THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 8, pp. 5317–5326, February 19, 2010

The C-terminal Repeat Domain of Spt5 Plays an Important Role in Suppression of Rad26-independent Transcription Coupled Repair*

Received for publication, November 5, 2009, and in revised form, December 17, 2009 Published, JBC Papers in Press, December 30, 2009, DOI 10.1074/jbc.M109.082818

Baojin Ding, Danielle LeJeune, and Shisheng Li[1]

From the Department of Comparative Biomedical Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

In eukaryotic cells, transcription coupled nucleotide excision repair (TCR) is believed to be initiated by RNA polymerase II (Pol II) stalled at a lesion in the transcribed strand of a gene. Rad26, the yeast homolog of the human Cockayne syndrome group B (CSB) protein, plays an important role in TCR. Spt4, a transcription elongation factor that forms a complex with Spt5, has been shown to suppress TCR in *rad26*Δ cells. Here we present evidence that Spt4 indirectly suppresses Rad26-independent TCR by protecting Spt5 from degradation and stabilizing the interaction of Spt5 with Pol II. We further found that the C-terminal repeat (CTR) domain of Spt5, which is dispensable for cell viability and is not involved in interactions with Spt4 and Pol II, plays an important role in the suppression. The Spt5 CTR is phosphorylated by the Bur kinase. Inactivation of the Bur kinase partially alleviates TCR in *rad26*Δ cells. We propose that the Spt5 CTR suppresses Rad26-independent TCR by serving as a platform for assembly of a multiple protein suppressor complex that is associated with Pol II. Phosphorylation of the Spt5 CTR by the Bur kinase may facilitate the assembly of the suppressor complex.

Nucleotide excision repair (NER)[2] is a conserved DNA repair mechanism capable of removing a variety of helix-distorting lesions, such as UV-induced cyclobutane pyrimidine dimers (CPDs) (1). NER can be grouped into two pathways: global genomic repair (GGR), which refers to repair throughout the genome, and transcription coupled repair (TCR), which refers to a repair mechanism that is dedicated to the transcribed strand of actively transcribed genes (2). In the yeast *Saccharomyces cerevisiae*, Rad7, Rad16 (3), and Elc1 (4) are specifically required for GGR, but dispensable for TCR. Rad7 and Rad16 form a complex that binds specifically to UV-damaged DNA in an ATP-dependent manner and has DNA-dependent ATPase activity (5). Elc1 has been shown to be a component of a ubiquitin ligase that contains Rad7 and Rad16, and is responsible for regulating the levels of Rad4 protein in response to UV damage (6, 7). It has also been suggested that Elc1 is a component of another ubiquitin ligase complex, which contains Ela1, Cul3, and Roc1 but not Rad7 and Rad16 (8, 9). The role of Elc1 in GGR may not be subsidiary to that of Rad7 and Rad16 (4).

The mechanistic details of TCR are relatively well understood in *Escherichia coli.* The transcription repair coupling factor Mfd targets the transcribed strand for repair by recognizing a stalled RNA polymerase and actively recruiting the NER machinery to the transcription blocking lesion as it dissociates the stalled RNA polymerase (10). Conversely, the TCR mechanisms in eukaryotes appear to be extremely complicated (for reviews, see Refs. 11 and 12). In mammalian cells, Cockayne syndrome group A (CSA) and B (CSB) proteins are specifically required for TCR, but dispensable for GGR (13–16). Like its human homolog CSB, the yeast Rad26 plays an important role in TCR but is dispensable for GGR (17). Both human CSB (18) and yeast Rad26 (19) are DNA-stimulated ATPases and play roles in transcription elongation (20, 21). However, TCR in yeast is not solely dependent on Rad26, as a substantial extent of TCR still occurs in cells lacking Rad26 (22–24). Rpb9, a nonessential subunit of RNA polymerase II (Pol II), has also been shown to play a role in TCR (22, 23, 25, 26).

Mutations in the *SPT4* and *SPT5* genes in yeast were originally isolated as suppressors of the Ty insertion mutations that interfere with adjacent gene transcription (27). When the Ty sequence is inserted in the upstream region of a gene, the transcription signal directs transcription from the Ty promoter and interferes with normal transcription of the adjacent gene. A mutation in *SPT4* or *SPT5* attenuates the aberrant transcription, restoring transcription from the normal site. The *SPT4* gene is dispensable (28), whereas the *SPT5* gene is essential (29) for cell viability. Immunoprecipitation studies showed that Spt4 and Spt5 form a complex, which physically interacts with Pol II (30). Yeast cells lacking Spt4 show reduced efficiency of Pol II elongation through GC-rich DNA sequences and a general decrease in Pol II processivity (31, 32). These proteins are conserved eukaryotic transcription-elongation factors and are generally required for normal development and viral gene expression in multicellular eukaryotes (33). In mammalian cells, the Spt4-Spt5 complex, which is also called DRB sensitivity inducing factor, and represses transcription elongation at the early elongation-recessive elongation transition (34, 35). Phosphorylation of the C-terminal repeat region of Spt5 plays a key role in converting the complex from a repressor to a positive regulator of transcription (36, 37).

Interestingly, it was shown that deletion of *spt4* alleviates the requirement of Rad26 for TCR in yeast, indicating that Spt4

* This work was supported by National Science Foundation Grant MCB-0745229.

[1] To whom correspondence should be addressed. Tel.: 225-578-9102; Fax: 225-578-9895; E-mail: shli@vetmed.lsu.edu.

[2] The abbreviations used are: NER, nucleotide excision repair; 5-FOA; 5-fluoroorotic acid; CHX, cycloheximide; CPD, cyclobutane pyrimidine dimer; CTR, C-terminal repeat; GGR, global genomic nucleotide excision repair; Pol II, RNA polymerase II; TCR, transcription coupled nucleotide excision repair; CSA and -B, Cockayne syndrome group A and B.

TABLE 1
Yeast strains used in this study

Strain	Genotype[a]	Ref./source
BJ5465	*MATa ura3–52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1*	74
CR18	As BJ5465, but *rad7Δ rad26Δ*	75
CR78	As BJ5465, but *rad7Δ rad26Δ spt4::LEU2*	This study
BD4	As BJ5465, but *rad7Δ rad26Δ* (*SPT5*-3×FLAG)	This study
BD7	As BJ5465, but (*SPT5*-3×FLAG)	This study
BD9	As BJ5465, but *rad7::URA3* (*SPT5*-3×FLAG)	This study
BD10	As BJ5465, but *rad7Δ rad26Δ* (*SPT5*-3×FLAG)	This study
BD13	As BJ5465, but *spt4::LEU2* (*SPT5*-3×FLAG)	This study
BD14	As BJ5465, but *rad7Δ spt4::LEU2* (*SPT5*-3×FLAG)	This study
BD15	As BJ5465, but *rad7Δ rad26Δ spt4::LEU2* (*SPT5*-3×FLAG)	This study
BD16	As CR18, but [pGAL-SPT5]	This study
BD17	As CR78, but [pGAL-SPT5]	This study
BD21	As BJ5465, but [pGAL-SPT5]	This study
BD56	As CR18, but *spt5::KanMX* [pRS416-SPT5, pSPT5]	This study
BD57	As CR18, but *spt5::KanMX* [pRS416-SPT5, pSPT5/CTRΔ]	This study
BD58	As CR18, but *spt5::KanMX* [pRS416-SPT5, pSPT5/641-1063Δ]	This study
BD59	As CR18, but *spt5::KanMX* [pRS416-SPT5, pSPT5/422-1063Δ]	This study
BD60	As CR18, but *spt5::KanMX* [pRS416-SPT5, pSPT5/1–244Δ]	This study
BD61	As CR18, but *spt5::KanMX* [pRS416-SPT5, pSPT5/1–421Δ]	This study
BD62	As CR18, but *spt5::KanMX* [pRS416-SPT5, pSPT5/1–640Δ]	This study
BD63	As BD56, but [pRS416-SPT5] removed	This study
BD64	As BD57, but [pRS416-SPT5] removed	This study
BD94	As CR18, but *bur2::URA3*	This study
BD95	As BD64, but *bur2::URA3*	This study
BD96	As BD63, but *bur2::URA3*	This study

[a] Genomic genes tagged with 3×FLAG are indicated in parentheses; plasmids contained in strains are indicated in brackets.

suppresses Rad26-independent TCR (38). Until now, whether and/or how Spt5 is involved in the suppression is unclear. Like the *spt4 deletion*, an Spt5 point mutation, *spt5–194*, results in the Spt$^-$ phenotype (*i.e.* unable to suppress Ty insertion mutations) and is sensitive to the nucleotide depletion drug 6-azauracil, indicating that *spt4* deletion and *spt5–194* mutation may cause similar deficiencies in transcription elongation (30, 39). In addition, *spt5–194* combined with an *spt4* mutation leads to synthetic lethality (39). However, unlike *spt4*Δ, the *spt5–194* mutation does not suppress UV sensitivity of *rad16*Δ *rad26*Δ cells (38). This observation led to the proposition that, unlike Spt4, Spt5 may not play a role in suppressing Rad26-independent TCR or that, despite the shared phenotypes with *spt4*Δ, the specific *spt5–194* mutation may not lead to a defect in the suppression. In this paper, we present evidence that Spt4 indirectly suppresses Rad26-independent TCR by protecting Spt5 from degradation and stabilizing the interaction of Spt5 with Pol II. We further found that the C-terminal repeat (CTR) domain of Spt5, which contains 15 copies of a 6-amino acid sequence that can be phosphorylated by the Bur kinase, plays an important role in suppressing Rad26-independent TCR.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast strains used in this study are listed in Table 1. Wild type yeast strain BJ5465 (*MATa ura3–52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1*) was obtained from the American Type Culture Collection. All deletion mutants were made in BJ5465 background and confirmed by PCR analysis, using procedures described previously (22). *URA3*, *LEU2*, and *KanMX* were used to replace the gene to be deleted. In some cases, the *URA3* gene that had replaced a gene was further knocked-out as described previously (22). Nucleotides (with respect to the starting codon ATG) +14 to +288, +51 to +2400, +214 to +1454, +58 to +2297, and +204 to +730 were deleted for *SPT4*, *SPT5*, *RAD7*, *RAD26*, and *BUR2* genes, respectively. Strains with their genomic genes tagged with three consecutive FLAG (3xFLAG) sequences were created using PCR products amplified from plasmid p3FLAG-KanMX, as described previously (40).

A plasmid overexpressing 3xFLAG-tagged Spt5 under control of the *GAL10* promoter was created using vector pESC-URA (Fig. 2*A*). Two consecutive FLAG sequences were inserted in-frame downstream of the FLAG sequence (downstream of the *GAL10* promoter) present in the original pESC-URA vector to create a vector encoding 3xFLAG. The *SPT5* gene coding sequence was amplified by PCR and inserted in-frame downstream of the 3xFLAG sequence to create plasmid pGAL-SPT5 (Fig. 2*A*).

A single-copy centromeric plasmid with the *URA3* gene as a selection marker and encoding the wild type Spt5 protein was created by using the plasmid pRS416 (41). The full-length of the *SPT5* gene encompassing the 5′ promoter, the coding region, and the 3′ terminator was amplified by PCR and inserted between BamHI and EagI sites of pRS416 to create plasmid pRS416-SPT5. A single-copy centromeric plasmid with the *LEU2* gene as a selection marker and encoding the full-length or truncated Spt5 proteins were created by using plasmid pRS415 (Fig. 4, *A* and *B*) (41). The promoter, full-length or truncated coding sequences, and the terminator of the *SPT5* gene, and the 3 consecutive Myc sequences (3xMyc) were amplified by PCR and ligated into pRS415 to create plasmids expressing the full-length or CTR-deleted Spt5 (Fig. 4*B*).

Shuffling of Plasmids Encoding Different Spt5 Truncates—Yeast cells were transformed with plasmid pRS416-SPT5 and the genomic *SPT5* gene was then deleted using a standard procedure as described above. The specific deletion of the genomic *SPT5* gene was confirmed by PCR using primer pairs specific for the genomic *SPT5* gene and the plasmid-borne *SPT5* gene. pRS415-based plasmids encoding the full-length or truncated Spt5 were transformed into the yeast cells whose genomic *SPT5* gene had been deleted and complemented with pRS416-SPT5.

The transformed cells were cultured in medium containing uracil but not leucine to select the *LEU2* plasmids and allow the loss of the *URA3* plasmid. A centromeric plasmid generally has a loss rate of 1% per generation and shows virtually no segregation bias (42). The cultures were then spotted onto plates containing 5-fluoroorotic acid (5-FOA), which is toxic to cells with a functional *URA3* gene (43), to select for cells that had lost plasmid pRS416-SPT5.

Repair Analysis of UV-induced CPDs—Yeast cells were grown at 30 °C in minimal medium containing 2% glucose (SD) or 2% galactose (SG, for Spt5 overexpression) to late log phase ($A_{600} \approx 1.0$), irradiated with 80 J/m^2 of 254 nm UV and incubated in YPD medium (2% peptone, 1% yeast extract and 2% glucose) or YPG medium (2% peptone, 1% yeast extract and 2% galactose) in the dark at 30 °C. At different times of the repair incubation, aliquots were removed and the genomic DNA was isolated using a hot SDS procedure as described previously (22).

The gene fragments of interest were 3′-end labeled with [α-^{32}P]dATP using a procedure described previously (44, 45). Briefly, ~1 μg of total genomic DNA was digested with restriction enzyme(s) to release the fragments of interest and incised at CPD sites with an excess amount of T4 endonuclease V (Epicentre). Excess copies of biotinylated oligonucleotides, which are complementary to the 3′-end of the fragments to be labeled, were mixed with the sample. The mixture was heated at 95 °C for 5 min to denature the DNA and then cooled to an annealing temperature of around 50 °C. The annealed fragments were attached to streptavidin magnetic beads (Invitrogen), labeled with [α-^{32}P]dATP (PerkinElmer Life Sciences), and resolved on sequencing gels. The gels were exposed to a PhosphorImager screen (Bio-Rad). The signal intensities at gel bands corresponding to CPD sites were quantified using Quantity One software (Bio-Rad).

Whole Cell Extract Preparation and Immunoprecipitation—Yeast cells were cultured at 30 °C in minimal medium containing 2% glucose or galactose (to induce a gene under the control of the *GAL10* promoter) to late log phase and harvested. For measuring cellular levels of proteins of interest, whole cell extracts were prepared using a trichloroacetic acid method (46). The harvested cells from a 5-ml culture were resuspended in 300 μl of 20% trichloroacetic acid and broken by vertexing them with acid-washed glass beads. The proteins in the lysates were pelleted by centrifugation, washed with ice-cold 80% acetone, and dissolved in 100 μl of 2× SDS-PAGE gel loading buffer (47).

To examine the effect of Spt4 on Spt5 degradation, *spt4*Δ and *SPT4*$^+$ cells expressing 3xFLAG-tagged Spt5 were cultured to late log phase. Cycloheximide (CHX), a potent protein synthesis inhibitor (48), was added to the cultures to a final concentration of 500 μg/ml to completely stop protein synthesis (46). At different times following the addition of CHX, cells were harvested and whole cell extracts were prepared using the trichloroacetic acid method as described above.

For immunoprecipitation, the harvested cells from a 25-ml culture were washed with and resupended in 0.5 ml of immunoprecipitation buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.4 mM Na_4VO_3, 10 mM $Na_4P_2O_7$, 10 mM sodium fluoride, 0.5% Nonidet P-40, 1% Triton X-100, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors) (46). The cells were broken with acid-washed glass beads, and cell debris was removed by centrifugation at 20,000 × *g* for 10 min at 4 °C. Fifty μl of the lysate was saved as an "input." The remaining lysate was added with 15 μg of anti-FLAG (M2) (Sigma), anti-Myc (Sigma), 8WG16 (Neoclone), or H14 (Covance) antibodies, which recognize FLAG tag peptide, Myc tag peptide, the hypophosphorylated and hyperphosphorylated C-terminal heptapeptide repeats of Rpb1, respectively (49). The mixture was incubated at 4 °C overnight with gentle rotation. Protein A- or G-coated agarose beads (Sigma) were added to the mixture and incubated at 4 °C for 3 h with gentle rotation. The beads were washed twice with immunoprecipitation buffer containing 0.5 M NaCl and twice with immunoprecipitation buffer containing 150 mM NaCl. Bound proteins were eluted by boiling the beads in 50 μl of 2× SDS-PAGE gel loading buffer.

Treatment of Immunoprecipitated Pol II Complexes with λ Phosphatase—Pol II complex was immunoprecipitated from yeast cells using antibody H14 as described above. Protein A- or G-coated agarose beads attached with the immunoprecipitates were resuspended in 100 μl of dephosphorylation reaction buffer (50 mM HEPES, 100 mM NaCl, 2 mM dithiothreitol, 0.01% Brij-35, 1 mM $MnCl_2$). Four hundred units of λ protein phosphatase (New England Biolabs) were added to the sample. Following 30 min of incubation at 30 °C, 100 μl of 2× SDS-PAGE gel loading buffer was added to the sample. Proteins were eluted from the beads by boiling for 5 min.

Western Blot—Proteins in whole cell extracts, immunoprecipitation inputs, immunoprecipitated samples, or phosphatase-treated samples were resolved on an SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Rpb1 was probed with 8WG16 or H14 antibodies. 3xFLAG- and 3xMyc-tagged proteins were probed with anti-FLAG M2 antibody (Sigma) and anti-Myc antibody (Sigma), respectively. Blots were incubated with SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce), and the protein bands were detected using a chemiluminescence scanner (Fluorchem 8800, Alpha Innotech). Band intensities were quantified using AlphaEaseFC 4.0 software.

Northern Blot—Yeast cells were cultured to late log phase under the same conditions as those used for NER analysis. Total RNA was isolated using a hot acidic phenol method, as described (50). The RNA was fractionated on formaldehyde-agarose gels (47), transferred onto Hybond-N$^+$ membranes (GE Healthcare), and hybridized with radioactive probes generated using the Prime-It® II Random Primer Labeling Kit (Stratagene).

UV Sensitivity Assay—Yeast cells were grown at 30 °C in minimal medium to saturation, and sequential 10-fold dilutions were made. The diluted samples were spotted onto YPD plates. When the spots had dried, the plates were irradiated with different doses of 254 nm UV light. The plates were incubated at 30 °C for 3–7 days in the dark prior to being photographed.

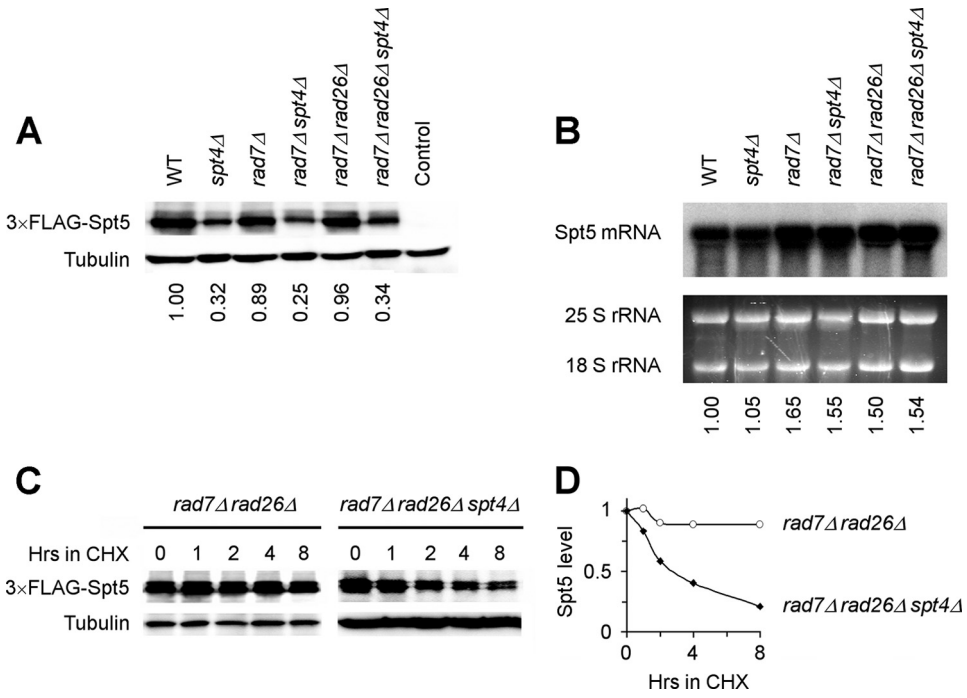

FIGURE 1. **Spt4 protects Spt5 from degradation.** *A*, Western blot showing cellular levels of Spt5 expressed from the genomic *SPT5* gene tagged with 3xFLAG in different strains. The *control* lane contains a sample prepared from the wild type strain whose *SPT5* gene was not tagged. The tubulin bands serve as internal loading controls. The *numbers at the bottom* indicate relative cellular levels of the tagged Spt5 in the different strains (the level in wild type cells is set as 1). *B*, Northern blot showing Spt5 mRNA levels in the different strains. The *lower panel* shows an ethidium bromide-stained agarose gel containing the resolved total RNA before being blotted onto a membrane. The *numbers at the bottom* indicate relative cellular levels of Spt5 mRNA in the different strains (the level in wild type cells is set as 1). The 25 S and 18 S rRNA bands serve as internal loading controls. *C*, Western blot showing levels of the tagged Spt5 at different times following the addition of CHX in *rad7*Δ *rad26*Δ and *rad7*Δ *rad26*Δ *spt4*Δ strains. As the steady state level of the tagged Spt5 in *spt4*Δ cells was lower than that in *SPT4*$^+$ cells, the amounts of cell extracts from *rad7*Δ *rad26*Δ *spt4*Δ cells were loaded more than those from *rad7*Δ *rad26*Δ cells. Tubulin bands serve as internal loading controls. *D*, plot showing relative levels of the tagged Spt5 at different times following the addition of CHX.

RESULTS

Spt4 Protects Spt5 from Degradation and Stabilizes the Interaction of Spt5 with Pol II—It has been shown that Spt4 partially suppresses Rad26-independent TCR, as deletion of *SPT4* reinstates TCR in *rad26*Δ cells (38). Spt4 forms a complex with Spt5 in yeast (30, 39) and human cells (34). In yeast, the *SPT4* gene is dispensable (28), whereas the *SPT5* gene is essential for cell viability (29). We wondered if Spt4 suppresses Rad26-independent TCR directly or through or together with Spt5. To this end, we first examined if Spt4 affects the cellular level of Spt5. Three consecutive FLAG sequences (3xFLAG) were tagged to the coding sequence of the genomic *SPT5* gene in different yeast mutants. The 3xFLAG tag did not cause any noticeable deficiency to the cells (not shown). As shown in Fig. 1*A*, the cellular level of Spt5 in *spt4*Δ cells was about ⅓ of that in *SPT4*$^+$ cells, regardless of the presence of the GGR factor Rad7 or the TCR factor Rad26. However, the *SPT5* mRNA levels were similar between *spt4*Δ and *SPT4*$^+$ cells (Fig. 1*B*), indicating that the lower cellular level of Spt5 in *spt4*Δ cells was not caused by a decreased transcription of the *SPT5* gene. For an unknown reason, *SPT5* mRNA levels were somewhat higher in *rad7*Δ cells than in *RAD7*$^+$ cells (Fig. 1*B*, compare *first* and *second lanes* with the *third* to *sixth lanes*). We then tested whether Spt5 was degraded faster in *spt4*Δ cells. The level of Spt5 barely changed in *SPT4*$^+$ cells during an 8-h incubation after protein synthesis was completely suppressed by the addition of the protein synthesis inhibitor CHX (Fig. 1, *C* and *D*). In contrast, the Spt5 level decreased dramatically in *spt4*Δ cells under the same incubation conditions (Fig. 1, *C* and *D*). These results indicate that Spt4 protects Spt5 from degradation.

To test whether overexpression of Spt5 in *spt4*Δ cells could compensate for the absence of Spt4, we created a multicopy (with 2-μm replication origin) plasmid (pGAL-SPT5) expressing the 3xFLAG-tagged Spt5 under the control of the galactose inducible *GAL10* promoter (Fig. 2*A*). The plasmid was transformed into different yeast mutant strains. Upon galactose induction, the plasmid-encoded 3xFLAG-tagged Spt5 was expressed at cellular levels that were 3–6 times of those of the genomically encoded 3xFLAG-tagged Spt5 (Fig. 2*B*, compare *lanes 1* and *4*, *2* and *5*, and *3* and *6*).

It has been shown that the Spt4-Spt5 complex is associated with Pol II (30). The elongation form of Pol II is hyperphosphorylated at serines 2 and 5 of the C-terminal heptapeptide repeats ($Y_1S_2P_3T_4S_5P_6S_7$) of Rpb1, whereas the non-elongation form of Pol II is hypophosphorylated at the repeats (51). Antibody 8WG16 recognizes the serine 2 unphosphorylated repeats, whereas H14 recognizes the serine 5 phosphorylated repeats (49). We immunoprecipitated 3xFLAG-tagged Spt5 with an anti-FLAG antibody. The presence of the hypo- and hyperphosphorylated forms of Pol II in the immunoprecipitates were examined by using antibodies 8WG16 and H14, respectively. As can be seen in Fig. 2*C*, slightly more hyperphosphorylated Pol II was coimmunoprecipated than hypophosphorylated Pol II, suggesting that Spt5 may have a slight preference for binding to the elongation form of Pol II.

To examine whether Spt4 affects the binding of Spt5 to Pol II, the hypo- and hyperphosphorylated Pol II were immunoprecipitated from different yeast mutants using antibodies 8WG16 and H14, respectively. The level of Spt5 associated with the hyperphosphorylated Pol II was much lower in *spt4*Δ cells than in *SPT4*$^+$ cells (Fig. 2*D*, compare *lanes 1* and *2*). Overexpression of Spt5 increased its binding to the hyperphosphorylated Pol II, especially in *spt4*Δ cells (Fig. 2*D*, compare *lanes 2* and *4*). However, the level of Spt5 associated with the hyperphosphorylated Pol II in *spt4*Δ cells overexpressing the tagged Spt5 was still somewhat lower than that in *SPT4*$^+$ cells normally expressing the genomically tagged Spt5 (Fig. 2*D*, compare *lanes 1* and *4*), although the cellular level of the overexpressed Spt5 in the *spt4*Δ cells is higher (~1.5-fold) than that in *SPT4*$^+$ cells nor-

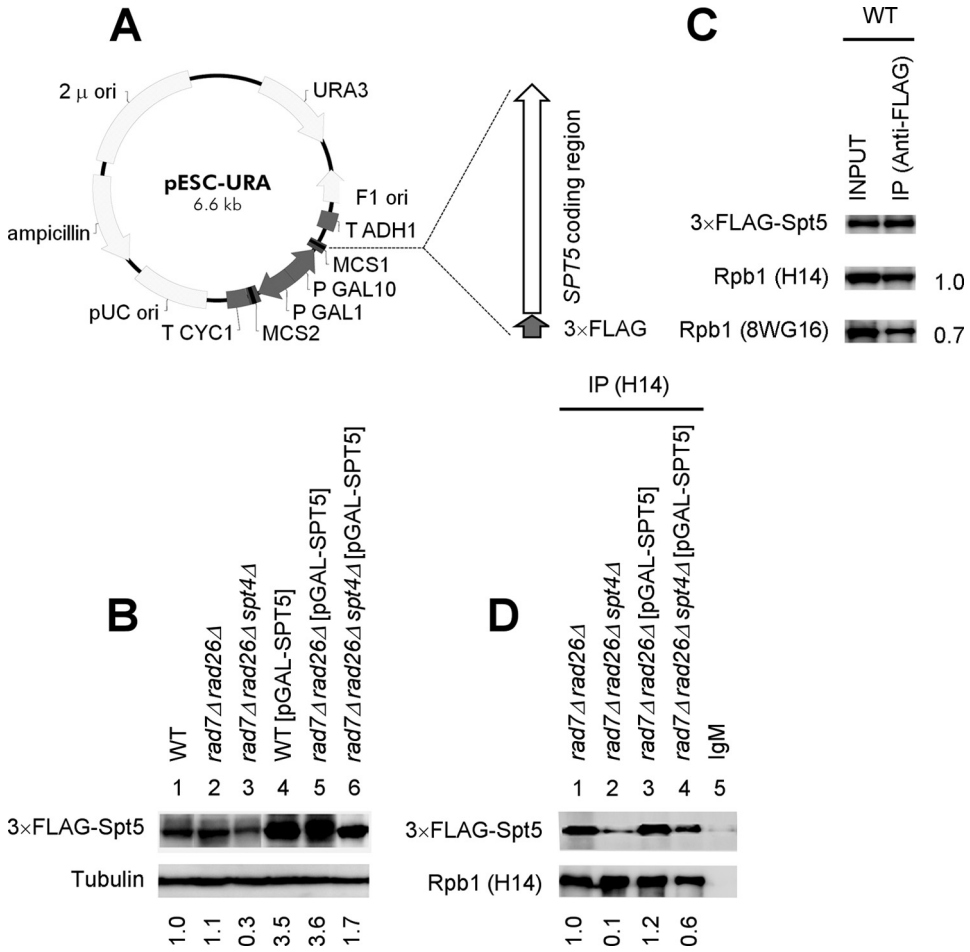

FIGURE 2. **Spt4 stabilizes the interaction of Spt5 with Pol II.** *A*, schematic of plasmid pGAL-SPT5 overexpressing 3xFLAG-tagged Spt5. *B*, Western blot showing cellular levels of 3xFLAG-tagged Spt5 in different strains cultured in a galactose medium (to induce overexpression of Spt5 encoded by plasmid pGAL-SPT5). Strains containing plasmid pGAL-SPT5 are indicated in *brackets*. The tubulin bands serve as internal loading controls. The *numbers at the bottom* indicate relative cellular levels of the tagged Spt5 in the different strains (the level in wild type cells is set as 1). *C*, binding of Spt5 to hyperphosphorylated (recognized by H14) and hypophosphorylated (recognized by 8WG16) Pol II. 3xFLAG-tagged Spt5-associated protein complexes were immunoprecipitated from wild type cells by using an anti-FLAG (M2) antibody, and probed with antibodies H14 and 8WG16 on Western blots. *Numbers on the right* of the blots indicate relative levels of hyperphosphorylated and hypophosphorylated Rpb1 co-immunoprecipitated with the tagged Spt5 (normalized to the respective immunoprecipitation inputs). *D*, binding of Spt5 to Pol II in different mutants. Pol II complexes were immunoprecipitated from different mutant cells cultured in a galactose medium by using the H14 antibody. *Lane 5* was a mock immunoprecipitated sample by using nonspecific mouse IgM. Rpb1 and co-immunoprecipitated 3xFLAG-tagged Spt5 were probed with H14 and anti-FLAG (M2) antibodies, respectively, on Western blots. Strains containing plasmid pGAL-SPT5 are indicated in *brackets*. *Numbers below* the blots indicate relative levels of 3xFLAG-tagged Spt5 co-immunoprecipitated (normalized the immunoprecipitated Rpb1) (the level in *rad7*Δ *rad26*Δ cells is set as 1).

mally expressing the genomically tagged Spt5 (Fig. 2*B*, compare *lanes 2* and *6*). We observed similar trends of Spt5 binding to the hypophosphorylated Pol II (recognized by 8WG16) in the different yeast mutants (not shown). These results indicate that the decreased binding of Spt5 to Pol II (both hypo- and hyperphosphorylated forms) in *spt4*Δ cells is due to both a lower cellular level of Spt5 and a decreased interaction between Spt5 and Pol II.

*Overexpression of Spt5 Suppresses Rad26-independent TCR in spt4*Δ *Cells*—Next, we determined if overexpression of Spt5 can suppress Rad26-independent TCR in *spt4*Δ cells. In yeast, TCR can be exclusively analyzed in *rad7*Δ (or *rad16*Δ and *elc1*Δ) cells, as these cells are defective in GGR (3, 4, 24). In agreement with previous studies (*e.g.* Ref. 22), TCR initiates ~40 nucleotides upstream of the transcription start site in the *RPB2* gene (Fig. 3*A*). In *rad7*Δ *rad26*Δ cells, little TCR can be seen in the coding region of the *RPB2* gene except for a short region immediately downstream of the transcription start site (Fig. 3*A*). In agreement with the previous report (38), the TCR rate is significantly faster in *rad7*Δ *rad26*Δ *spt4*Δ cells than in *rad7*Δ *rad26*Δ cells (Fig. 3, *B*, *C*, and *E*), indicating that Spt4 can indeed suppress Rad26-independent TCR. The TCR rate in *rad7*Δ *rad26*Δ *spt4*Δ cells overexpressing Spt5 is similar to that in *rad7*Δ *rad26*Δ cells (Fig. 3, *B*, *D*, and *E*), indicating that overexpression can suppress Rad26-independent TCR in the absence of Spt4.

The CTR Domain of Spt5 Is Dispensable for Cell Viability—Our results described above indicate that Spt5 may play a direct role in suppressing Rad26-independent TCR, whereas Spt4 may be indirectly involved in the suppression by protecting Spt5 from degradation and stabilizing the binding of Spt5 to Pol II. We next asked which domain(s) of Spt5 is involved in the suppression. Based on the results of human Spt5 domain mapping (35, 52) and the prediction with Pfam and STRING software (53, 54), yeast Spt5 consists of several distinct domains: an N-terminal acidic region, an N-terminal NusG (NGN), four KOW, and the C-terminal region that contains 15 6-amino acid repeats (CTR) (Fig. 4*A*). We used a plasmid shuffling technique to map the functions of the different domains of Spt5. A series of single-copy centromeric *LEU2* (pRS415 (41)) plasmids encoding full-length or different truncated Spt5 proteins that are tagged with 3xMyc and are under control of the native Spt5 promoter were created (Fig. 4*B*). These plasmids were transformed into yeast cells whose genomic *SPT5* gene was deleted and complemented with a single-copy centromeric *URA3* (pRS416 (41)) plasmid encoding the full-length Spt5 (pRS416-SPT5). The transformed cells were cultured in medium containing uracil but not leucine to select for the *LEU2* plasmids and allow the loss of pRS416-SPT5. The cultures were then spotted onto plates containing 5-FOA, which is toxic to cells with a functional *URA3* gene (43). Therefore, only those cells that had lost plasmid pRS416-SPT5 were able to grow on 5-FOA plates. As shown in Fig. 4*C*, cells

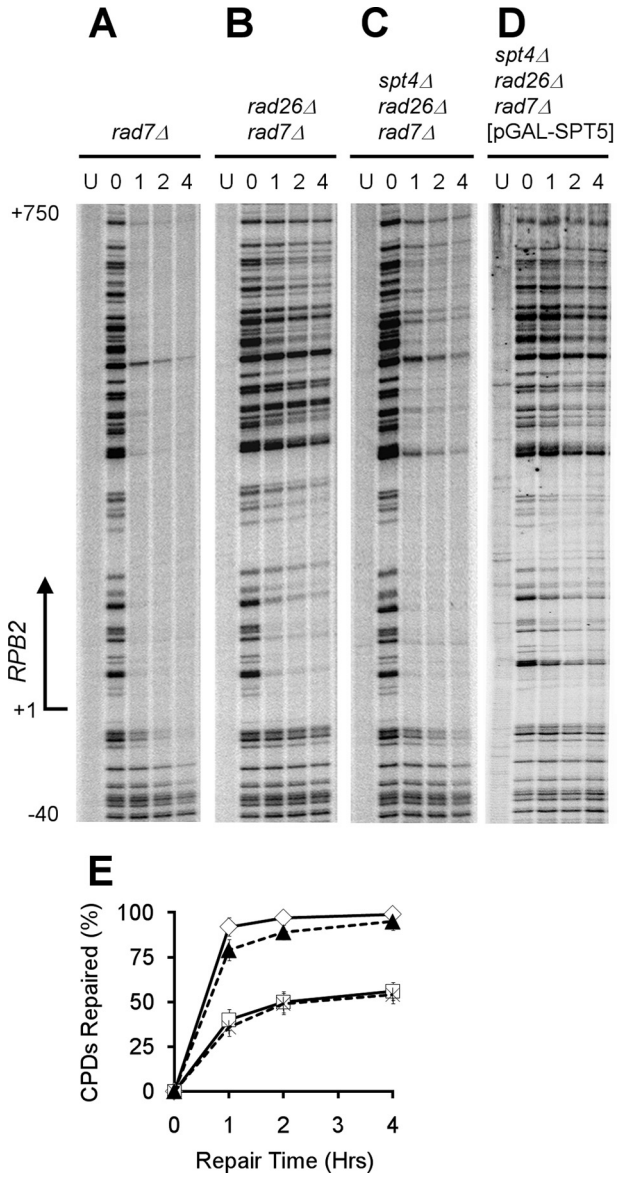

FIGURE 3. **Overexpression of Spt5 suppresses TCR in *spt4*Δ *rad26*Δ cells.** *A–D*, DNA sequencing gels showing TCR in the *RPB2* gene in galactose cultures (to induce overexpression of Spt5 encoded by plasmid pGAL-SPT5 (Fig. 2*A*)). *Brackets at the top* of *panel D* indicate plasmid pGAL-SPT5 contained in the strain. The lanes are DNA samples from unirradiated (*U*) and UV-irradiated cells following different times (hours) of repair incubation as indicated at the *top of the gels*. The *arrow on the left* of the gels marks the transcription start site. *D*, plot showing the mean (±S.D.) of percent CPDs repaired in the transcribed region of the *RPB2* gene in *rad7*Δ (*open diamond*), *rad7*Δ *rad26*Δ (*open square*), *rad7*Δ *rad26*Δ *spt4*Δ (*solid triangle*), and *rad7*Δ *rad26*Δ *spt4*Δ [pGAL-SPT5] (*asterisk*) cells.

transformed with *LEU2* plasmids encoding the full-length or the CTR-deleted Spt5 were able to grow on the 5-FOA plates, whereas those transformed with the *LEU2* plasmids encoding the other Spt5 truncates were not (Fig. 4*C*). This indicates that the CTR domain is dispensable, whereas all other domains of Spt5 examined here are essential for cell viability.

Deletion of the Spt5 CTR Domain Does Not Affect Its Binding to Spt4 or Pol II—To determine the role of the Spt5 CTR domain in its bindings to Spt4 and Pol II, we conducted a series of immunoprecipitation assays. A 3xFLAG was tagged to the genomic *SPT4* gene in cells whose genomic *SPT5* gene was deleted and complemented with the single-copy centromeric *LEU2* (pRS415) plasmid encoding 3xMyc-tagged full-length (wild type) or CTR-deleted (CTRΔ) Spt5 (Fig. 4, *A* and *B*). The Pol II complex Spt4 and Spt5 were immunoprecipited by using H14, anti-FLAG, and anti-Myc antibodies, respectively. Rpb1, the 3xFLAG-tagged Spt4, and 3xMyc-tagged Spt5 in the immunoprecipitation inputs and immunoprecipitates were detected on Western blot. Deletion of the Spt5 CTR domain did not appear to affect the cellular levels of Rpb1, Spt4, and Spt5 (Fig. 4*D*, *INPUT*). Furthermore, the deletion did not affect the levels of Rpb1, Spt4, and Spt5 in the immunoprecipitates (Fig. 4*D*, *IP*), indicating that the deletion does not affect the binding of Spt5 to Spt4 or Pol II.

The CTR Domain of Spt5 Is Involved in Suppression of Rad26-independent TCR—Due to the reinstatement of TCR, *rad7*Δ *rad26*Δ *spt4*Δ cells are about 10 times more UV-resistant than *rad7*Δ *rad26*Δ cells (Fig. 5). Interestingly, *rad7*Δ *rad26*Δ *spt5*Δ cells expressing the CTR-deleted Spt5 (Fig. 4, *A* and *B*) (*rad7*Δ *rad26*Δ *spt5*Δ + pSPT5-CTRΔ) are as UV-resistant as *rad7*Δ *rad26*Δ *spt4*Δ cells (Fig. 5). On the other hand, *rad7*Δ *rad26*Δ *spt5*Δ cells expressing the full-length Spt5 (Fig. 4, *A* and *B*) (*rad7*Δ *rad26*Δ *spt5*Δ + pSPT5) are as UV-sensitive as *rad7*Δ *rad26*Δ cells (Fig. 5). This indicates that, like Spt4, the Spt5 CTR may suppress Rad26-independent TCR.

We then directly analyzed TCR in *rad7*Δ *rad26*Δ *spt5*Δ cells expressing the full-length and CTR-deleted Spt5. The TCR rate in cells expressing the CTR-deleted Spt5 was significantly faster than that in cells expressing the full-length Spt5 (Fig. 6), indicating that the Spt5 CTR domain is indeed involved in suppression of Rad26-independent TCR.

Phosphorylation of the Spt5 CTR Domain by Bur Kinase Plays a Role in Suppression of Rad26-independent TCR—The Spt5 CTR domain contains 15 6-amino acid repeats with the consensus sequence of S(A/T)WGG(A/Q) (29). The Ser and Thr residues in these repeats are potential phosphorylation sites. We noticed that the yeast Spt5 protein can show duplicate bands on a Western blot, especially if the SDS-PAGE gel was run long enough (Fig. 7*A*). The slower migrating band of Spt5 tends to be much stronger in the immunoprecipitated Pol II complex, suggesting that this band may be a form of Spt5 that preferably associates with Pol II. Treatment of the immunoprecipitated Pol II complex with a phosphatase eliminates the slower migrating band (Fig. 7*A*), indicating that the band is the phosphorylated Spt5. Deletion of the Spt5 CTR domain eliminates the slower migrating band (Fig. 7*B*), indicating that the phosphorylation occurs in the CTR.

In human cells, Spt5 can be phosphorylated by positive transcription elongation factor b (P-TEFb), which is composed of Ctk9 and a cyclin subunit (34, 36). In yeast, two cyclin-dependent kinases are homologous to human Ctk9 (55, 56). The yeast Ctk1 has been shown to phosphorylate serine 2 of the heptapeptide repeats of the Rpb1 C-terminal domain (57, 58). The activity of Bur1 kinase is dependent on its cyclin partner Bur2. *bur1* and *bur2* mutations cause nearly identical spectra of phenotypes (59). However, *Bur1* is essential for cell viability, whereas *Bur2* is not. As can be seen in Fig. 7*C*, the slower migrating band reflecting the phosphorylated Spt5 cannot be detected in *bur2*Δ cells. This indicates that the Bur kinase is

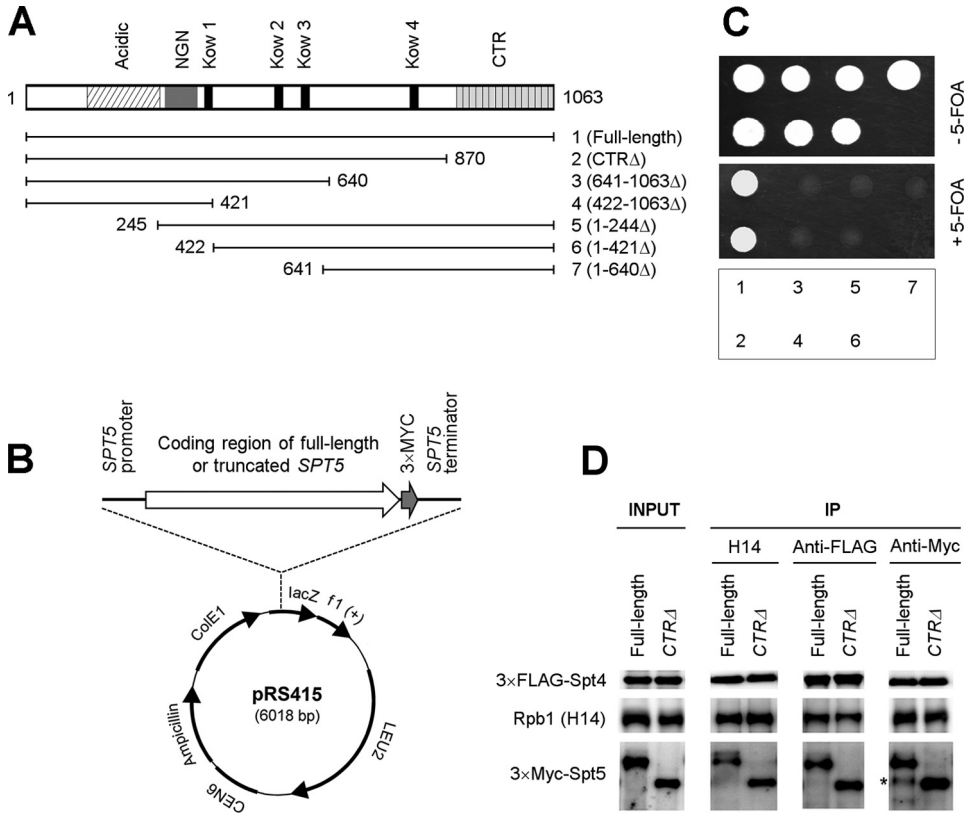

FIGURE 4. **The Spt5 CTR is not essential for cell viability and does not affect the interactions of Spt5 with Spt4 and Pol II.** *A*, schematic of the Spt5 protein. *Bars 1–7* below the schematic indicates full-length or truncated Spt5 encoded by plasmids. *B*, structure of plasmids expressing the full-length or truncated Spt5. *C*, growth of cells whose genomic *SPT5* gene was deleted and transformed with a *URA3* plasmid encoding the full-length Spt5 ([*pRS416-SPT5*]) and a *LEU2* plasmid encoding the full-length or truncated Spt5 (*1–7*, as shown in *panels A* and *B*) on 5-FOA plates. *D*, coimmunoprecipitation of the full-length or CTR-deleted Spt5 with Pol II and Spt4. Cells whose genomic *SPT5* gene was deleted and *SPT4* gene was tagged with 3xFLAG, and bearing the *LEU2* plasmid encoding the full-length or CTR-deleted Spt5 with a 3xMyc tag were cultured to log phase. Pol II, 3xFLAG-tagged Spt4, and 3xMyc-tagged Spt5 were immunoprecipitated from the cells with H14, anti-FLAG, and anti-Myc antibodies, respectively. The levels of Pol II, 3xFLAG-tagged Spt4, and 3xMyc-tagged full-length or CTR-deleted Spt5 in the immunoprecipitates were probed with the respective antibodies on Western blots.

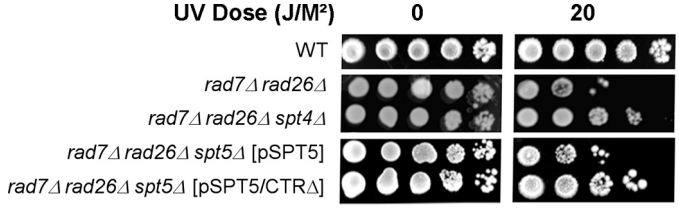

FIGURE 5. **Deletion of the Spt5 CTR increases the UV resistance of *rad7*Δ *rad26*Δ cells to the same extent as *spt4*Δ.** Saturated cultures of yeast strains were sequentially 10-fold diluted and spotted onto YPD plates. When the spots had dried, the plates were irradiated with the indicated doses of 254 nm UV light. The plates were incubated at 30 °C for 3–5 days in the dark prior to being photographed. Strains containing a single-copy plasmid encoding the full-length ([*pSPT5*]) or the CTR-deleted ([*pSPT5/CTR*Δ]) Spt5 are indicated in *brackets*.

responsible for phosphorylation of Spt5 at the CTR, in agreement with two recent reports (60, 61).

We then attempted to determine whether phosphorylation of the Spt5 CTR domain by the Bur kinase plays a role in suppression of Rad26-independent TCR. Although the *bur2*Δ cells grew extremely slowly (with a doubling time of ~8 h as opposed to ~2.5 h for *BUR2*$^+$ cells) (data not show), they showed significantly faster TCR than did the isogenic *BUR*$^+$ cells (compare Figs. 6*A* and 8, *A* and *C*), especially during the initial hour of the repair incubation. This indicates that the Bur kinase plays a role in suppression of Rad26-independent TCR, especially during the initial period of the repair incubation. However, the TCR rate in *bur*Δ cells expressing the full-length Spt5 (*rad7*Δ *rad26*Δ *spt5*Δ *bur2*Δ + pSPT5) was somewhat slower than that in isogenic *bur*Δ cells expressing the CTR-deleted Spt5 (*rad7*Δ *rad26*Δ *spt5*Δ *bur2*Δ + pSPT5-CTRΔ), especially during the later time period of the repair incubation (Fig. 8). This indicates that phosphorylation of the Spt5 CTR domain by the Bur kinase may be partially responsible for suppression of Rad26-independent TCR. In other words, besides phosphorylation, other components of the Spt5 CTR may also play a significant role in suppression of Rad26-independent TCR.

DISCUSSION

In this paper, we show that the nonessential CTR domain of Spt5 plays an important role in suppression of Rad26-independent TCR. We also present evidence that the interacting partner of Spt5, Spt4, indirectly suppresses Rad26-independent TCR by protecting Spt5 from degradation and by stabilizing the interaction between Spt5 and Pol II.

TCR is generally believed to be initiated by stalling an RNA polymerase at a lesion on the transcribed strand of a gene (12). In principle, a high level of transcription may facilitate TCR. Indeed, the transcription elongation function of Rpb9 is involved in TCR in yeast cells (26). The human CSB and yeast Rad26 enhance transcription elongation by Pol II (20, 21). However, TCR is not always positively correlated with transcription. For example, in *E. coli* the transcription factor Fis stimulates transcription of the tRNA gene *tyrT* to a very high level and at the same time suppresses TCR in this gene (62). It was proposed that, during very high level transcription, an RNA polymerase may arrive at the site of a downstream RNA polymerase stalled at a lesion before the downstream RNA polymerase can initiate or finish the TCR process, resulting in suppression of TCR (63).

Cells carrying mutations in *SPT4* and *SPT5* genes display phenotypes associated with defects in transcription elongation (39), and the gene products are thought to be involved directly in transcription elongation (30, 32). However, the suppression of Rad26-independent TCR by Spt4-Spt5 does not seem to be achieved simply by stimulating transcription. First, the Spt4-

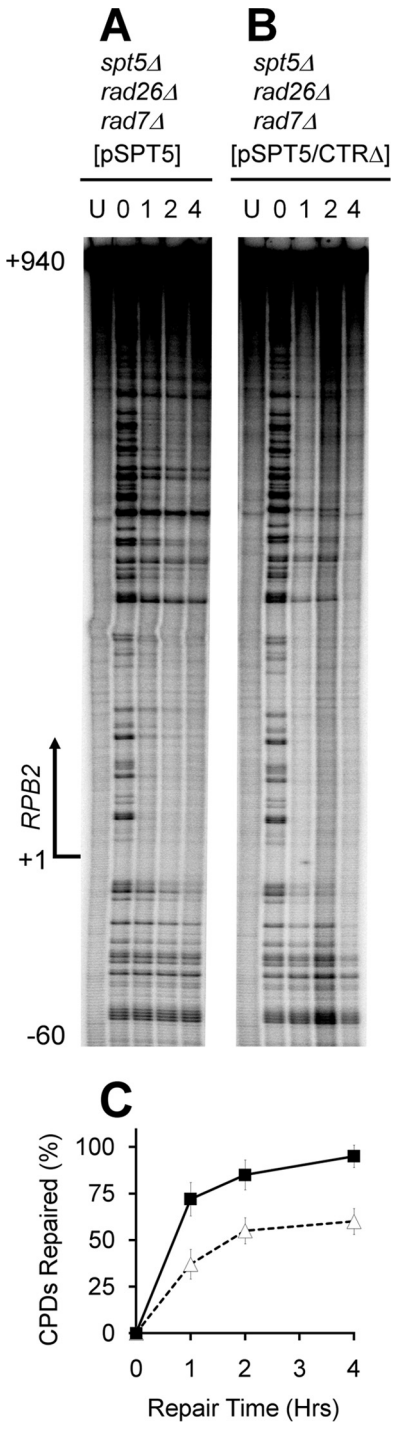

FIGURE 6. **Deletion of the Spt5 CTR increases Rad26-independent TCR.** *A* and *B*, DNA sequencing gels showing TCR in the *RPB2* gene. *Brackets at the top* indicate single-copy plasmid encoding the full-length ([*pSPT5*]) or CTR-deleted ([*pSPT5/CTR*Δ]) Spt5. The lanes are DNA samples from unirradiated (*U*) and UV-irradiated cells following different times (hours) of repair incubation as indicated at the *top* of the gels. The *arrow on the left* of the gels marks the transcription start site. *C*, plot showing the mean (±S.D.) of percent CPDs repaired in the transcribed region of the *RPB2* gene in *rad7*Δ *rad26*Δ *spt5*Δ ([*pSPT5*]) (*open triangle*) and *rad7*Δ *rad26*Δ *spt5*Δ ([*pSPT5/CTR*Δ]) (*solid square*) cells.

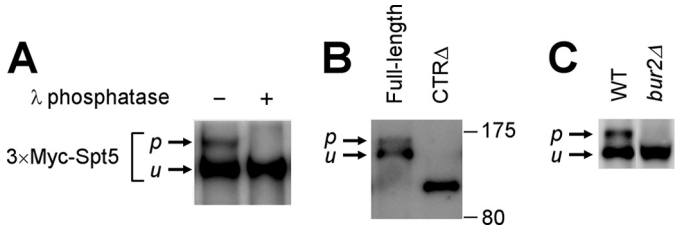

FIGURE 7. **The Spt5 CTR is phosphorylated by the Bur kinase.** *A*, Western blot showing phosphorylation of Spt5. 3xMyc-tagged Spt5 was co-immunoprecipitated with Pol II using antibody H14, treated or mock-treated with λ phosphatase, and probed with an anti-Myc antibody on a Western blot. *B*, deletion of the Spt5 CTR abolishes phosphorylation. 3xMyc-tagged full-length and CTR-deleted Spt5 was coimmunoprecipitated with Pol II by using H14 antibody and probed with an anti-Myc antibody a Western blot. *Numbers on the right* of the blot indicate approximate positions of molecular mass standards (in kDa). *C*, deletion of *bur2* abolishes Spt5 phosphorylation. 3xMyc-tagged Spt5 was co-immunoprecipitated with Pol II from *BUR2*$^+$ and *bur2*Δ cells using antibody H14, and probed with an anti-Myc antibody a Western blot. *p* and *u* on the *left* of each of the blots mark phosphorylated and unphosphorylated Spt5, respectively.

Spt5 complex does not seem to stimulate Pol II transcription to a level that is high enough to suppress Rad26-independent TCR. The galactose-induced *GAL1–10* genes are among the most robustly transcribed genes by Pol II in yeast (64). However, TCR occurs very rapidly (*i.e.* not suppressed) in these genes in *rad16*Δ *rad26*Δ (*SPT4*$^+$ *SPT5*$^+$) cells (22, 23). In contrast, TCR is much slower (*i.e.* largely suppressed) in the much more slowly transcribed *RPB2* gene in the same cells (22, 23). Second, *spt4* and *spt5* mutations that cause similar deficiency in transcription elongation have different effects on suppression of Rad26-independent NER. For example, the *spt5–194* mutation, which is due to S324F substitution of the Spt5 protein (65), shares similar deficiency in transcription to *spt4*Δ (30) or deletion of the Spt5 CTR domain (29). However, unlike *spt4*Δ or deletion of the Spt5 CTR, the *spt5–194* mutation does not seem to alleviate TCR in *rad16*Δ *rad26*Δ cells (38).

Although the exact binding site of Spt4-Spt5 on Pol II is currently unclear, it is predicted that this site is on the Rpb4-Rpb7 subcomplex that is dissociable from the 10-subunit core Pol II (66). In the absence of Rpb4-Rpb7, Pol II has an open confirmation, whereas in the presence of this subcomplex, Pol II assumes a closed conformation (67, 68). Interestingly, deletion of *rpb4* also reinstates TCR in *rad26*Δ cells (22). Therefore, it is likely that Spt4-Spt5 and Rpb4-Rpb7 function together to suppress Rad26-independent TCR.

In human cells, the NGN and Kow domains of Spt5 have been shown to interact with Spt4 and Pol II, respectively (35, 52). Similar to that of the human Spt5, the NGN domain of the yeast Spt5 is involved in interaction with Spt4 (65). The CTR domain of Spt5 does not seem to be involved in these interactions. Consistent with these studies, we found that deletion of the Spt5 CTR does not affect the interaction with either Spt4 or Pol II (Fig. 4*D*).

How does the CTR of Spt5 suppress Rad26-independent TCR? It was found recently that the Spt5 CTR is a platform for the association of proteins that promote both transcription elongation and histone modification in transcribed regions (61). One protein complex recruited by the Spt5 CTR is PAF (61). Interestingly, deletion of PAF also reinstates TCR in *rad16*Δ *rad26*Δ cells.[3] PAF plays an important role for recruitment of many factors involved in transcription elongation, such as COMPASS, FACT, and Rad6/Bre1 (69, 70). Therefore, the

[3] D. LeJeune, B. Ding, and S. Li, unpublished results.

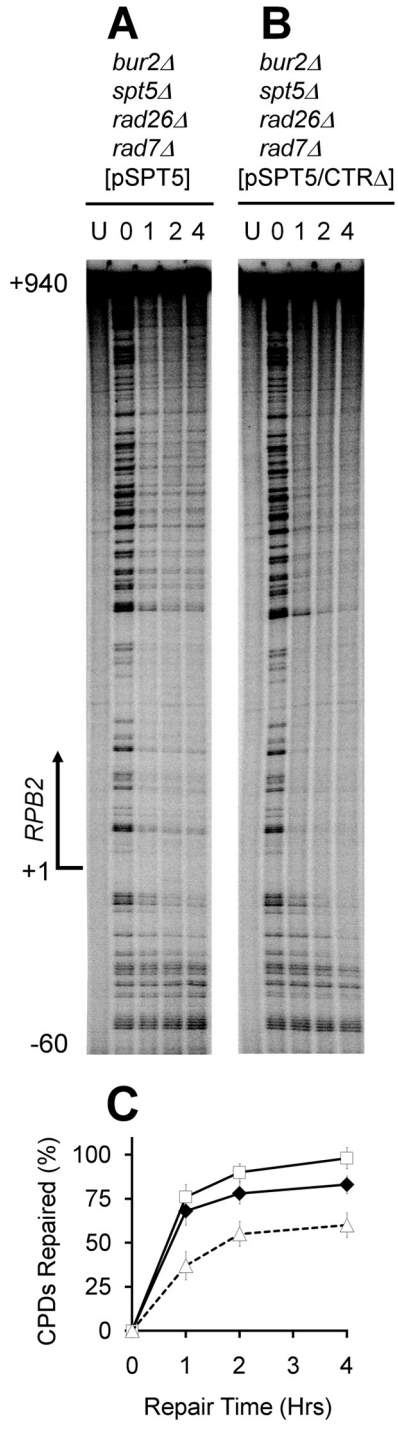

FIGURE 8. **Effects of deletions of *bur2* and the Spt5 CTR on Rad26-independent TCR.** *A* and *B*, DNA sequencing gels showing TCR in the *RPB2* gene. *Brackets at the top* indicate single-copy plasmid encoding the full-length ([*pSPT5*]) or CTR-deleted ([*pSPT5/CTR*Δ]) Spt5. The lanes are DNA samples from unirradiated (*U*) and UV-irradiated cells following different times (hours) of repair incubation as indicated at the *top* of the gels. The *arrow on the left* of the gels marks the transcription start site. *C*, plot showing the mean (±S.D.) of percent CPDs repaired in the transcribed region of the *RPB2* gene in *rad7*Δ *rad26*Δ *spt5*Δ *bur2*Δ ([*pSPT5*]) (*solid diamond*) and *rad7*Δ *rad26*Δ *spt5*Δ *bur2*Δ ([*pSPT5/CTR*Δ]) (*open square*) cells. As comparison, the repair data in *rad7*Δ *rad26*Δ *spt5*Δ ([*pSPT5*]) cells (from Fig. 6*C*) are also plotted (*open triangle*).

role of the Spt5 CTR in suppressing Rad26-independent TCR-NER may be achieved by serving as a platform for assembly of a megasuppressor complex that is associated with Pol II. This megasuppressor complex may suppress Rad26-independent TCR by preventing Pol II from either efficiently "sensing" a lesion or recruiting the NER machinery.

*bur2*Δ cells grow much slower than Spt5 CTR-deleted cells, indicating more pleiotropic roles for the Bur kinase in transcription. In addition to phosphorylation of the Spt5 CTR, Bur2 also plays a minor role in phosphorylating serine 2 of the heptapeptide repeats of the Rpb1 CTD (71). Phosphorylation of the Spt5 CTR by the Bur kinase may not be solely responsible for suppression of Rad26-independent TCR by the Spt5 domain, as TCR rate in *rad7*Δ *rad26*Δ *bur2*Δ cells expressing the full-length Spt5 is somewhat slower than that in the same cells expressing the CTR-deleted Spt5 (Fig. 8). It is possible that phosphorylation of the Spt5 CTR may enhance but not be solely responsible for the CTR to recruit other TCR suppressors, such as PAF.

In *RAD26*$^+$ cells, TCR does not appear to be affected by deletion of either *spt4* (38) or the Spt5 CTR (not shown). It seems that the suppression of TCR by Spt4-Spt5 is specifically antagonized by Rad26. Rpb9, a nonessential subunit of Pol II that also plays an important role in TCR (22, 23), does not antagonize the suppression of TCR by Spt4-Spt5.[4] One important question that remains to be answered is how Rad26 antagonizes the suppression of TCR by Spt4-Spt5. Rad26 (19) and its human counterpart CSB (18) are members of the SWI2/SNF2 family of ATPases, and both are involved in transcription elongation by Pol II (20, 21). Rad26 (72) and CSB (73) appear to dynamically associate with Pol II, especially upon DNA damage. One explanation is that Rad26 may somehow displace Spt4-Spt5 (and possibly other suppressors) from Pol II through either competitive binding to Pol II or remodeling the Pol II complex stalled at a lesion, making Spt4-Spt5 unable to suppress TCR. We are testing this hypothesis.

Acknowledgments—We thank Dr. Toshio Tsukiyama for supplying plasmid p3FLAG-KanMX and Dr. Grant Hartzog for communications.

REFERENCES

1. Friedberg, E. C., Walker, G. C., Siede, W., Wood, R. D., Schultz, R. A., and Ellenberger, T. (2006) *DNA Repair and Mutagenesis*, 2nd Ed., ASM Press, Washington, D. C.
2. Hanawalt, P. C. (2002) *Oncogene* **21,** 8949–8956
3. Verhage, R., Zeeman, A. M., de Groot, N., Gleig, F., Bang, D. D., van de Putte, P., and Brouwer, J. (1994) *Mol. Cell. Biol.* **14,** 6135–6142
4. Lejeune, D., Chen, X., Ruggiero, C., Berryhill, S., Ding, B., and Li, S. (2009) *DNA Repair* **8,** 40–50
5. Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1998) *J. Biol. Chem.* **273,** 6292–6296
6. Gillette, T. G., Yu, S., Zhou, Z., Waters, R., Johnston, S. A., and Reed, S. H. (2006) *EMBO J.* **25,** 2529–2538
7. Ramsey, K. L., Smith, J. J., Dasgupta, A., Maqani, N., Grant, P., and Auble, D. T. (2004) *Mol. Cell. Biol.* **24,** 6362–6378
8. Ribar, B., Prakash, L., and Prakash, S. (2006) *Mol. Cell. Biol.* **26,** 3999–4005
9. Ribar, B., Prakash, L., and Prakash, S. (2007) *Mol. Cell. Biol.* **27,** 3211–3216
10. Selby, C. P., and Sancar, A. (1993) *Science* **260,** 53–58
11. Fousteri, M., and Mullenders, L. H. (2008) *Cell Res.* **18,** 73–84

[4] B. Ding and S. Li, unpublished results.

12. Hanawalt, P. C., and Spivak, G. (2008) *Nat. Rev. Mol. Cell Biol.* **9,** 958–970
13. Lommel, L., and Hanawalt, P. C. (1991) *Mutat. Res.* **255,** 183–191
14. Troelstra, C., van Gool, A., de Wit, J., Vermeulen, W., Bootsma, D., and Hoeijmakers, J. H. (1992) *Cell* **71,** 939–953
15. van Hoffen, A., Natarajan, A. T., Mayne, L. V., van Zeeland, A. A., Mullenders, L. H., and Venema, J. (1993) *Nucleic Acids Res.* **21,** 5890–5895
16. Venema, J., Mullenders, L. H., Natarajan, A. T., van Zeeland, A. A., and Mayne, L. V. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87,** 4707–4711
17. van Gool, A. J., Verhage, R., Swagemakers, S. M., van de Putte, P., Brouwer, J., Troelstra, C., Bootsma, D., and Hoeijmakers, J. H. (1994) *EMBO J.* **13,** 5361–5369
18. Selby, C. P., and Sancar, A. (1997) *J. Biol. Chem.* **272,** 1885–1890
19. Guzder, S. N., Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1996) *J. Biol. Chem.* **271,** 18314–18317
20. Lee, S. K., Yu, S. L., Prakash, L., and Prakash, S. (2001) *Mol. Cell. Biol.* **21,** 8651–8656
21. Selby, C. P., and Sancar, A. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 11205–11209
22. Li, S., and Smerdon, M. J. (2002) *EMBO J.* **21,** 5921–5929
23. Li, S., and Smerdon, M. J. (2004) *J. Biol. Chem.* **279,** 14418–14426
24. Verhage, R. A., van Gool, A. J., de Groot, N., Hoeijmakers, J. H., van de Putte, P., and Brouwer, J. (1996) *Mol. Cell. Biol.* **16,** 496–502
25. Li, S., Chen, X., Ruggiero, C., Ding, B., and Smerdon, M. J. (2006) *J. Biol. Chem.* **281,** 36643–36651
26. Li, S., Ding, B., Chen, R., Ruggiero, C., and Chen, X. (2006) *Mol. Cell. Biol.* **26,** 9430–9441
27. Winston, F., Chaleff, D. T., Valent, B., and Fink, G. R. (1984) *Genetics* **107,** 179–197
28. Malone, E. A., Fassler, J. S., and Winston, F. (1993) *Mol. Gen. Genet.* **237,** 449–459
29. Swanson, M. S., Malone, E. A., and Winston, F. (1991) *Mol. Cell. Biol.* **11,** 3009–3019
30. Hartzog, G. A., Wada, T., Handa, H., and Winston, F. (1998) *Genes Dev.* **12,** 357–369
31. Mason, P. B., and Struhl, K. (2005) *Mol. Cell* **17,** 831–840
32. Rondón, A. G., García-Rubio, M., González-Barrera, S., and Aguilera, A. (2003) *EMBO J.* **22,** 612–620
33. Winston, F. (2001) *Genome Biol.* **2,** REVIEWS1006
34. Wada, T., Takagi, T., Yamaguchi, Y., Ferdous, A., Imai, T., Hirose, S., Sugimoto, S., Yano, K., Hartzog, G. A., Winston, F., Buratowski, S., and Handa, H. (1998) *Genes Dev.* **12,** 343–356
35. Yamaguchi, Y., Wada, T., Watanabe, D., Takagi, T., Hasegawa, J., and Handa, H. (1999) *J. Biol. Chem.* **274,** 8085–8092
36. Yamada, T., Yamaguchi, Y., Inukai, N., Okamoto, S., Mura, T., and Handa, H. (2006) *Mol. Cell* **21,** 227–237
37. Zhu, W., Wada, T., Okabe, S., Taneda, T., Yamaguchi, Y., and Handa, H. (2007) *Nucleic Acids Res.* **35,** 4064–4075
38. Jansen, L. E., den Dulk, H., Brouns, R. M., de Ruijter, M., Brandsma, J. A., and Brouwer, J. (2000) *EMBO J.* **19,** 6498–6507
39. Swanson, M. S., and Winston, F. (1992) *Genetics* **132,** 325–336
40. Gelbart, M. E., Rechsteiner, T., Richmond, T. J., and Tsukiyama, T. (2001) *Mol. Cell. Biol.* **21,** 2098–2106
41. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
42. Lundblad, V. (2004) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 13.4.1–13.4.10, John Wiley & Sons, Inc., New York
43. Boeke, J. D., LaCroute, F., and Fink, G. R. (1984) *Mol. Gen. Genet.* **197,** 345–346
44. Li, S., and Waters, R. (1996) *Carcinogenesis* **17,** 1549–1552
45. Li, S., Waters, R., and Smerdon, M. J. (2000) *Methods* **22,** 170–179
46. Chen, X., Ruggiero, C., and Li, S. (2007) *Mol. Cell. Biol.* **27,** 4617–4625
47. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
48. Schindler, D., and Davies, J. (1975) *Methods Cell Biol.* **12,** 17–38
49. Palancade, B., and Bensaude, O. (2003) *Eur. J. Biochem.* **270,** 3859–3870
50. Collart, M. A., and Oliviero, S. (2004) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 13.12.1–13.12.5, John Wiley & Sons, Inc., New York
51. Phatnani, H. P., and Greenleaf, A. L. (2006) *Genes Dev.* **20,** 2922–2936
52. Ivanov, D., Kwak, Y. T., Guo, J., and Gaynor, R. B. (2000) *Mol. Cell. Biol.* **20,** 2970–2983
53. Finn, R. D., Tate, J., Mistry, J., Coggill, P. C., Sammut, S. J., Hotz, H. R., Ceric, G., Forslund, K., Eddy, S. R., Sonnhammer, E. L., and Bateman, A. (2008) *Nucleic Acids Res.* **36,** D281–D288
54. Jensen, L. J., Kuhn, M., Stark, M., Chaffron, S., Creevey, C., Muller, J., Doerks, T., Julien, P., Roth, A., Simonovic, M., Bork, P., and von Mering, C. (2009) *Nucleic Acids Res.* **37,** D412–D416
55. Buratowski, S. (2005) *Curr. Opin. Cell Biol.* **17,** 257–261
56. Wood, A., and Shilatifard, A. (2006) *Cell Cycle* **5,** 1066–1068
57. Keogh, M. C., Podolny, V., and Buratowski, S. (2003) *Mol. Cell. Biol.* **23,** 7005–7018
58. Patturajan, M., Conrad, N. K., Bregman, D. B., and Corden, J. L. (1999) *J. Biol. Chem.* **274,** 27823–27828
59. Yao, S., Neiman, A., and Prelich, G. (2000) *Mol. Cell. Biol.* **20,** 7080–7087
60. Liu, Y., Warfield, L., Zhang, C., Luo, J., Allen, J., Lang, W. H., Ranish, J., Shokat, K. M., and Hahn, S. (2009) *Mol. Cell. Biol.* **29,** 4852–4863
61. Zhou, K., Kuo, W. H., Fillingham, J., and Greenblatt, J. F. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 6956–6961
62. Li, S., and Waters, R. (1997) *J. Mol. Biol.* **271,** 31–46
63. Selby, C. P., and Sancar, A. (1994) *Microbiol. Rev.* **58,** 317–329
64. Lohr, D., Venkov, P., and Zlatanova, J. (1995) *FASEB J.* **9,** 777–787
65. Guo, M., Xu, F., Yamada, J., Egelhofer, T., Gao, Y., Hartzog, G. A., Teng, M., and Niu, L. (2008) *Structure* **16,** 1649–1658
66. Aloy, P., Böttcher, B., Ceulemans, H., Leutwein, C., Mellwig, C., Fischer, S., Gavin, A. C., Bork, P., Superti-Furga, G., Serrano, L., and Russell, R. B. (2004) *Science* **303,** 2026–2029
67. Armache, K. J., Mitterweger, S., Meinhart, A., and Cramer, P. (2005) *J. Biol. Chem.* **280,** 7131–7134
68. Bushnell, D. A., and Kornberg, R. D. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 6969–6973
69. Krogan, N. J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M. A., Dean, K., Ryan, O. W., Golshani, A., Johnston, M., Greenblatt, J. F., and Shilatifard, A. (2003) *Mol. Cell* **11,** 721–729
70. Krogan, N. J., Kim, M., Ahn, S. H., Zhong, G., Kobor, M. S., Cagney, G., Emili, A., Shilatifard, A., Buratowski, S., and Greenblatt, J. F. (2002) *Mol. Cell. Biol.* **22,** 6979–6992
71. Qiu, H., Hu, C., and Hinnebusch, A. G. (2009) *Mol. Cell* **33,** 752–762
72. Jansen, L. E., Belo, A. I., Hulsker, R., and Brouwer, J. (2002) *Nucleic Acids Res.* **30,** 3532–3539
73. Fousteri, M., Vermeulen, W., van Zeeland, A. A., and Mullenders, L. H. (2006) *Mol. Cell* **23,** 471–482
74. Jones, E. W. (1991) *Methods Enzymol.* **194,** 428–453
75. Ding, B., Ruggiero, C., Chen, X., and Li, S. (2007) *DNA Repair* **6,** 1661–1669