Evidence that Moderate Eviction of Spt5 and Promotion of Error-Free Transcriptional Bypass by Rad26 Facilitates Transcription Coupled Nucleotide Excision Repair

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Abstract

Transcription coupled repair (TC-NER) is a subpathway of nucleotide excision repair triggered by stalling of RNA polymerase at DNA lesions. It has been suspected that transcriptional misincorporations of certain nucleotides opposite lesions that result in irreversible transcription stalling might be important for TC-NER. However, the spectra of nucleotide misincorporations opposite UV photoproducts and how they are implicated in transcriptional stalling and TC-NER in the cell remain unknown. Rad26, a low abundant yeast protein, and its human homolog CSB have been proposed to facilitate TC-NER in part by positioning and stabilizing stalling of RNA polymerase II (RNAPII) at DNA lesions. Here, we found that substantial AMPs but no other nucleotides are transcriptionally misincorporated and extended opposite UV photoproducts and adjacent bases in Saccharomyces cerevisiae. Rad26 does not significantly affect either the misincorporation or extension of AMPs. At normally low or moderately increased levels, Rad26 promotes error-free transcriptional bypass and TC-NER of UV photoproducts. However, Rad26 completely loses these functions when it is overexpressed to ~1/3 the level of RNAPII molecules. Also, Rad26 does not directly displace RNAPII but constitutively evicts Spt5, a key transcription elongation factor and TC-NER repressor, from the chromatin. Our results indicate that transcriptional nucleotide misincorporation is not implicated in TC-NER, and moderate eviction of Spt5 and promotion of error-free transcriptional bypass of DNA lesions by Rad26 facilitates TC-NER.

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Introduction

Nucleotide excision repair (NER) is a multistep process that removes bulky and/or helix-distorting DNA lesions, such as ultraviolet (UV)-induced dipyrimidine photoproducts, cyclobutane pyrimidine dimers (CPDs), and 6–4-photoproducts [1,2]. Transcription coupled NER (TC-NER) is a subpathway of NER that is dedicated to rapid removal of lesions in the transcribed strand of active genes [3–9]. Global genomic NER (GG-NER) is the other subpathway of NER that removes lesions throughout the genome [10]. The two NER subpathways differ only in the early lesion recognition steps but share the same NER factors in the later lesion verification, dual incision and excision, repair synthesis, and ligation steps [1,2].

In eukaryotic cell, TC-NER is believed to be triggered by stalling RNA polymerase II (RNAPII) [5,8,9]. UV photoproducts inhibit transcription elongation, and recovery of RNA synthesis occurs only after the lesions are repaired [11]. Bulky DNA lesions, such as CPDs and cisplatin adducts, have also been shown to efficiently stall RNAPII in vitro [12–14]. On a template containing a TT CPD, purified RNAPII incorporates AMP (A) opposite the 3’ T of the TT CPD [15–17]. However, primarily UMP (U) and, to a much lesser extent, A can be incorporated...
opposite the 5’ T of the TT CPD. Incorporation of A opposite the 5’-T enables transcriptional bypass of the CPD, whereas misincorporation of U at this site results in irreversible stalling of RNAPII. It has therefore been proposed that misincorporation of nucleotide(s) that leads to transcription stalling may be important for TC-NER. To date, however, the spectra of nucleotide misincorporations opposite UV photoproducts and how they are implicated in transcriptional stalling and TC-NER in the cell remain unknown.

It has been well known that Rad26, a low-abundant DNA-dependent ATPase that is homologous to the human Cockayne syndrome B (CSB), plays an important role in TC-NER in Saccharomyces cerevisiae [18]. However, Rad26 is completely or partially dispensable for TC-NER in yeast cells lacking Rpb4 [19], Spt4 [20], certain domains of Spt5 (which is essential for cell viability) [21,22], and any subunit of the 5-subunit RNAPII associated factor complex (PAF0) [23]. Rpb4 is a non-essential subunit of RNAPII and forms a subcomplex with Rpb7, an essential subunit of RNAPII [24]. Spt4 and Spt5 are transcription elongation factors that form a complex [25]. It appears that the coordinated interactions of these factors with RNAPII hold the complex in a closed conformation that is highly competent for transcription elongation but intrinsically repressive to TC-NER [5]. Rad26 appears to facilitate TC-NER by antagonizing the repression. However, the underlying mechanism for the antagonization remains not well understood.

RNAPII has recently been shown to be dissociated from the chromatin after the step of dual incision of a transcription-blocking lesion [26]. However, how RNAPII behaves before the dual incision step that allows the trapped lesion to be recognized and repaired by the NER machinery has been enigmatic, although multiple models have been proposed [5,8,9]. Ubiquitination and degradation of Rpb1, the largest subunit of RNAPII [27,28], and TFIIS-facilitated backtracking of RNAPII [29,30] have been shown to play no role in TC-NER. In vitro studies have shown that both the yeast Rad26 [31] and human CSB [32] promote transcription elongation, facilitate transcriptional bypass of intrinsic pausing/arrest sequences, and resolve backtracking of RNAPII. However, neither Rad26 nor CSB is able to promote transcriptional bypass of a TT CPD in vitro. It has therefore been proposed that Rad26 and CSB might facilitate TC-NER in part by positioning and stabilizing stalling of RNAPII at lesion sites [31–33]. To date, however, no in vivo evidence has been available to support this proposition.

To gain insights into the TC-NER mechanisms, we performed high-resolution mappings of transcription across and TC-NER of UV photoproducts in yeast cells with well-controlled combinations of gene deletions and/or expressions. Here, we present evidence that transcriptional nucleotide misincorporation is not implicated in TC-NER, and moderate eviction of Spt5 and promotion of error-free transcriptional bypass of DNA lesions by Rad26 facilitates TC-NER.

Results

The ATPase activity of Rad26 is essential for its TC-NER function

Rad7 is essential for GG-NER but plays no role in TC-NER [2]. To specifically test the role of the ATPase activity of Rad26 in TC-NER, we transformed rad7Δ rad26Δ cells (Supplemental Table S1) with single-copy plasmids expressing the 3× FLAG-tagged wild-type (pRAD26) and helicase motifs la (pRAD26Hla), VI (pRAD26HVI) and Ia and VI (pRAD26Hla-VI) mutant Rad26 under the native RAD26 gene promoter (Fig. 1a and Supplemental Table S2). The expression levels of the wild-type and helicase motif mutant Rad26 proteins were similar (Fig. 1b). To compare the level of the plasmid-expressed Rad26 to those of the endogenous Rad26 and RNAPII, we tagged the genomic RAD26 and RPB2 (encoding the second largest subunit of RNAPII) genes with 3× FLAG. It appeared that the level of the plasmid-expressed Rad26 was ~3/4 of that of the endogenously expressed Rad26 and ~1/350 of that of the Rpb2 in the cell (Fig. 1c). It has been previously estimated that about 90 and 30,000 molecules of Rad26 and RNAPII, respectively, are present in a yeast cell [34,35]. Our estimation of the ratio of Rad26 to RNAPII appears to be within the range of previous estimations.

The ATPase activities of the immunoprecipitated helicase motif mutant Rad26 were similar to that of mock immunoprecipitated samples (from rad7Δ rad26Δ cells containing the empty vector) (Fig. 1d), indicating that the mutant Rad26 proteins have no ATPase activity. The UV resistance of rad7Δ rad26Δ cells can be restored by pRAD26, but not by pRAD26Hla, pRAD26HVI, or pRAD26Hla-VI (Fig. 1e), indicating that the ATPase activity of Rad26 is essential for its TC-NER function. To confirm this notion, we directly measured TC-NER of CPDs. As expected, in rad7Δ rad26Δ cells containing an empty vector, repair of CPDs was defective in the transcribed strand of the RPB2 gene, except for a short region of ~50 nucleotides (nt) immediately downstream of the transcription start site (from +1 to +50) (Fig. 2a). TC-NER in this short region has been known to be independent of Rad26 [19]. TC-NER was not restored upon complementation of the cells with pRAD26Hla, but was fully restored upon complementation with pRAD26 (Figs. 2a–c and 3a), indicating that the ATPase activity of Rad26 is indeed essential for its TC-NER function.
Only at normally low or moderately increased activities can Rad26 facilitate TC-NER.

In vitro studies demonstrating the roles of the yeast Rad26 and human CSB in regulating transcription across a DNA lesion generally use high levels of these factors [31–33]. However, the cellular level of Rad26 is much lower than that of RNAPII (Fig. 1c). Also, different from its purified form, RNAPII is associated with a number of transcription elongation factors that repress TC-NER in vivo [5]. We therefore tested how TC-NER might be affected by different levels of Rad26 in the cell. We transformed rad7Δ rad26Δ cells with plasmids expressing Rad26 under its native promoter (pRAD26), the strong galactose-inducible GAL10 promoter (pGAL-RAD26), or the moderately strong Cu2+-inducible CUP1 promoter (pCU-RAD26) (Supplemental Table S2). The ATPase activity (relative to the immunoprecipitated plasmid-expressed wild-type and helicase motif mutant Rad26). The ATPase activities were calculated by comparing the ADP/ATP ratios of the different samples. The ATPase activity of Rad26 is autoinhibited by the N-terminal leucine latch motif (Fig. 1a) and deletion or
mutation of the leucine latch motif of Rhp26, the Schizosaccharomyces pombe homolog of the S. cerevisiae Rad26, has been shown to increase the ATPase activity ~4- to 5-fold [36]. To test the role of the leucine latch in TC-NER, we also transformed rad7Δ rad26Δ cells with plasmids expressing the leucine latch mutant Rad26 (Rad26LM) under its native promoter (pRAD26LM), the GAL10 promoter (pGAL-RAD26LM), or the CUP1 promoter (pCU-RAD26LM) (Supplemental Table S2). Rad26 and Rad26LM were expressed to similar levels under all the different promoters (Fig. 4a–c). After 4 h of galactose induction of the cells containing pGAL-RAD26 and pGAL-RAD26LM, the Rad26 and Rad26LM proteins reached the highest levels (Fig. 4b), which were ~120 times the normal expression level (Fig. 4d). At this high level of overexpression, the number of Rad26 or Rad26LM molecules would be ~1/3 of that of RNAPII molecules. Within 1 h of Cu²⁺ (CuSO₄) induction of the cells containing pCU-RAD26 and pCU-RAD26LM, the Rad26 or Rad26LM proteins reached the highest levels (Fig. 4c), which were ~20 times the normal expression level (Fig. 4d).

The rad7Δ rad26Δ cells containing pRAD26 and pRAD26LM showed similar TC-NER rates (Figs. 2c, d and 3a), indicating that, under the normally low expression level, the increased ATPase activity conferred by the leucine latch mutation does not dramatically affect TC-NER. The CuSO₄-induced cells containing pCU-RAD26 also showed normal TC-NER (Figs. 2e and 3a), indicating that a
g, h and 3a), indicating that the highly (~120 fold) CuSO4-induced cells containing pCU-RAD26LM not significantly affect TC-NER. Surprisingly, the moderate (~20 fold) overexpression of Rad26 did not significantly affect TC-NER. Only at normally low or moderately increased activities can Rad26 efficiently promote transcriptional bypass of UV photoproducts.

Based on in vitro studies, it has been proposed that Rad26 and its human homolog CSB might facilitate TC-NER in part by positioning and stabilizing stalling of RNAPII at lesion sites [31–33]. To determine if the modulation of TC-NER by the different activities of Rad26 was achieved by modulating transcription stalling at UV photoproducts, we fine-mapped transcription on a UV damaged template in the cell. To avoid interference by preexisting RNAs, we analyzed galactose-induced transcripts of the genomic GAL10 gene, which has undetectable leakage transcription in the absence of galactose [37]. Like its human homolog XPA, Rad14 plays an important role in lesion recognition/verification and is essential for both TC-NER and GG-NER [2]. Elc1 is a component of a ubiquitin ligase complex that is required for ubiquitination and degradation of RNAPII upon DNA damage [38] but plays no role in TC-NER [27]. To prevent repair of UV photoproducts and degradation of RNAPII during the transcription analyses (which would skew the transcription results), we used rad7Δ rad26Δ rad14Δ elc1Δ cells (Supplemental Table S1) containing an empty vector, pRAD26, pCU-RAD26, or pCU-RAD26LM (Supplemental Table S2). The cells were induced with CuSO4 to moderately overexpress Rad26 (which enables the TC-NER function) or Rad26LM (which disables the TC-NER function) in cells containing pCU-RAD26 or pCU-RAD26LM, UV irradiated (240 J/m²) to produce ~1 CPDs/kb of single-stranded DNA and switched to galactose medium to induce transcription of the GAL10 gene.

Full-length and nascent GAL10 transcripts became detectable in all unirradiated cells shortly after galactose induction (Supplemental Fig. S2). However, mostly nascent and only a little full-length GAL10 transcripts could be detected in the UV-irradiated cells (Supplemental Fig. S2), indicating stalling of transcription by UV photoproducts.

We then sequenced the nascent GAL10 transcripts that were ≤800 nt long (Fig. 5). To exclude sequencing artifacts, the transcripts were ligated to an single-stranded DNA adapter that contains a region of random nucleotides, which serves as a Unique Molecular Identifier (UMI) so that PCR duplicates and sequencing errors (which are typically 0.1%–1% for next-generation sequencing) can be removed/corrected [39] (Fig. 5c). Equal amounts of barcoded sequencing libraries prepared from different cell samples were combined, and the

![Fig. 3. CPDs remaining in the RPB2 gene. (a) percent CPDs remaining in the transcribed strand (nt +60 to +900) of the RPB2 gene in rad7Δ rad26Δ cells containing the indicated plasmids. CPDs located in the short region immediately downstream of the transcription start site were excluded for calculating the CPDs remaining, as TC-NER in this short region is independent of Rad26. Only the error bars (S.D.) for cells containing pCU-RAD26 (pink symbols) and pGAL-RAD26 (cyan symbols) are shown for clarity. (b) Percent CPDs remaining in the non-transcribed strand of the RPB2 gene in rad26Δ cells containing the indicated plasmids.](image-url)
fragments with the insert sizes of ≤800 nt were gel purified and sequenced. The length distributions of the nascent \textit{GAL10} transcripts were similar among the unirradiated cells containing the different plasmids (Fig. 6a, d, g, and j). At 20 min of galactose induction, the length distribution was somewhat more toward the longer transcripts in the UV-irradiated cells containing \textit{pRAD26} or \textit{pCU-RAD26} than those containing the empty vector or \textit{pCU-RAD26LM} (Fig. 6b, e, h, and k). At 40 min of galactose induction, the length distribution shifted more toward the longer transcripts in the UV-irradiated cells containing \textit{pRAD26} (Fig. 6, compare e and f) or \textit{pCU-RAD26LM} (Fig. 6, compare h and i). Indeed, in the cells containing \textit{pRAD26} or \textit{pCU-RAD26}, the log$_2$ ratio of the numbers of longer (>250 nt) transcripts at 40 min to those at 20 min of galactose induction were generally >0 (indicating increase of the numbers of longer transcripts), whereas the log$_2$ ratio of the numbers of shorter (<250 nt) transcripts was generally <0 (indicating decrease of the numbers of shorter transcripts) (Fig. 6n and o). However, the length distribution did not change significantly with time in the cells containing either the empty vector (Fig. 6b, c, and m) or \textit{pCU-RAD26LM} (Fig. 6k, l, and p). These results indicate that, only at normally low or moderately increased activities, can \textit{Rad26} promote transcriptional bypass of UV lesions in the cell.

UV photoproducts occur at di-pyrimidine sites with the frequencies in the order of (3′→5′) TT > TC > CC [40]. We analyzed transcripts ended opposite the di-pyrimidine sites and flanking sequences within the first 550-nt region of the transcribed strand of the \textit{GAL10} gene. Transcription may also stall at certain intrinsic pausing sites. However, the intrinsic stalling is generally transient, as evidenced by the fact that within 5 min of induction, full-length \textit{GAL10} mRNA can be detected in unirradiated cells (Supplemental Fig. S2). Also, once \textit{GAL10} transcription is fully induced, the distribution of nascent transcripts ended at different sites along the \textit{GAL10} genes will not change in unirradiated cells (although the total transcripts will increase with time until a steady state is reached). Therefore, by comparing the counts of nascent \textit{GAL10} transcripts ended opposite the di-pyrimidine sites and flanking sequences between UV-irradiated and -unirradiated samples, we will be able to assess how transcription may be stalled by UV photoproducts. At 20 min of galactose induction, the nascent \textit{GAL10} transcripts ended opposite the purines 3′ to

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**Fig. 4.** Levels of expressions of \textit{Rad26} and \textit{Rad26LM}. (a–c) Western blots showing levels of \textit{Rad26} and \textit{Rad26LM} in \textit{rad7Δ rad26Δ} cells containing the indicated plasmids at different times after additions of galactose and CuSO$_4$. (d) Western blot showing levels of \textit{Rad26} and \textit{Rad26LM} after dilutions of the protein extracts. The protein extracts diluted were prepared from cells containing \textit{pGAL-RAD26} after 4-h galactose induction and from cells containing \textit{pCU-RAD26} after 1 h of CuSO$_4$ induction. The asterisk indicates a non-specific band. Rpb1 serves as loading control.
the di-pyrimidine sites (A/G−1) were significantly higher in all the UV-irradiated cells than in the unirradiated ones (Fig. 7a, c, e, and g), indicating stalling of transcription by the UV photoproducts. Significant transcription stalling could also be seen opposite the 3′ C (C1) in CT (Fig. 7c), and the 3′ T (T1) in TT (Fig. 7g). At 40 min of galactose induction, stalling of the GAL10 transcription opposite the A/G−1, C1, and T1 persisted (the log2 ratios did not significantly change) in UV-irradiated cells containing the empty vector or pCU-RAD26LM but disappeared (the log2 ratios decreased to near 0) in those containing pRAD26 or pCU-RAD26 (Fig. 7b, d, f, and h). These results, together with our above observations (Fig. 6), indicate that only at normally low or moderately increased activities can Rad26 promote transcriptional bypass of UV photoproducts in the cell. These findings are surprising because neither Rad26 [31] nor CSB [32] is able to promote transcriptional bypass of a TT CPD in vitro. Also, while the positioning and stabilization of RNAPII at lesions by Rad26 and CSB may be important for initial lesion detection [31,32], our results indicate that the function for promoting transcriptional bypass of DNA lesions is required for Rad26 to facilitate TC-NER.

Only AMPs are substantially misincorporated and extended opposite UV photoproducts and adjacent bases; Rad26 does not significantly affect either misincorporation or extension of AMPs but at normally low or moderately increased activities promotes error-free transcriptional bypass of UV photoproducts

Previous in vitro studies have shown that U misincorporation opposite the 5′ T of a TT CPD results in irreversible stalling of RNAPII, which was suspected to be important for elicitation of TC-NER [15,16]. To determine if incorporation or misincorporation of specific nucleotides is required for transcriptional stalling at UV photoproducts and TC-NER in the cell, we analyzed the nucleotides at the 3′ ends of nascent GAL10 transcripts. Significant misincorporations of AMPs (As) could be seen at the 3′ ends of the nascent transcripts opposite A/G−1 3′ to all dipyrimidine sites, C1 in CC and CT, and A/G + 1 5′ to TT in all the UV-irradiated cells containing
the different plasmids (Fig. 8, pink symbols). Also, significant AA misincorporations were seen at the 3′ ends of the nascent transcripts opposite C2 in CC (the 5′ A opposites C1 and the 3′ A opposites C2), T2 in CT (the 5′ A opposites C1 and the 3′ A opposites T2), T1 in TT (the 5′ A opposites A/G − 1 and the 3′ A opposites T1), and N + 2 5′ to TT (the 5′ A opposites A/G + 1 and the 3′ A opposites N + 2) in all the UV-irradiated cells containing the different plasmids (Fig. 8, cyan symbols). No significant misincorporation of other nucleotides could be seen at the 3′ ends of the nascent transcripts, including misincorporations of Us opposite the 5′ To fT Ta ss e e nb yp r e v i o u s in vitro studies [15–17].

To understand if transcriptional bypass of UV photoproducts involves misincorporations of certain nucleotides, we analyzed misincorporated nucleotides in the internal regions (i.e., not at the ends) of the nascent GAL10 transcripts. At 20 min of galactose induction, significant misincorporations of As could be seen opposite A/G − 1 3′ to all dipyrimidine sites, C1 in CC and CT and A/G + 1 5′ to TT in all the UV-irradiated cells containing the different plasmids (Fig. 9a, c, e, and g, pink symbols). At 40 min of galactose induction, the frequencies of A misincorporations were somewhat lower opposite certain sites (not always statistically significant) in cells containing pRAD26 and pCU-RAD26 than those containing the empty vector or pCU-RAD26LM (Fig. 9b, d, f, and h). Misincorporations of other nucleotides were rare and not significantly different between UV-irradiated and unirradiated cells (Fig. 9). These results indicate that UV photoproducts can be transcriptionally bypassed following misincorporations of As regardless of Rad26 activities. Taken together, our results also indicate that Rad26, at normally low or moderately increased activities, primarily promotes error-free transcriptional bypass of UV photoproducts.
The inability of a high level of Rad26 activity to facilitate transcriptional bypass of UV photoproducts is not due to displacement of RNAPII

Why a high level of Rad26 activity, like in the absence of Rad26, is unable to facilitate transcriptional bypass of DNA lesions and TC-NER? Under physiological conditions, the RNAPII complex stalled at a lesion is extremely stable [41]. We wondered if a high level of Rad26 activity could displace RNAPII from the UV damaged template, thereby disabling transcriptional bypass of DNA lesions and TC-NER. We compared the levels of Rpb1, the largest subunit of RNAPII, in the chromatin and non-chromatin

Fig. 7. Transcription stalling opposite UV photoproducts and flanking sequences. (a–h) Log₂ ratio (+UV/−UV) of counts of nascent GAL10 transcripts ended opposite CC (a and b), CT (c and d), TC (e and f), TT (g and h), and flanking sequences. Plasmids contained in the rad7Δ rad26Δ rad14Δ elc1Δ cells are shown in different colors as indicated at the bottom. A/G − 1 and A/G + 1 are purines immediately 3’ and 5’ to the di-pyrimidine sites, respectively. N − 2 and N + 2 are the second nucleotide 3’ and 5’ to the di-pyrimidine site, respectively, and are neither within nor neighbored by di-pyrimidine sites. The normalized counts from the UV-irradiated cells were divided by the corresponding ones from the unirradiated cells. Bars represent the means of log₂ count ratios of all transcripts (with or without nucleotide misincorporation at the 3’ ends) ended opposite all the relevant positions within the first 550-nt region of the transcribed strand of the GAL10 gene. Single and double asterisks indicate that the counts are significantly different between UV-irradiated and -unirradiated cells (p values < 0.01 and 0.05, respectively; paired and two-tailed Student’s t test).
Fig. 8. Nucleotides at the 3’ ends of nascent GAL10 transcripts opposite UV photoproducts and flanking sequences. (a–h) Count differences (between +UV and −UV) of nucleotides at the 3’ ends of nascent GAL10 transcripts opposite CC (a and b), CT (c and d), TC (e and f), TT (g and h), and flanking sequences. Plasmids contained in the rad7Δ rad26Δ rad14Δ elc1Δ cells are indicated at the top. A/G − 1 and A/G + 1 are purines immediately 3’ and 5’ to the di-pyrimidine sites, respectively. N − 2 and N + 2 are the second nucleotide 3’ and 5’ to the di-pyrimidine site, respectively, and are neither within nor neighbored by di-pyrimidine sites. The counts of transcripts with matched (M, gray bars) or misincorporated nucleotides (A, C, G, U, or AA, shown in different colors as indicated at the bottom) at the 3’ ends were normalized to 10^5 total reads across the nucleotide positions (which include all reads that are the same length or longer than the transcripts). The counts from the UV-irradiated cells were subtracted by the corresponding ones from the unirradiated cells. Bars represent the means of count differences of the 3’ end nucleotides opposite all the relevant positions within the first 550-nt region of the transcribed strand of the GAL10 gene. Single and double asterisks with colors matching those of the 3’ end nucleotides indicate that the counts are significantly different between UV-irradiated and -unirradiated cells (p values < 0.01 and 0.05, respectively; paired and two-tailed Student’s t test).
fractions in galactose-induced rad7Δ rad26Δ rad14Δ elc1Δ cells containing an empty vector with those containing pGAL-RAD26LM (Supplemental Fig. S3).

As expected, histone H3 was primarily present in the chromatin fraction, whereas the cytoplasmic protein GAPDH was primarily present in the supernatant (Supplemental Fig. S3B). The levels of Rpb1 in chromatin and supernatant fractions in the UV-irradiated cells containing pGAL-RAD26LM were similar to those in the cells containing the empty vector, and the levels did not change significantly with time (Supplemental Fig. S3C and D). These results indicate that the inability of a high level of Rad26 activity to facilitate transcriptional bypass of UV photoproducts and TC-NER is not due to displacement of RNAPII from the template.

Rad26 evicts Spt5 from chromatin

Rad26 has recently been shown to bind to the clamp and stalk (Rpb4/7) regions of RNAPII in vitro [31]. These binding sites on RNAPII partially overlap with those of Spt5 [22,31], a key transcription elongation factor that coordinates the repression of TC-NER [5]. Rad26 may modulate TC-NER by affecting the interaction of Spt5 with RNAPII in the cell. To test this possibility, we compared the levels of Spt5 in the chromatin and non-chromatin fractions

Fig. 9. Nucleotide misincorporations in the internal region of nascent GAL10 transcripts opposite UV photoproducts and flanking sequences. (a–h) Count differences (between +UV and −UV) of misincorporated nucleotides in the internal regions of nascent GAL10 transcripts opposite CC (a and b), CT (c and d), TC (e and f), TT (g and h), and flanking sequences. Plasmids contained in the rad7Δ rad26Δ rad14Δ elc1Δ cells are indicated at the top. A/G − 1 and A/G + 1 are purines immediately 3’ and 5’ to the di-pyrimidine sites, respectively. N − 2 and N + 2 are the second nucleotide 3’ and 5’ to the di-pyrimidine site, respectively, and are neither within nor neighbored by di-pyrimidine sites. The counts of misincorporated nucleotides (A, C, G, U, shown in different colors as indicated at the bottom) were normalized to 10^5 total reads across the respective nucleotide positions (which include all reads that are the same length or longer than the transcripts). The counts from the UV-irradiated cells were subtracted by the corresponding ones from the unirradiated cells. Bars represent the means of count differences of the misincorporated nucleotides opposite all the relevant positions within the first 550-nt region of the transcribed strand of the GAL10 gene. Pink single and double asterisks indicate that the counts of misincorporated As are significantly different between UV-irradiated and -unirradiated cells (p values < 0.01 and 0.05, respectively; paired and two-tailed Student’s t test).
in rad7Δ rad26Δ rad14Δ elc1Δ cells containing an empty vector or those expressing different levels of Rad26 or Rad26LM. Indeed, the levels of Spt5 associated with chromatin were inversely correlated with the levels of Rad26 activity, and UV irradiation did not significantly affect the chromatin association (Fig. 10a and b). This is in line with the previous report showing that Rad26 is recruited to the coding sequences of genes in a transcription-dependent but DNA-lesion-independent manner [42]. Our results indicate that Rad26 constitutively evicts Spt5 from chromatin and the degree of the eviction is correlated with the activities of Rad26.

Discussion

We, for the first time, analyzed the spectra of nucleotides transcriptionally incorporated opposite UV photoproducts and how they are implicated in transcriptional stalling, bypass, and TC-NER in the cell. We found that only As but no other nucleotides are significantly misincorporated into transcripts and extended opposite both the 3' and 5' bases in a UV photoproducts. We also observed significant A misincorporations and extensions opposite the bases immediately 3' to all pyrimidine dimers and 5' to TTs. These findings suggest that nucleotide misincorporations and extensions by RNAPII opposite UV photoproducts and adjacent bases in the cell follow the A-rule as commonly known for error-prone DNA polymerases [43]. Furthermore, we found that different Rad26 activities do not significantly affect either misincorporation or extension of As. Taken together, our results indicate that promotion of TC-NER of UV photoproducts by Rad26 does not require nucleotide misincorporations that result in irreversible transcriptional stalling.

In vitro studies have demonstrated that Rad26 [31] and CSB [32] are able to resolve backtracking of RNAPII but cannot enable transcriptional bypass of a TT CPDs. We found that, at moderate activities but not at high activities, Rad26 promotes transcriptional bypass of UV photoproducts in vitro. It is possible that the levels of Rad26 and CSB used by the in vitro studies are too high to allow for transcriptional bypass of the UV photoproduct. RNAPII is associated with multiple transcription elongation factors. It is also possible that transcriptional bypass of UV photoproducts may require the participation of these transcription elongation factors after being remodeled by moderate activities of Rad26.

Rad26 has recently been shown in vitro to bind to the DNA upstream of the RNAPII elongation complex and sits between the RNAPII clamp and stalk (Rpb4/7 regions [31]). These binding sites on RNAPII overlap with those of Spt5 [22,31]. Based the previous reports and our findings here, we propose the following model for how Rad26 facilitates TC-NER. In the absence of Rad26, a DNA lesion is trapped in the RNAPII complex, which is stabilized by coordinated actions of Spt5 and other transcription elongation factors, and TC-NER is repressed (Fig. 11a). At normally low or moderately increased activities, Rad26 may transiently bind to and “loosen” the RNAPII complex by competing with Spt5, which enables trans-lesion transcription leading to exposure, dual incision, and excision of DNA lesions behind the RNAPII complex (Fig. 11b). The excised fragment containing the DNA lesion may dissociate from the chromatin along with RNAPII as shown in human cells [26]. However, RNAPII does not dissociate from the chromatin if TC-NER does not proceed past the dual incision step (e.g., in NER-defective rad14Δ cells). In view of the fact that TC-NER is very rapid, whereas the Rad26-mediated trans-lesion transcription appears to be fairly slow in the absence of NER (in rad14Δ cells), it is likely that TC-NER may be able to initiate at an early stage of the trans-lesion transcription. It is also possible that Rad26 promotes trans-lesion synthesis more rapidly in the presence of the NER machinery. At a high level of activity, achieved by overexpressing the Rad26 or Rad26LM to ~1/3 or 1/17 the level of RNAPII molecules, respectively, Rad26 may severely disrupt the binding of Spt5 (and other transcription elongation factors) to RNAPII, resulting in impairments of trans-lesion transcription and repression of TC-NER (Fig. 11c). It is quite unlikely that the overexpression of Rad26, especially that driven by the GAL10 promoter, will cause dramatic Rad26 misfolding because the overexpressed
Rad26 appeared to be more active in evicting Spt5 from chromatin (Fig. 10). Still, we cannot rule out the possibility that a certain fraction of the overexpressed Rad26 is misfolded and not functional for TC-NER. It should be noted that, instead of facilitating TC-NER, enhanced transcriptional by-pass of DNA lesions accomplished by Rpb1 mutations has been shown to actually attenuate TC-NER [44]. Therefore, Rad26 may facilitate TC-NER not just by promoting transcriptional bypass of UV photoproducts. Rad26 may also simultaneously promote the recruitment of NER factors by weakening the binding of Spt5 and other TC-NER repressors to RNAPII. This explains why Rad26 becomes completely or partially dispensable for TC-NER in the absence of TC-NER repressors [5]. However, it remains to be elucidated as to how NER proteins are recruited once the RNAPII moves past the lesion. Among all the known NER factors, only TFIIH appears to directly interact with RNAPII during transcription initiation. To date, there is no evidence that TFIIH directly interacts with RNAPII during transcription elongation (where TC-NER mainly occur). If a true transcription–repair coupling factor (i.e., a matchmaker between RNAPII and the NER machinery) exists in yeast remains to be determined.

A previous report showed that overexpression of Rad26 increased repair of CPDs in both the transcribed and nontranscribed strands of the RPB2 gene, indicating that both TC-NER and GG-NER may be enhanced upon Rad26 overexpression [45]. However, we observed that TC-NER was unaffected when Rad26 was moderately overexpressed and attenuated when Rad26 was highly overexpressed. We also did not observe significant change of GG-NER upon moderate or high levels of Rad26 overexpression. The discrepancy between the previous report and our findings here might be due to the difference of Rad26 overexpression levels, which were not quantitatively measured in the previous study.

The promotion of transcriptional bypass of DNA lesions by Rad26 may be analogous to that of the human CSB, which has been shown in vitro to...
promote the addition of one more nucleotide opposite the 3’ T of a TT CPD [32]. If CSB at a certain level of activity promotes transcription bypass of DNA lesions in human cells remains to be tested. Rad26 may also be analogous to the Escherichia coli Mfd in resolving stalling of an RNA polymerase at lesions by pushing the RNA polymerase forward [4,6,7]. However, unlike Rad26, which promotes transcriptional bypass of DNA lesions, Mfd displaces the polymerase from the template. Also unlike Rad26, which does not seem to directly recruit NER factors, Mfd has been shown to recruit the bacterial NER machinery by directly interacting with Uvra [4,6,7].

Materials and Methods

Yeast strains and plasmids

Yeast strains and plasmids used are listed in Supplemental Tables S1 and S2, respectively. Strains with their genomic genes tagged with three consecutive FLAG (3× FLAG) sequences were created by using PCR products amplified from plasmid p3FLAG-KanMX, as described previously [46].

Measurement of protein expression

Cells were cultured in synthetic minimal medium containing 2% lactate, 2% glycerol, and 2% ethanol (LGE) at 30 °C to late log phase (A600 ≈ 1.0). Galactose and CuSO4 were added to the cultures to final concentrations of 2% and 1 mM, respectively. Aliquots were taken at different times of further incubation. Whole-cell protein extracts were prepared from the aliquots by using the procedure as described previously [47]. Proteins of interest were detected by Western blot with antibodies against the FLAG tag (M2, Sigma, for 3× FLAG tagged proteins), Rpb1 (6WG16, Biologend; H14, Enzo Life Sciences), histone H3 (182,926, Abcam), GAPDH (184,193, Abcam), and Tubulin (GTX76511, GeneTex).

Assays of Rad26 ATPase activity

Cells expressing 3× FLAG-tagged wild-type and helicase motif mutant Rad26 from 25 ml of log phase culture were washed with and resuspended in 0.5 ml of IP buffer [50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.4 mM Na2VO3, 10 mM Na3P2O7, 10 mM NaF, 0.5% NP-40, 1% Triton X-100, 0.1% SDS, 0.2 mM PMSF, protease inhibitors]. After the addition of 0.5 ml of glass beads, the cells were lysed by 8 × 30-s pulses of bead beating. Cell debris was removed by centrifugation at 20,000g for 10 min at 4 °C. Fifteen micrograms of the anti-FLAG antibody was added to the lysate, and the mixture was incubated at 4 °C overnight with gentle rotation. Protein A-coated agarose beads (Sigma) were added to the mixture and incubated at 4 °C for 3 h with gentle rotation. After being washed twice with IP buffer containing 0.5 M of NaCl and twice with IP buffer, the beads were incubated with 0.5 μCi of [α-32P]ATP in 20 μl of a reaction buffer [50 mM Tris–HCl (pH 8.0), 5 mM MgCl2, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 5% glycerol, 1 mg/ml of sonicated heat-denatured salmon sperm DNA] at 37 °C for 30 min. One microliter of the reaction was spotted onto a thin-layer chromatography (TLC) plate (Sorbent Technology) and air dried. The TLC plate was developed in 750 mM potassium phosphate (pH 3.5) and exposed to a phosphorimager screen.

Measurement of RNAPII and Spt5 associations with chromatin

Cells were cultured in LGE medium (see above) to late log phase. Galactose or CuSO4 was added to the cultures to a final concentration of 2% or 1 mM to induce Rad26 or Rad26LM under the GAL10 or CUP1 promoter, respectively. After 4 h of further incubation for galactose-induced cells or 1 h of further incubation for CuSO4-induced cultures, the cultures were irradiated with 240 J/m2 of 254 nm UV and incubated in YPG (1% yeast extract, 2% peptone, and 2% galactose) with or without 1 mM CuSO4 at 30 °C. Aliquots of 30 ml were taken at different times of the incubation. For measuring association of RNAPII with chromatin, cells from the aliquots were directly pelleted. For measuring association of Spt5 with chromatin, cells from the aliquots were fixed with 1% formaldehyde for 30 min, quenched with 125 mM glycine, washed, and then pelleted. The cell pellet from each aliquot was mixed with 0.5 ml ice-cold cell lysis buffer [20 mM Hepes (pH 8.0), 60 mM KCl, 15 mM NaCl, 10 mM MgCl2, 1 mM CaCl2, 10 mM N-butyric acid, 0.8% Triton X-100, 0.25 M sucrose, 2.5 mM spermidine, 0.5 mM spermine, 2× concentrated protease inhibitor cocktail (P8125, Sigma-Aldrich), 1 mM PMSF, and 20 mM ribonucleoside-vanadyl complex (New England Bio-labs)] and 0.5-ml acid washed beads. Cells were lysed by 8 × 30-s pulses of bead-beating. Residual intact cells (P1) were removed by centrifugation at 500g for 5 min (Supplemental Fig. S3A). The supernatant (S1) was centrifuged at 2000g for 20 min. The pellet (P2) was washed once with cell lysis buffer and twice with wash buffer [20 mM Hepes (pH 7.6), 450 mM NaCl, 7.5 mM MgCl2, 10% glycerol, 1% NP-40, 0.5 M sucrose, 1 ml DTT, 0.125 mM PMSF, 2× concentrated of protease inhibitor, 20 mM ribonucleoside-vanadyl complex] (Supplemental Fig. S3A). To concentrate proteins from the supernatant fractions, an equal volume of phenol was added. After vortexing and centrifugation, the phenol phase was transferred to a
fresh tube. Proteins in the phenol phase were precipitated by mixing with 5 volumes of methanol containing 0.1 M of ammonium acetate and centrifugation at 16,000g for 30 min. The protein pellets were dissolved in SDS-PAGE gel loading buffer. To reverse formaldehyde crosslinks, the samples were boiled for 20 min. Rpβ1, Sp5, histone H3, and GAPDH in the chromatin and supernatant fractions were analyzed by Western blot.

**Repair analysis of UV-induced CPDs**

Cells were cultured in LGE medium (see above) at 30 °C to late log phase, and galactose and CuSO₄ were added to the cultures to final concentrations of 2% and 1 mM, respectively. After 4 h of further incubation, the cultures of cells containing pGAL-RAD26 or pGAL-RAD26LM were harvested. After 1 h of further incubation, all other cultures were harvested. UV irradiation (120 J/m² of 254 nm UV), 1 h of further incubation, all other cultures were harvested. After 4 h of further incubation, the cultures of cells containing pGAL-RAD26 or pGAL-RAD26LM were harvested. After 1 h of further incubation, all other cultures were harvested. UV irradiation (120 J/m² of 254 nm UV), repair incubation and repair analyses of CPDs of the harvested cells were performed as described previously [44].

**Analysis of transcription in the genomic GAL10 gene**

Cells were grown in LGE medium (see above) to late log phase, and CuSO₄ was added to the cultures to a final concentration of 1 mM. After 1 h of further induction, the cells were irradiated with 240 J/m² of 254 nm UV. The irradiated and unirradiated cells were pelleted and resuspended in YPG medium containing 1 mM CuSO₄ and incubated at 30 °C. Aliquots were taken at different times of the incubation, and total RNA was isolated by using a hot acidic phenol method [48].

For Northern blot analysis, the total RNA was resolved on formaldehyde agarose gels and transferred onto Hybond-N⁺ membranes (GE Healthcare) [49]. The GAL10 transcripts were probed with a GAL10 riboprobe in vitro transcribed from plasmid pGAL10b in the presence of [α-³²P] UTP (Supplemental Table S2) [49].

The procedure for creating a next-generation sequencing library of GAL10 transcripts is outlined in Fig. 5. One picomoles of a biotinylated oligonucleotide (5′-ACTTTGTAACTGAGCTGTCATTTA-TATTGAAT-biotin) that is complementary to the 5′ end of the GAL10 mRNA was mixed with 10 μg of total RNA in a volume of 100 μl. The mixture was heated at 95 °C for 10 min and 50 °C for 5 min. Ten microliters of streptavidin magnetic beads (Dynabeads M-280 streptavidin, Life Technologies) was mixed with the sample and incubated at room temperature for 30 min. The beads were sequentially washed with MBS [100 Mops, 1 M NaCl, and 5 mM EDTA (pH 8.0)], 0.1 × MBS containing 0.5% SDS, 0.1 × MBS, and H₂O. GAL10 transcripts now attached to the beads were ligated to a 5′ phosphorylated and 3′ blocked adapter containing a UMI of 12 random nucleotides (Ns) (5′-phosphate-CTGACNNNNNNNNAGATCGGAA-GAGCGTCTGTG-inverted dT). The ligation was carried out in a 20-μl reaction containing 25 pmol of the UMI-containing adapter and 20 units of T4 RNA ligase (New England Biolabs) for 5 h at room temperature. The ligated GAL10 transcripts were hybridized to a reverse transcription primer (5′-ACACGACGCTCT) by incubating the beads in 10 μl of 0.1 × MBS containing 100 pm of the primer at 37 °C for 20 min. The beads were sequentially washed with 0.1 × MBS containing 0.5% SDS, 0.1 × MBS, and H₂O. The GAL10 transcripts were then reverse transcribed into DNA fragments by incubating the beads in a 10-μl reaction containing 200 units of ProtoScript II reverse transcriptase (New England Biolabs) for 5 h at 42 °C. The reverse-transcribed DNA fragments were amplified by 20 cycles of PCR. Illumina-barcoded sequencing adapters were added to the amplified fragments by 8 cycles of PCR using primers containing the adapter sequences at the 5′ ends. The sequencing libraries prepared from different samples were combined, and the fragments with the insert sizes of no more than 800 bp were gel purified and sequenced 300 bp from both ends. We obtained a total of ~20 million reads for each of two biological repeats.

The sequencing reads from different samples were sorted based on their sequencing barcodes by using FASTQ/A Barcode Splitter and aligned to the GAL10 gene sequence by using BWA-MEM. The sequencing errors were corrected, and PCR duplicates were removed based on the UMI sequences attached to the GAL10 transcripts by sequentially using GroupReadsByUmi and CallMolecularConsensusReads (Fgbio, Fulcrumgenomics). The corrected reads were aligned to the GAL10 gene sequence by using Bowtie2. The reads with a mapping quality of <30 were removed by using Samtools View. The number of transcripts with a specific length and 3′ nucleotide was counted and normalized to the total number of reads that are the same length or longer than the transcripts, and the length distribution and nucleotide(s) frequencies at the 3′ ends of the transcripts were obtained by using custom scripts. The numbers of nucleotide misincorporations in an internal site (i.e., not at the 3′ ends) of the GAL10 transcripts were counted by using Pysamstats and normalized to the total number of reads across the site.

**Statistical analyses**

All experiments presented in this paper were repeated 1–3 times, depending on reproducibility and necessity. Student’s t tests were used for statistical analyses.
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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2019.02.010.

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Abbreviations used:
CPD, cyclobutane pyrimidine dimer; GG-NER, global genomic nucleotide excision repair; NER, nucleotide excision repair; RNAPII, RNA polymerase II; TC-NER, transcription coupled nucleotide excision repair; TLC, thin-layer chromatography; UV, ultraviolet.

References

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Rad26 in transcriptional lesion bypass and TC-NER


