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Transitions in the coupling of transcription and nucleotide excision repair within RNA polymerase II-transcribed genes of *Saccharomyces cerevisiae*

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ABSTRACT **The molecular mechanism of transcription-coupled nucleotide excision repair in eukaryotes is poorly understood. The identification of the dual role of basal transcription factor TFIIH in DNA repair and transcription provided a plausible link between both processes. However, TFIIH is not part of the elongating transcription complex, suggesting that additional components are required to recruit TFIIH when RNA polymerase II (RNAPII) stalls at the site of DNA damage. Previously, we have shown that the yeast Rad26 protein is involved in transcription-coupled DNA repair. This paper describes the differential contribution of the Rad26 protein to efficient removal of UV-induced cyclobutane pyrimidine dimers (CPDs) from transcribed DNA. Two distinct regions within the transcribed strand of RNAPII-transcribed genes are identified that differ in their requirement for the *RAD26* gene product. Using high-resolution repair analysis, we determined the *in vivo* repair kinetics of cyclobutane pyrimidine dimers positioned around the transcription initiation site of RNAPII-transcribed genes *RPB2* and *URA3*. Although transcription-coupled repair is severely reduced in *rad26* mutants, lesions positioned in a small region immediately downstream of transcription initiation are efficiently removed in the absence of Rad26. The observed transition in repair characteristics is abrupt and in excellent agreement with the region where TFIIH dissociates from RNAPII *in vitro*, strongly suggesting an inverse correlation between TFIIH association and Rad26 requirement. These data suggest that a transcription repair coupling factor (Rad26/CSB) is required for efficient repair only during the elongating stages of RNAPII transcription.**

DNA lesions that block DNA or RNA polymerase (RNAP) can be lethal to cells by interfering with replication or by depriving cells from the synthesis of essential proteins. The latter impediment might be circumvented by targeting repair proteins toward actively transcribed DNA regions, thereby enhancing the rate of damage removal from important DNA sequences. Indeed, it has been found that UV-induced lesions are repaired preferentially from transcribed DNA sequences compared with nontranscribed regions in *Escherichia coli*, yeast, and mammals (1–3). In the prokaryote *E. coli*, the Mfd protein has been identified as the coupling factor between DNA repair and transcription (4). The Mfd protein's capability to displace RNAP stalled at a lesion, together with the ability to bind the UvrA protein (5), component of the *E. coli* repair machinery, suggest that the Mfd protein functions in recruiting repair proteins specifically to lesions in transcribed DNA. Mutating the *mfd* gene leads to a reduced rate in removal of UV photoproducts from the transcribed strand of the *lacI* gene (6, 7). Furthermore, spectra of UV-induced mutations in wild-type and *mfd* strains revealed a pronounced mutational shift from dipyrimidines in the nontranscribed strand (wild type) to dipyrimidines in the transcribed strand (mfd^-) (8).

In eukaryotes, the molecular basis for transcription-coupled DNA repair is not known yet. The multiprotein complex TFIIH has been identified to participate in both nucleotide excision repair (NER) and in the initiation of transcription of RNA polymerase II (RNAPII)-transcribed genes (9–11). The obligatory loading of TFIIH onto promoter sites during transcription initiation provides an obvious scenario for a direct coupling of RNAPII transcription and NER, if TFIIH remains associated during transcription elongation. However, using an *in vitro* competition assay, Zawel *et al.* (12) demonstrated that although TFIIH is indeed associated with the RNAPII complex during the first steps of nascent mRNA synthesis, this factor is released from the transcription machinery between positions +30 and +68, and no TFIIH was detected in isolated stalled elongation complexes (12, 13). This suggests that additional components are required to re-recruit TFIIH toward RNAPII stalled at the site of DNA damage. Likely candidates for this function are the Cockayne syndrome (CS) group A and B gene products (14, 15). Cells from patients suffering from CS fail to repair transcribed DNA preferentially (16). This lack of transcription-coupled repair (TCR) is accompanied by an increase in UV sensitivity. Although the observed defects resulting from a mutation in these genes resemble the mfd^- phenotype in *E. coli*, it is unknown whether these proteins act identically to the Mfd protein at the molecular level.

Recently, the yeast homologs of the *CSA* and *CSB* genes, designated *RAD28* and *RAD26*, respectively, have been cloned (17, 18). Although gene specific repair analyses have revealed that Rad28 is not neccesary for efficient repair of transcribed DNA (17), the influence of Rad26 in TCR was noticable when the yeast *RPB2* locus was examined (18). For this locus, the repair rate of the transcribed strand was reduced to almost the level of the nontranscribed strand in *rad26*-disrupted cells.

In this study, we have analyzed repair around the transcription initiation site of different RNAPII-transcribed genes at nucleotide resolution in a *rad26* and a *rad28* genetic background. This high-resolution repair mapping reveals that the influence of Rad26 on efficient removal of cyclobutane pyrimidine dimers (CPDs) is nonuniform throughout the transcribed strand of RNAPII-transcribed genes. In *rad26* mutants, a transition from fast to slow repair in the transcribed strand coincides with the previously reported region where TFIIH dissociates from the RNAPII complex *in vitro* (11). These data

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Abbreviations: CPD, cyclobutane pyrimidine dimer; RNAP, RNA polymerase; RNAPII, RNA polymerase II; NER, nucleotide excision repair; CS, Cockayne syndrome; TCR, transcription-coupled repair; TRCF, transcription-repair coupling factor.

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suggest that a transcription repair coupling factor (Rad26/CSB) is not necessary in regions where TFIIH is still associated with the transcription machinery.

MATERIALS AND METHODS

Strains. The *Saccharomyces cerevisiae* NER-proficient (RAD^+) strain used for this study is W303–1B, genotype: *MATα ho can1–100 ade2–1 trp1–1 leu2–3,112 his3–11,15 ura3–1*. The *rad7*, *rad26*, *rad7rad26*, *rad28*, and *rad14* disruptions were introduced into this background by one-step gene replacement. For repair analysis on the *URA3* locus, strain W303–1B was rendered *URA3* by transformation of a linear PCR fragment containing the complete locus. Uracil prototrophs were checked by Sanger sequencing for proper recombination at its chromosomal position. Subsequently, disruption mutants were introduced as described above. The NER deficient *rad3–2* mutation was analyzed in *S. cerevisiae* strain YR3–3. All strains were kept on selective yeast nitrogen base medium (0.67% yeast nitrogen base/2% glucose/2% Bacto agar) supplemented with the appropriate markers. Cells were grown in complete medium (yeast extract peptone: 1% yeast extract/2% Bacto Peptone/2% glucose) at 28°C under vigorous shaking.

CPD Analysis. Cells diluted in chilled PBS 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 before DNA isolation. DNA samples were purified on CsCl gradients (19). DNA samples (25 μg) were digested with appropriate endonucleases and precipitated, and *RPB2* or *URA3* fragments were isolated and end-labeled as described previously (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 cpms were loaded on 6% denaturing acrylamide gels alongside Maxam–Gilbert sequencing reactions to identify the CPD positions. After drying, autoradiograms were prepared from the gels.

Quantification of Repair Rates. Serial dilutions of Maxam–Gilbert sequencing reactions were used to determine the linear range of the Kodak X-Omat-AR scientific imaging films used. From each experiment, multiple autoradiograms were obtained with different exposure times to allow signal determination within the linear range these imaging films for each individual CPD. Autoradiograms were scanned using an LKB Ultrascan XL densitometer (Pharmacia) and analyzed using ImageMaster software (Pharmacia). Background levels were subtracted, and gel-band intensities were corrected for loading variations. Quantification data were obtained from experiments carried out in triplicate. 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.

Maxam–Gilbert Sequencing Reactions. Maxam–Gilbert sequencing ladders were obtained according to standard procedures (21) using PCR fragments identical to the chromosomal DNA fragment that is analyzed. After chemical modification and piperidine cleavage the fragments were ^{32}P-labeled identically to the studied chromosomal fragments.

RESULTS

TCR in *RPB2* Promoter Proximal Region Is Rad26 Independent. Two subpathways of NER have been postulated, i.e., TCR, responsible for fast repair of the transcribed strand, and global genome repair, required for repair of lesions that are not repaired by the TCR pathway (22, 23). Gene-specific repair analysis has shown that the yeast Rad7 and Rad16 proteins, which are essential for repair of nontranscribed DNA, also can contribute to repair of the transcribed strand, but this feature is only observed when TCR is hampered. To account for the contribution of the Rad7/Rad16 dependent pathway in our analysis, the role of *RAD26* in coupling repair to transcription was analyzed in both *RAD7* and isogenic *rad7*Δ genetic background.

Cells were UV-irradiated at a dose of 70 J/m^2. After incubation to allow repair, DNA was isolated and specific DNA fragments of interest were isolated, labeled, and incised 5′ of the dimer with T4endoV. Repair of CPDs was analyzed by separation of the T4endoV-cleaved DNA fragments on denaturing PAGE.

Fast repair of the *RPB2*-transcribed strand starts immediately downstream of the transcription initiation site and is not dependent on global genome repair proteins Rad7 and Rad16 (Fig. 1 *a* and *b* and ref. 20). Repair of CPDs positioned in the transcribed strand is severely reduced in both *rad26*Δ (data not shown) and in *rad26*Δ*rad7*Δ cells (Fig. 1*c*), indicating a function for Rad26p in the efficient repair of lesions from transcribed DNA, in agreement with previous findings (18, 23). However, considerable repair is observed for lesions positioned directly downstream of the transcription initiation site both in *rad26* single mutants (data not shown) and in *rad26rad7* double-mutants (Fig. 1*c*). After 20 min, 60–80% of CPDs at these positions (arrows in Fig. 1*c*) were repaired, whereas lesions more downstream are not repaired at all within this timeframe. This *RAD26*-independent TCR shows a clear and abrupt transition from fast to slow repair approximately 50 bases downstream of the start of transcription.

Repair Analysis of the *URA3* Locus. To determine whether the observed repair characteristics for the *RPB2* locus hold true for other RNAPII-transcribed genes, we have analyzed the *URA3* locus as a second repair target. A schematic representation of repair analysis in the repair-proficient strain as well as the *rad7*Δ, *rad26*Δ, and *rad7*Δ*rad26*Δ double-mutants is depicted in Fig. 2.

First, the *URA3* locus allows us to map the start of TCR with respect to transcription initiation in detail because photoproducts were detected at position +2 in the *URA3* transcribed strand. In wild-type RAD^+ yeast cells, fast repair of the transcribed strand is observed starting at this position and for dipyrimidine sites more downstream (Fig. 2*a*). This fast repair is not dependent on proteins Rad7 (Fig. 2*b*) and Rad16 (data not shown). In contrast, dinucleotides at position −11 and more upstream are repaired with moderate, heterogeneous rates in RAD^+ cells and are totally dependent on Rad7 and Rad16 because repair of these lesions is completely abolished in *rad7* or *rad16* mutants. These data indicate that fast repair of the transcribed strand starts immediately downstream of transcription initiation, implying a key function for RNAPII in efficient recognition of UV-induced dimers. The observation that individual dinucleotide positions in the transcribed strand are repaired with uniform rates in the *RPB2* gene as well as in the *URA3* gene strengthens this hypothesis. Furthermore, CPDs in both transcribed strands are repaired equally efficiently ($t_{½}$ values of 8–9 min).

Fig. 2*c* clearly shows the reduction in repair efficiency of the transcribed strand due to a mutation in *rad26*. The contribution of the Rad26 protein in TCR is best illustrated in a *rad7* or *rad16* genetic background (23). As for the *RPB2* locus, a residual efficient TCR is observed in approximately the first 40 bp of the transcribed strand in *rad26* (Fig. 2*c*) and *rad26rad7* genetic background (Figs. 2*d* and 3*a*). Repair of the nontranscribed strand was examined to determine whether this residual repair was indeed strand specific. Previously, we have reported that repair of the *RPB2* nontranscribed strand is

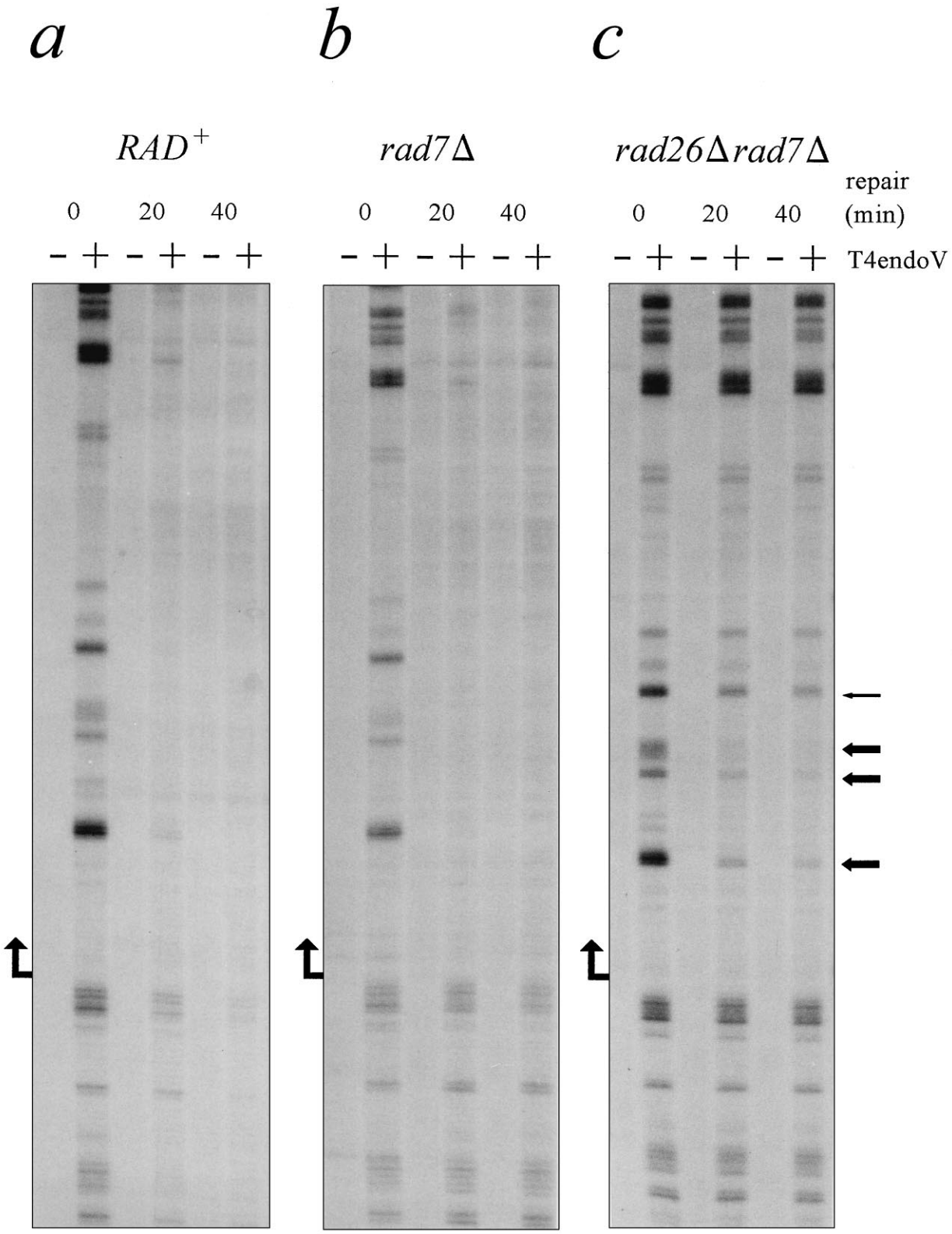

FIG. 1. Repair of UV-induced CPDs at single nucleotide resolution along the transcription initiation site of the *S. cerevisiae RPB2* locus. Data are for the template DNA strand, nucleotides −40 to +200 with respect to the start site of transcription. (*a*) Wild-type RAD$^+$, (*b*) isogenic *rad7*Δ, and (*c*) *rad7*Δ*rad26*Δ cells were irradiated with 70 J/m^2, and repair was allowed for 0, 20, and 40 min. The large arrow indicates the major transcription initiation site and the direction of transcription. Samples mock-treated or treated with the dimer-specific enzyme T4endoV are denoted − and +, respectively.

completely dependent on the Rad7 or Rad16 proteins, including lesions positioned within 50 bp downstream of transcription initiation (20). Also for *URA3*, no repair is observed for lesions in the nontranscribed strand in both *rad7* (data not shown) and *rad7rad26* cells (Fig. 3*b*). Together these data indicate that for both loci *RAD26*-independent repair is transcription-coupled and confined to a small defined region immediately downstream of transcription initiation.

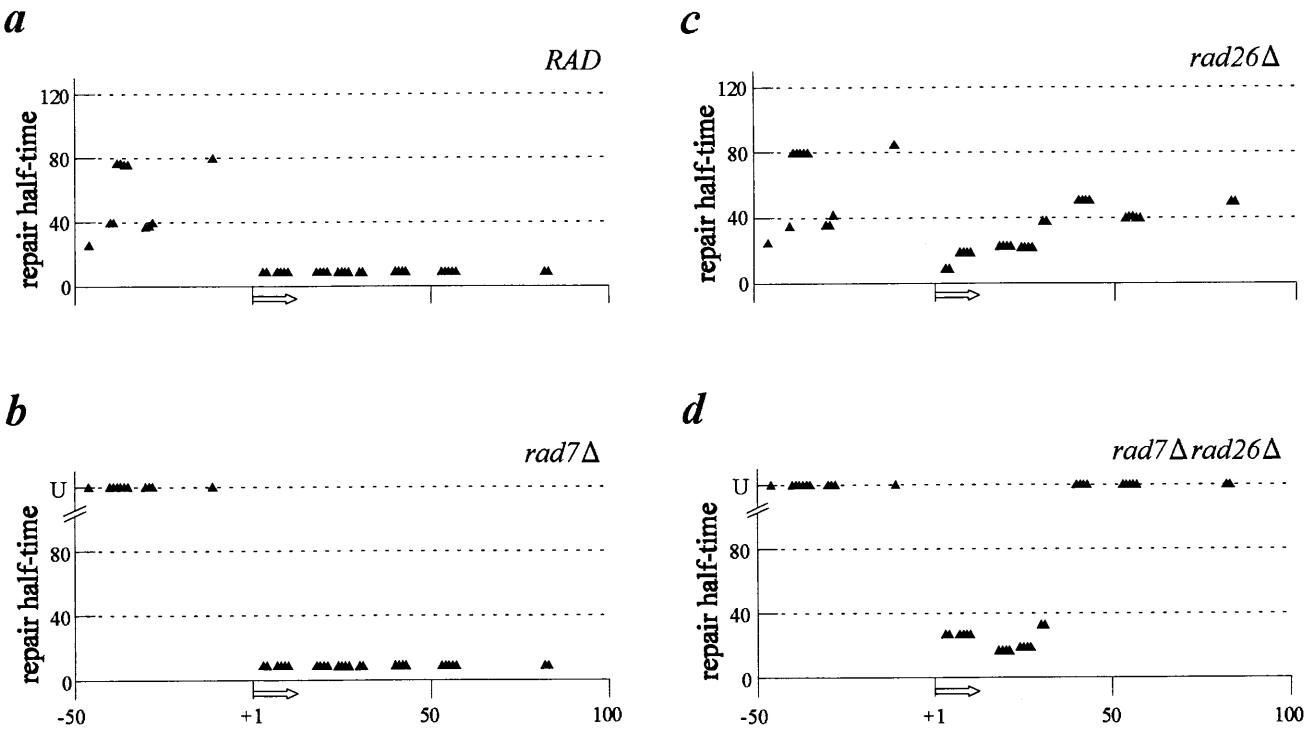

FIG. 2. Graphic representation of quantified repair rates for the template strand of the *URA3* locus from position −50 to +100 in (*a*) repair proficient *RAD*$^+$ background and in isogenic (*b*) *rad7*Δ, (*c*) *rad26*Δ, and (*d*) *rad7*Δ*rad26*Δ mutant strains. Repair half-time values, determined as the time at which 50% of the initial CPD signal was removed, were calculated for each individual CPD position and depicted above its corresponding dipyrimidine position. Repair $t_{1/2}$ = U indicates that CPDs were unrepaired after 2 hr of incubation.

Rad28 Is Not Involved in TCR of UV-Induced CPDs. Previously, using gene-specific repair analysis it was found that a deletion of the yeast *CSA* homolog, *RAD28* does not affect TCR (17). However, because in those experiments repair rates are avaraged over kilobase-length DNA fragments, a subtle influence on TCR could not be excluded, especially because a small, but significantly enhanced, frequency of UV-induced mutations was observed in *rad28* mutants. Repair rates for CPDs in the transcribed strand of the *URA3* (Fig. 4*a*) and *RPB2* (data not shown) are identical in *rad28*Δ compared with the isogenic background strain W303–1B, excluding a role of Rad28 in TCR of the transcribed strand of RNAPII transcribed genes.

***RAD26*-Independent TCR Is NER.** As a control, we analyzed dimer removal in a *rad14* genetic background. Rad14 is one of the core NER components essential for a reconstituted *in vitro* NER assay (24). Dimer removal was absolutely absent in these cells (Fig. 4*b*), indicating that the RAD26-independent TCR in the promoter proximal region downstream of transcription initiation still requires this component and therefore can be qualified as NER. Additionally, to investigate the requirement for TFIIH, we analyzed repair in a *rad3–2* genetic background. These cells carry a mutation in one of the subunits of TFIIH, *RAD3*, which specifically abrogrates the NER activity of TFIIH without disturbing its function in RNAPII transcription (25). In this mutant, no repair was observed at all (Fig. 4*c*), in both the *URA3* and *RPB2* gene including the regions immediately downstream of transcription initiation. Together, these data indicate that *RAD26*-independent TCR is due to NER and requires the repair activity of TFIIH.

DISCUSSION

The molecular basis for the TCR phenomenon is thought to originate in an efficient recruitment of repair proteins toward RNAP stalled at the site of DNA damage. In support of this hypothesis, lesions that block RNAP are a substrate for TCR (26) whereas lesions that do not halt transcription are repaired with rates comparable to lesions situated in nontranscribed DNA sequences (27). Other observations that corroborate the proposed antenna function for the RNAP in NER are: (*i*) the requirement for ongoing transcription (28, 29); (*ii*) the onset of efficient repair directly downstream of transcription initiation, and (*iii*) the uniform rate at which differently positioned dimers in transcribed DNA are repaired (ref. 20, this study). The latter suggests an identical rate-limiting recognition mechanism. In *E. coli*, a specific factor that targets repair proteins toward stalled RNAPs has been identified (5). This protein, designated TRCF for transcription-repair coupling factor, is able to displace the polymerase molecule and delivers the NER machinery directly to the damaged DNA strand via its interaction with UvrA.

The Rad26 protein is a likely candidate to function as a TRCF in yeast. This gene, which was cloned by homology with the human CSB gene (18), has been implicated in the removal of lesions specifically from the transcribed strand as was shown for its human counterpart (16). Both the Rad26 and CSB proteins have been purified to homogeneity and shown to contain DNA-dependent ATPase activity (30, 31) as expected from the presence of helicase motifs belonging to the SWI/SNF family of DNA-dependent ATPases (32). The notion that some members of this gene family are involved in remodeling DNA-protein interactions supports the proposed role for a TRCF to modulate contacts of the elongating transcription complex with the DNA at sites of base damage (33, 34). However, in contrast to the *E. coli* TRCF, the CSB protein is not able to displace the RNAPII from the DNA *in vitro* (31). In agreement with the *E. coli* model, CSB can interact with components of the human NER pathway TFIIH and XPA (31, 35); however, these interactions have not been reported for the yeast Rad26 protein (30).

Our results indicate that Rad26 is not required for efficient TCR of lesions positioned within approximately 50 bases from the transcription initiation site in the transcribed strand. The

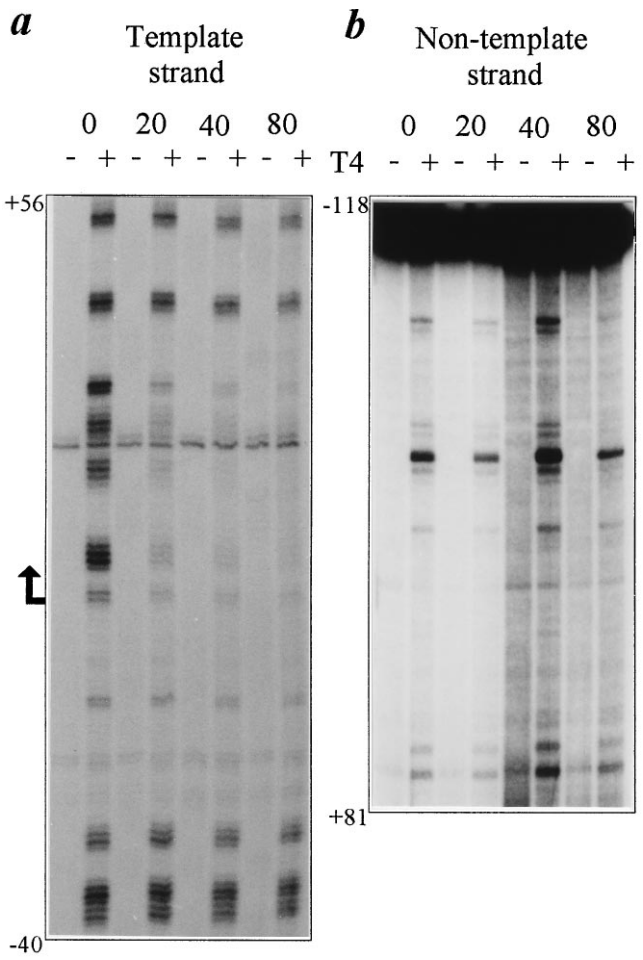

FIG. 3. Repair of UV-induced CPDs at single nucleotide resolution along the template (*a*) and the nontemplate strand (*b*) of the *S. cerevisiae URA3* locus in a *rad26rad7* strain. Cells were irradiated with 70 J/m^2, and repair was allowed for 0, 20, 40, and 80 min. The large arrow indicates the major transcription initiation site and the direction of transcription. Samples mock-treated or treated with the dimer-specific enzyme T4endoV are denoted − and +, respectively. More DNA was present in *b*. t = 40.

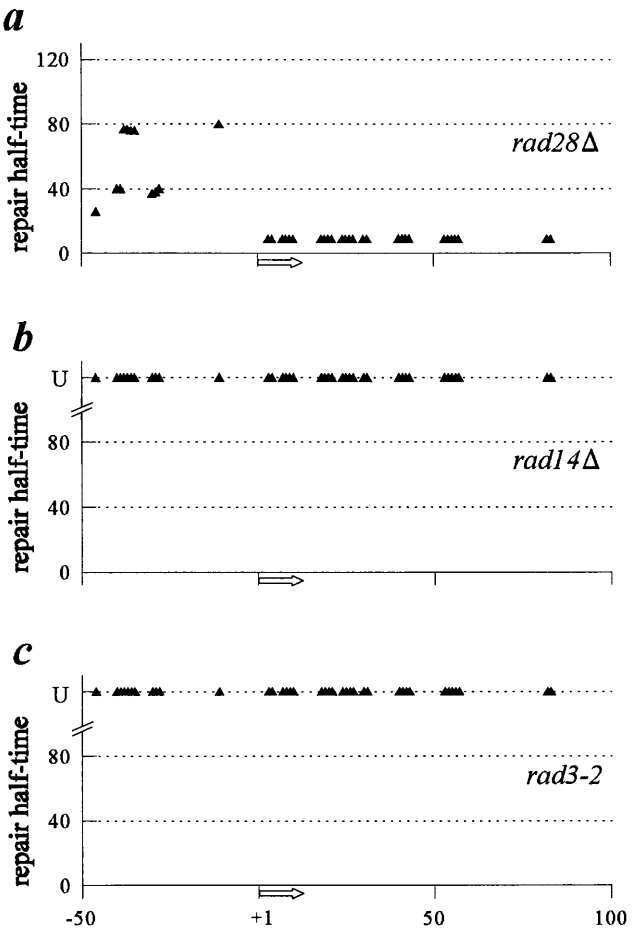

FIG. 4. Graphic representation of NER for the *URA3* transcribed strand in (*a*) *rad28*, (*b*) *rad14*, and (*c*) *rad3–2* genetic background. Data for the *rad28* mutant were obtained with time samples of 0, 5, 10, 15, and 20 min of incubation after irradiation whereas 0, 20-, 40-, 60-, and 120-min time samples were used for repair analysis of the *rad14* and *rad3–2* mutants. Repair half-time values, determined as the time at which 50% of the initial CPD signal was removed were calculated for each individual CPD position and depicted above its corresponding dipyrimidine position. Repair $t_{1/2}$ = U indicates that CPDs were unrepaired after 2 hr of incubation.

position of the abrupt change in repair characteristics is in excellent agreement with the region where TFIIH dissociates from RNAPII *in vitro*, strongly suggesting an inverse correlation between TFIIH association and Rad26 requirement. Whether TFIIH dissociates from the RNAPII complex *in vivo* is not clear. Several studies indicate a transition between transcription initiation and transcription elongation, which is influenced by the ability of TFIIH to phosphorylate the highly conserved carboxyl-terminal repeat domain (CTD) of RNAPII (36–38). Whereas only the nonphosphorylated form of RNAPII can enter a preinitiation complex, the phosphorylated form is responsible for RNA synthesis (39, 40). Although the process of promoter clearance is not yet fully understood, it has been suggested that CTD phosphorylation triggers the release of RNAPII paused close to the start site. In support of this model, distinct activated and nonactivated RNAPII complexes have been found (41, 42). RNAPII complexes are engaged at the 5′ end before activation and depend on CTD phosphorylation to travel to the 3′ end of a gene. If indeed such a transition from initiation to elongation is accompanied by the release of TFIIH from the transcription machinery as was seen *in vitro*, this would provide an obvious explanation for our results. The association of TFIIH with the transcription machinery during the first steps of nascent mRNA synthesis obviates a TRCF in this region and a deficiency in TCR due to a defective coupling factor will only be observed downstream of the position where TFIIH is released. In this hypothesis, Rad26 functions, analogous to the *E. coli* TRCF, as coupling factor to recruit TFIIH, alone or associated with other repair proteins in a so-called repairosome (43), toward RNAPII complexes stalled at the site of the damage.

Although the TRCF model could explain the observed repair transition, other explanations for this phenomenon are not excluded. Because the transition of fast to slow repair is observed in the region where the transition from initiation to elongation might occur *in vivo*, our data are also compatible with a role of Rad26 in this switch, leading to a TCR deficiency downstream of this point as a consequence of an alteration in the transcription process in the *rad26* mutant. An impediment in the onset of efficient transcription elongation will lead to an inability to detect lesions by the RNAPII. Therefore, a defect in TCR might not neccesarily be due to a defective coupling or coupling factor but result from a deficiency or reduction in transcription elongation. Two observations hint at a direct role for the Rad26 human homolog CSB in the transcription process. Cells of CSB patients fail to recover mRNA synthesis after treatment with *N*-acetoxy-2-acetylaminofluorene (44). This mutagen results primarily in dG-C8-AF adducts that are not repaired strand-specifically in normal human fibroblasts.

Repair of these lesions was unaffected in CS cells, ruling out defective TCR as the cause of the impaired recovery of mRNA synthesis. Another study involved the ubiquitination status of RNAPII (45). Fibroblast cells exposed to UV-irradiation or cisplatin treatment exhibit ubiquitination of the large subunit of Pol II (Pol II LS). This phenomenon is dependent on the CSA and CSB gene products, suggesting a direct link between the transcription mechanism and CS.

Although a transcription hypothesis might explain the complex clinical phenotypes of CS patients, which are hard to reconcile with a defect in DNA repair only, direct evidence for a function of Rad26 or CSB in general transcription has not been reported despite extensive research. A defect in general transcription is not supported by the notion that mutations in *rad26* do not result in a pronounced phenotype, apart from a TCR defect, and CS does permit apparent normal embryogenesis and tolerates postnatal growth albeit with limited vigor.

We therefore favor the TRCF model, especially because it does not exclude an alteration in transcription, considering the endogenous barriers of this process, e.g., spontaneous base damage or natural pause sites. A TRCF function in alleviating natural occurring pause sites, either by RNAPII-DNA contact modification or via recruitment of TFIIH to facilitate resumption of mRNA transcription could result in a subtle transcription defect when Rad26 or CSB is mutated.

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