Yeast cells diluted in chilled phosphate-buffered saline were irradiated with 254 nm UV light (Philips T UV 30W) at a rate of 3.5 J/m²/s. Cells were collected by centrifugation, resuspended in growth medium and incubated for various times in the dark at 28°C prior to DNA isolation (33). DNA was purified on CsCl gradients (31).

Specific probes

Construction and isolation of single stranded M13 derived probes recognizing the *RPB2* gene was as described before (18). For construction of strand-specific rDNA probes a 1 kb *Eco*RI–*Mlu*I rDNA fragment from plasmid pGEM3-EM1 (gift of J. Venema) was cloned in both orientations in M13.

Single-stranded DNA was isolated according to Sambrook *et al.* (31) and used for primer extension to generate ³²P-labeled strand-specific probes as described earlier (14,18).

Table 1. *Saccharomyces cerevisiae* strains used

Strain	Genotype	Source[a]
W303-1B	*MATα ho can1-100 ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1*	R. Rothstein
W303236	*rad16Δ::URA3*[b]	This laboratory
MGSC102	*rad26Δ::HIS3*[b]	This laboratory
MGSC104	*rad7Δ::LEU2*[b]	This laboratory
MGSC101	*rad23Δ::URA3*[b]	This laboratory
MGSC131	*rad4Δ::URA3*[b]	This laboratory
MGSC132	*rad4Δ::URA3 rad7Δ::LEU2*[b]	This laboratory
MGSC133	*rad4Δ::URA3 rad26Δ::HIS3*[b]	This laboratory
MGSC139	*rad14Δ::LEU2*[b]	This laboratory
MGSC141	*rad4Δ::URA3 rad14Δ::LEU2*[b]	This laboratory
MG70/X9b-7B	*Matα gal ade2-1 rad4-4*	YGSC[c]
SF RAD1°	*MATa gal2 leu2-1,112 his4-58 ura3-52 pep4-3 rad1Δ*	R. Waters
SF RAD2-/2	*MATa gal2 leu2-1,112 his4-580 ura3-52 pep4-3 rad2::URA3*	R. Waters
YR3-3	*MATα leu2-3,112 ura3-52 can1 trp1Δ rad3-2*	L. Prakash

[a]Strains were gifts of the investigators mentioned or constructed in this laboratory.
[b]The remainder of the genotype is that of W303-1B.
[c]YGSC, Yeast Genetic Stock Centre.

Southern blot analysis showing the removal of endonuclease sensitive sites

Genomic DNA was cut with restriction endonuclease *Hin*dIII, which generates a 6.4 kb rDNA fragment. DNA samples were divided in two equal parts, one of which was incubated with T4 endonuclease V (T4endoV; isolated as described earlier: 34,35), and the other mock-treated, both were loaded on denaturing agarose gels as described by Bohr *et al.* (4). After electrophoresis the DNA was transferred to Hybond N+ (Amersham) and hybridized to strand-specific probes. After hybridization and data analysis the probe was removed by alkaline washing and subsequently the blot was hybridized to the probe recognizing the opposite strand.

The amount of hybridized labeled probe in each band on the Southern blots was quantified with a Betascope 603 blot analyser (Betagen) and used to calculate the amount of dimers per fragment according to the Poisson distribution as was described previously (4). After being scanned in the blot analyser, autoradiographs were prepared from the Southern blots.

RESULTS

The yeast rDNA cluster consists of 100–200 repeats of 9.1 kb each (26,27). Each repeat contains genes for 18, 5.8 and 25S rRNA that are transcribed by RNA pol I into a single 35S transcript that is post-transcriptionally processed into the separate rRNAs. Each unit also contains a 5S rRNA gene that is transcribed by RNA pol III (26,27). We have studied removal of dimers from
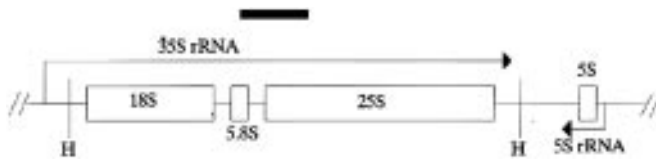
Figure 1. Map of yeast rDNA organization. One repeat unit is shown together with the relevant restriction sites (H: *Hin*dIII), probe (thick bar) and transcripts (arrows). The 35S rRNA is transcribed by RNA pol I, the 5S rRNA by RNA pol III.

both strands of a 6.4 kb *Hin*dIII fragment that comprises almost the whole RNA pol I transcribed region. A schematic map that shows relevant restriction sites, transcription units and the probe used for repair experiments is presented in Figure 1. Since probes recognizing rDNA detect all the repeats at the same time, it is important to note that only some of the copies are indeed transcribed at a given time, while the others are not active (28,29). Therefore a probe for the transcribed strand will recognize strands that are actually transcribed as well as template strands that are not currently transcribed. In addition, a fraction of both strands (correlating with the active copies) is non-nucleosomal. Under the conditions of the repair experiments it can be estimated that ~40–60% of the rDNA units are active (29).

Repair of rDNA is not dependent on *RAD26*

The efficiency of dimer removal from rDNA after irradiation with 70 J/m² of UV in cells of the repair proficient (*RAD+*) strain W303-1B was determined. Repair of the individual strands after various time points is shown in Figure 2. We find consistently that there is some strand-bias, the transcribed strand being repaired slightly more efficiently than the non-transcribed strand, but this difference is within the error margin of the experiment and therefore it is not clear whether it is real. The repair rate of both strands is comparable to removal of dimers from the non-transcribed strand of the RNA pol II transcribed *RPB2* gene (not shown, ref. 6).

The *RAD26* gene is the functional homolog of the human *ERCC6/CSB* gene, and is involved in transcription-coupled repair of the RNA pol II transcribed *RPB2* gene (14). However, for rDNA no significant difference is observed between repair in the *rad26* disruption mutant and the isogenic *RAD+* strain (Fig. 2). The slight strand-bias in favor of the transcribed strand that was observed for the *RAD+* strain is also found in the *rad26* mutant.

rDNA is not repaired in NER-deficient *rad1, 2, 3* and *14* mutants

To investigate whether NER plays a role in repair of rDNA in yeast we analysed repair of rDNA in mutants that are completely deficient in this process (1,2,36). Dimer removal from rDNA was analysed in a *rad14* disruption mutant, in which NER is totally absent since the damage recognizing protein Rad14 is not present (32). No repair of rDNA is observed in this mutant (Fig. 2D), demonstrating that *RAD14* is essential for removal of dimers from rDNA in yeast. Similar results were found for *rad1* (Fig. 2C), *rad2* and *rad3* mutants (not shown), as well as for a *rad23* mutant
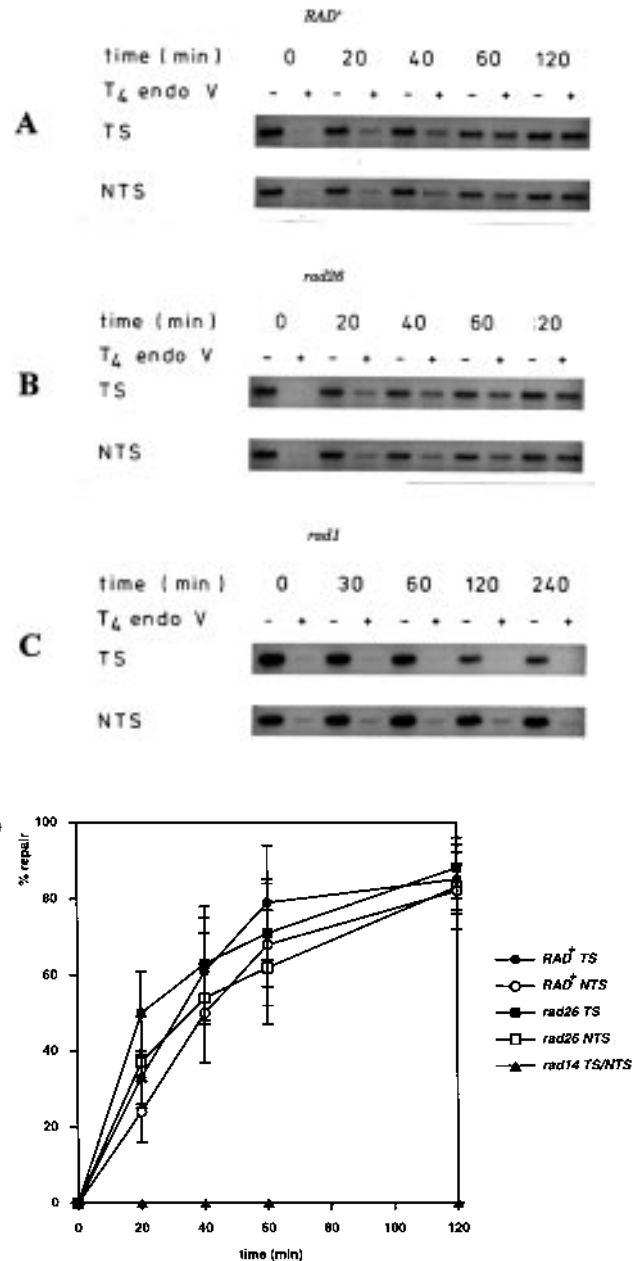
Figure 2. Removal of dimers from rDNA in *RAD+*, *rad26* and NER-deficient cells. (A–C) Representative Southern blots showing the removal of dimers from (**A**) strain W303-1B (*RAD+*), (**B**) strain MGSC102 (*rad26*), (**C**) strain SF RAD1° (*rad1*) after various repair periods. Times after UV are indicated, samples mock-treated or treated with the dimer-specific enzyme T4endoV are denoted by – and +, respectively. TS, transcribed strand; NTS, non-transcribed strand. Note that the low amount of signal in the treated (+) lanes compared to the signal in the non-treated (–) lanes for the NTS in the *rad1* strain does not increase with time, and therefore does not represent removal of dimers. (**D**) Repair as a function of time after quantification of the Southern blots with the Betascope blot analyser. Repair of both strands of rDNA in strains W303-1B (*RAD+*), MGSC102 (*rad26*) and MGSC139 (*rad14*) is shown. Symbols indicate the mean of several experiments, bars indicate standard deviations.

(37). These results demonstrate that dimer removal from rDNA in yeast is dependent on NER.

Strand-specific repair of rDNA in *rad7* and *rad16* mutants

Removal of dimers from both strands of rDNA was investigated in *rad7* or *rad16* disruption mutants and the results are shown in Figure 3. Repair of rDNA in these mutants is clearly strand-specific, the difference between both strands being significantly larger than in *RAD+* or *rad26* cells. The transcribed strand is repaired somewhat less completely than in *RAD+* cells or *rad26* mutants, while repair of the non-transcribed strand is significantly less complete and less efficient [statistical analysis of the raw data showed that the difference in repair between the transcribed and the non-transcribed strand in the *rad7* and *rad16* strains is significant (95% confidence limits)]. The strand-bias demonstrates that strand-specific repair of rDNA and therefore maybe transcription-coupled repair of RNA pol I transcribed genes is possible in yeast, in contrast to what has been reported for higher eukaryotic systems (24,25). Also the non-transcribed strand of rDNA can be repaired to a certain extent while *RAD7* or *RAD16* are absent, in contrast to other non-transcribed DNA sequences (6,18). Possibly, the non-nucleosomal structure of the active rDNA copies (29) allows repair of the non-transcribed strands in the absence of Rad7p and Rad16p. The percentage of the non-transcribed strand that is repaired in *rad7* and *rad16* mutants (~40%) is not in contradiction with the estimated amount of 'open' copies of rDNA (40–60%) under conditions used for repair experiments (29).

Repair of the transcribed strand of rDNA in *rad4* mutants

Repair of rDNA was analysed in a *rad4-4* mutant, which is defective in repair of the genome overall as well as for both strands of the *RPB2* gene (18). Quite unexpectedly, we found that in this mutant the transcribed strand of rDNA can still be repaired to ~50–60%, while the non-transcribed strand is not repaired at all (not shown). To make sure that this repair of the transcribed strand is not due to leakiness of the *rad4-4* allele, a *rad4* disruption mutant was constructed. As expected, this mutant is highly sensitive to UV and does not repair both strands of the *RPB2* gene (not shown). Also in this disruption mutant the transcribed strand of rDNA is repaired as shown in Figure 4. Since repair of rDNA in the *rad4* mutant is confined to 50–60% of the template strand, this may reflect transcription-coupled repair mediated by RNA pol I of the active copies during the repair period. Apparently the Rad4 protein that is generally assumed to be essential for NER, is dispensable for NER of the transcribed strand of rDNA *in vivo*.

DISCUSSION

We have studied repair of rDNA in *S.cerevisiae*. In this organism, rDNA is rather efficiently repaired (comparable to the non-transcribed strand of the *RPB2* gene) by NER, in contrast to the inefficient repair of rDNA in higher eukaryotes (24,25). We report that in yeast, strand-specific and therefore probably transcription-coupled repair of this class of RNA pol I transcribed genes exists, as is most clearly observed in *rad7*, *rad16* and especially in *rad4* mutants.

Only a small difference in repair of both rDNA strands is observed in *RAD+* cells, probably since many of the rDNA copies
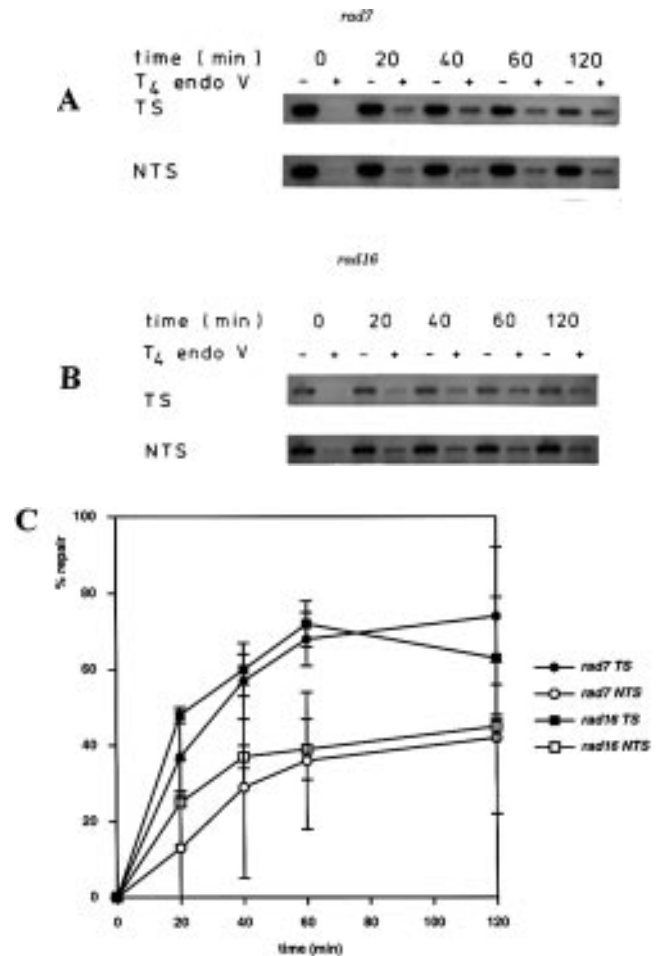
Figure 3. Repair of rDNA in *rad7* and *rad16* mutants. (A,B) Representative Southern blots showing the removal of dimers after various time points in (**A**) strain MGSC104 (*rad7*) and (**B**) strain W303236 (*rad16*). Time points after UV are indicated, samples mock-treated or treated with the dimer-specific enzyme T4endoV are denoted – and +, respectively. TS, transcribed strand; NTS, non-transcribed strand. Note that especially at the later time points the amount of signal in the treated (+) versus the mock-treated (–) lanes for the TS is higher (more repair) than for the NTS. (**C**) Repair of rDNA as a function of time after quantification of the Southern blots. Symbols indicate the mean of three experiments, bars indicate standard deviations.

are not active (29), thereby obscuring the more efficient repair of the transcribed strand of the active fraction. Transcription-coupled repair of this class of genes might also be less efficient than transcription-coupled repair of RNA pol II transcribed genes, because these processes may be mediated by different factors. The *RAD26* gene (14) is not involved in transcription-coupled repair of rDNA, whereas in human cells the Rad26p homolog CSB (13) does play a role in removal of dimers from rDNA (30). This may reflect the more general repair defect in Cockayne syndrome cells, that are disturbed in more than only transcription-coupled repair (12,30), while the yeast *rad26* mutant has a repair defect that seems to be confined to the transcribed strands of RNA pol II transcribed genes. Specific involvement of Rad26p in RNA pol II mediated transcription-coupled repair therefore most likely underlies the absence of an effect of the *rad26* mutation on rDNA repair. An alternative explanation comes from our recent observation that the effect of the *rad26* mutation is gene-specific and might depend on
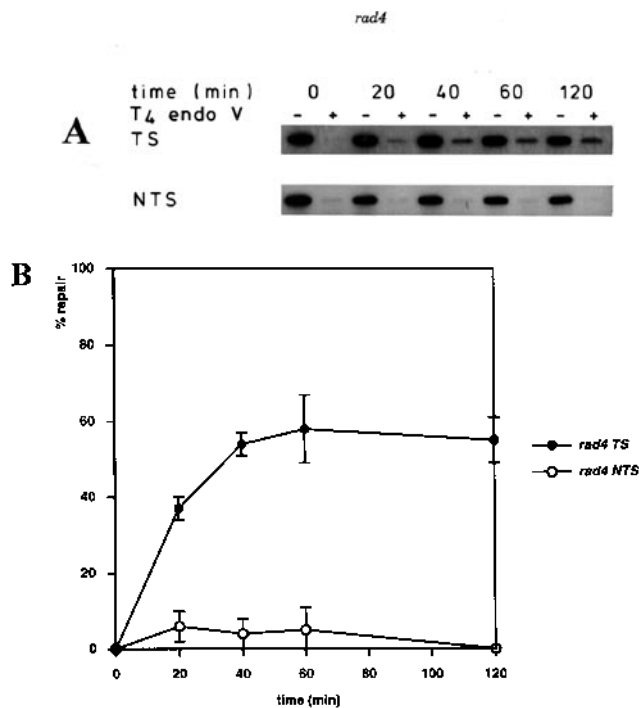
Figure 4. Repair of rDNA in a *rad4* mutant. (**A**) Southern blot showing the removal of dimers from rDNA in strain MGSC131 (*rad4*). Time points after UV are indicated, samples mock-treated or treated with the dimer-specific enzyme T4endoV are denoted by – and +, respectively. TS, transcribed strand; NTS, non-transcribed strand. (**B**) Repair as a function of time after quantification of the Southern blots. Symbols indicate the mean of three experiments, bars indicate standard deviations.

the level of transcription (6). Therefore the observation that rDNA repair is independent of *RAD26* might be due to the high level of transcription of the active rDNA copies (28,29).

The *rad7* and *rad16* mutants are completely deficient for removal of dimers from the non-transcribed strand of the *RPB2* and *GAL7* genes (6,18). In contrast, repair of non-transcribed strands of rDNA is only partly dependent on Rad7p and Rad16p. This could be due to the non-nucleosomal structure of the active rDNA genes, that might allow NER enzymes to exert their function on the non-transcribed strand of this DNA in the absence of Rad7p and Rad16p. Alternatively, some transcription by RNA pol III could come from the opposite direction (the 5S gene), causing transcription-coupled repair (mediated by RNA pol III) of the non-transcribed strand. However, we did not observe any transcripts derived from the non-transcribed strand on Northern blots with strand-specific probes, while transcripts from the transcribed strand were present in high amounts (data not shown). Moreover, it can be inferred from mutation spectra that targeting of repair enzymes to transcribed strands is probably not mediated by RNA pol III, since mutations in the *SUP4*-o gene that is transcribed by this polymerase are found mainly in the transcribed strand (38).

Most surprisingly, removal of dimers from rDNA occurs in a *rad4* mutant. This repair is confined to the transcribed strand. Dimer removal from the transcribed strand in the *rad4* mutant is conducted by *bona fide* NER, since in a *rad4rad14* mutant (Rad14p is essential for NER, 32) this rDNA repair is completely abrogated (data not shown). As expected, this repair is indepen-

dent of Rad7p and Rad26p, since in *rad4rad7* and *rad4rad26* double mutants the transcribed strand of rDNA is still repaired (data not shown). Until now Rad4p was considered essential for NER (both transcription-coupled repair and global genome repair), since no dimers are removed from both strands of an active gene as well as from the genome overall in a *rad4* mutant (18). The analysis of protein complexes suggested that Rad4p is part of the repairosome (19,39), but to date the molecular function of this protein has not been revealed. The Rad4 protein is essential for NER in a reconstituted yeast repair system (40) and cell-free extracts of *rad4* mutants are defective for NER (19). Apparently in the special case of RNA pol I transcribed DNA, NER can operate in the absence of this protein. Isogenic *rad4*, *rad14* and *rad4rad14* mutants are equally UV-sensitive (not shown), and therefore the Rad4p-independent repair of the transcribed strand of rDNA may be fortuitous rather than reflecting a biological important function. This phenomenon is not conserved in higher eukaryotes since an XP-C mutant, the proposed human counterpart of a *rad4* mutant on the basis of limited sequence homology between the yeast Rad4 and the human XPC proteins (41), is completely defective in repair of both rDNA strands (30). Strikingly, yeast *rad4* mutants and human XP-C cells *in vivo* seem to have reciprocal phenotypes regarding repair of RNA pol I transcribed versus RNA pol II transcribed genes: XP-C cells are only capable of repair of RNA pol II transcribed strands (16) while *rad4* mutants can only repair template strands that are transcribed by RNA pol I. Both yeast Rad4 and human XPC proteins seem to be essential for NER, as both are absolutely required in the respective reconstituted NER systems (40,42–44). Apparently, in the cell, NER can take place while these proteins are absent, but only at sites where transcription takes place, possibly by a—as yet unknown—component of the transcription machinery. The function of Rad4p in yeast is then supplied by RNA pol I transcription, while in human cells RNA pol II transcription overcomes the need for the NER-function of XPC.

The molecular function of Rad4p and XPC is still unknown, but clearly these proteins are not essential for the incision event of NER. The involvement of transcription to bypass the need for both yeast Rad4p and human XPC for NER *in vivo* makes it tempting to speculate that a function of these proteins might be during damage recognition, since Rad4p/XPC-independent NER seems to occur only at the site of transcription. Alternatively these proteins might have architectural roles, e.g. in building of a repairosome (39), or other important accessory functions during NER.

The findings described here reveal for the first time some similarity between the preferential repair phenotypes of the yeast *rad4* and human XP-C mutants (18). Since the interaction between Rad4p or XPC with the yeast and human Rad23 proteins, respectively, is also conserved (40,45), the hypothesis that Rad4p and XPC are indeed homologs (41), is supported. Both proteins are essential for the NER process but can in specific cases be replaced by components of transcription machineries. Yeast *rad7* and *rad16* mutants have a phenotype very similar to human XP-C mutants (18). This may be partly coincidental. In contrast to the essential function of XPC in reconstituted NER systems (42,43), the Rad7 and Rad16 proteins seem dispensable for NER in a highly purified system (40). Therefore Rad7p and Rad16p presumably have a specific function in repair of non-transcribed DNA (6), whereas XPC seems to have a more general function in NER. Identification of putative mammalian homologs of *RAD7* and

RAD16, that seem to be the real effectors of non-transcribed DNA repair, will therefore be highly interesting.

Our studies reveal some differences in repair of rDNA in yeast versus mammalian cells. These may be differences in efficiency rather than mechanistic differences since NER to date has been found to be highly conserved in eukaryotic species (1). Alternatively, a mechanistic divergence between the NER systems in yeast versus higher eukaryotes may be revealed. Summarized, we report that rDNA is repaired by NER in yeast, this repair can be strand-specific and probably transcription-coupled as revealed in specific NER mutants and finally our results demonstrate that Rad4p is not essential for NER in the special case of the RNA pol I transcribed strand of rDNA.

ACKNOWLEDGEMENTS

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