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MODULATION OF RAD26 AND RPB9 MEDIATED DNA REPAIR BY DIFFERENT PROMOTER ELEMENTS*

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Abstract

Rad26, the yeast homologue of human Cockayne syndrome group B protein, and Rpb9, a nonessential subunit of RNA polymerase II, have been shown to mediate two subpathways of transcription coupled DNA repair in yeast. Here we show that Rad26 and Rpb9 mediated repair in the yeast *GALI* gene are differently modulated by different promoter elements. The initiation site and efficiency of Rad26 mediated repair in the transcribed strand are determined by the upstream activating sequence (UAS), but not by the TATA or local sequences. The role of UAS in determining the Rad26 mediated repair is not through loading of RNA polymerase II or the transcriptional regulatory complex SAGA. However, both the UAS and TATA sequences are essential for confining Rad26 mediated repair to the transcribed strand. Mutation of the TATA sequence, which greatly reduces transcription, or deletion of the TATA or mutation of the UAS, which completely abolishes transcription, causes Rad26 mediated repair to occur in both strands. Rpb9 mediated repair only occurs in the transcribed strand, and is efficient only in the presence of both TATA and UAS sequences. Our results suggest that Rad26 mediated repair can be either transcription-coupled, provided that a substantial level of transcription is present, or transcription-independent, if the transcription is too low or absent. In contrast, Rpb9 mediated repair is strictly transcription-coupled, and is efficient only when the transcription level is high.

Nucleotide excision repair (NER) is a conserved DNA repair mechanism that removes a wide range of bulky DNA lesions including ultraviolet (UV) light induced cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts [for a recent review, see (1)]. One NER pathway, the so called global genomic repair (GGR), removes lesions throughout the genome including those in the nontranscribed strand (NTS) of an active gene. In mammalian cells, GGR is dependent on Xeroderma pigmentosum complementation group C (2,3) and damage-specific DNA-binding proteins (4). In *Saccharomyces cerevisiae*, GGR is dependent on Rad7 and Rad16 (5). Another NER pathway, termed transcription-coupled repair (TCR), is responsible for rapid repair in the transcribed strand (TS) of an active gene. An RNA polymerase stalled at a DNA lesion in the TS may serve as the initial signal for TCR (6). The TCR mechanism in *Escherichia coli* is best understood (7,8). However, in eukaryotes, the detailed biochemical mechanism of TCR has been elusive. In mammalian cells, it has been shown that the Cockayne syndrome group A (CSA) and B (CSB) proteins are required for TCR (9–12). In *S. cerevisiae*, Rad26, the yeast homologue of mammalian CSB (13), and Rpb9, a nonessential

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subunit of RNA polymerase II (Pol II) (14,15), have been shown to mediate two subpathways of TCR, respectively.

It seems that the Rad26 and Rpb9 mediated TCR subpathways have different efficiencies in different regions of a gene. Rpb9 mediated TCR operates more effectively in the coding region than in the region upstream of the transcription start site, whereas Rad26 mediated TCR operates equally well in the two regions. Contributions of Rad26 and Rpb9 to TCR may be different from one gene to another. In the constitutive *URA3* gene, TCR seems to be exclusively mediated by Rad26, except for a short region close to the transcription start site (16). In the constitutive gene *RPB2*, Rad26 is partially required (15,17–19), and both Rad26 and Rpb9 contribute to TCR (15). For the galactose induced *GALI-10* genes, Rad26 is almost dispensable, especially in the coding region, indicating that TCR in these genes is primarily mediated by Rpb9 (14,15).

Both Rad26 and Rpb9 mediated TCR seem to be confined to the TS of the galactose induced *GALI-10* genes, initiating at upstream sites that are ~ 100 nucleotides from the upstream activating sequence (UAS) (14). Interestingly, the initiation sites of the Rad26 and Rpb9 mediated TCRs are not correlated with either the transcription start sites or the key promoter elements, the TATA boxes (14).

At present, it is largely unknown how initiation and efficiency of Rad26 and Rpb9 mediated repairs are regulated in a gene. In this paper, we present evidence that the initiation site and efficiency of Rad26 mediated repair in the TS of the *GALI* gene are determined by the UAS, but not by TATA, local sequences or even active transcription. However, the UAS, TATA, and a substantial level of transcription are essential for confining the Rad26 mediated repair to the TS. In contrast, the Rpb9 mediated repair is always confined to the TS, and is efficient only in the presence of UAS, TATA and a high level of transcription.

EXPERIMENTAL PROCEDURES

Yeast strains and plasmids

The wild-type yeast strain Y452 (*MAT α* , *ura3-52*, *his3-1*, *leu2-3*, *leu2-112*, *cir^o*) and its isogenic *rad16*, *rad16 rpb9* and *rad16 rad26* deletion mutants were created as described previously (15). The *rad7*, *rad7 spt7*, *rad7 rad26 spt7* deletion mutants are derivatives of wild type strain BJ5465 (*MAT α* *ura3-52 trp-1 leu2 Δ 1 his3 Δ 200 pep4::HIS3 prb1 Δ 1.6R can1*) (20). Nucleotides (with respect to the starting codon ATG) +214 to +1454, +58 to +2297 and +11 to +4900 were deleted for the *RAD7*, *RAD26* and *SPT7* genes, respectively. (14,15). Moreover, in log-phase cells, the relative

PCR primers used for amplifying different *GALI-10* fragments are listed in Table 1. A 2kb normal *GALI-10* fragment, encompassing the UAS and 5' portions (0.7 kb) of each of the genes, was PCR amplified using primers 1 and 2. Primer pairs 1 and 4, and 2 and 3 were used to amplify two fragments, which were digested with *Sty* I and ligated to create a *GALI-10* fragment with the *GALI* TATA mutated from ATATAAAA (21) to CCATGGA. Primer pairs 1 and 6, and 2 and 5 were used to amplify another two fragments, which were digested with *Sph* I and ligated to create a *GALI-10* fragment with the UAS mutated (Table 2). Primer 1 was paired with primers 7, 8, 9 and 10 to amplify the *GALI-10* fragments with deletion from the *GALI* gene down to +14, -72, -111 and -185, respectively. All the *GALI-10* fragments were digested with *Hind* III and inserted in the *Hind* III site of shuttle vector pRS415 (22) (Fig. 1A). The plasmid constructs were propagated in *E. coli* and transformed into different yeast strains for transcription and repair analysis.

UV irradiation, repair incubation and DNA isolation

Yeast cells were grown at 28°C in minimal medium containing 2% galactose to late log phase ($OD_{600} \approx 1.0$), harvested and irradiated with 50 J/m² of 254 nm UV light. The cells were incubated in the same medium at 28°C for various times in the dark. Total DNA was isolated from the cells following repair incubation, using a glass beads method (15).

Mapping of NER

DNA fragments of interest were strand specifically end labeled with [α -³²P]dATP using the procedure described previously (23,24). Briefly, ~ 1 μ g of total DNA was digested with restriction endonuclease(s) to release the plasmid-borne *GALI* fragments of interest and incised at CPD sites with an excess amount of purified T4 endonuclease V (Epicentre). To specifically label the plasmid-borne *GALI* fragments, the incised total DNA was mixed with a DNA ladder (e.g., 2 Log DNA Ladder from New England Biolabs), resolved on a native agarose gel. The band corresponding to the sizes of the plasmid-borne *GALI* fragments were purified from the gel using the DNA ladder as carrier. Excess copies of biotinylated oligonucleotides, which are complementary to the 3' ends of the fragments to be labeled, were mixed with the sample. The mixture was heated to 95°C for 5 min to denature the DNA and then cooled to an annealing temperature. The annealed fragments were attached to streptavidin magnetic beads (Invitrogen) and the other fragments were removed by washing the beads at the annealing temperature. The attached fragments were labeled with [α -³²P]dATP (Perkin Elmer), resolved on sequencing gels. The dried gels were exposed to a Phosphorimager screen (GE Healthcare or Bio-Rad).

Northern blot analysis

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 (25). The RNA was fractionated by electrophoresis on formaldehyde-agarose gels (26), followed by transfer to Hybond N⁺ membranes (GE Healthcare). For, analysis of transcription driven by the plasmid-borne *GALI* promoter with different mutations or deletions, the pRS415 vector fragment (Fig. 1A, indicated by the open arch inside of the vector circle) was inserted into the multiple cloning site of plasmid pGEM-3Z (Promega). Radioactive RNA probes were made by *in vitro* transcription of the pRS415 fragment using T7 or SP6 RNA polymerases (26). Membranes were first hybridized with the respective probes. After exposure to Phosphorimager screens, the probes were stripped off by boiling the membranes in 1% SDS. The 25S rRNA on the stripped membranes was re-hybridized with probes generated from a 2.9 kb 25S DNA and exposed to Phosphorimager screens. The signal intensities of the 25S rRNA were served as loading controls.

Chromatin immunoprecipitation (ChIP) assay

ChIP was carried out as described previously with minor modifications (27,28). Cells were grown in minimal medium containing 2% galactose to late log phase ($A_{600} \approx 1.0$). Chromatin crosslinking was achieved by addition of 1.1 ml of 36.5% formaldehyde to 40 ml of culture (final concentration 1%). After 10 minutes of incubation, the action of formaldehyde was quenched with glycine (125 mM final concentration). Cells were resuspended in buffer A (50 mM HEPES pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1mM EDTA, and phosphatase and protease inhibitors) and lysed by vortexing with glass beads. The cell lysates were sonicated to shear the DNA to an average size of 500 bp and clarified by centrifugation at 16,000 $\times g$ for 10 minutes at 4°C. The supernatant was collected and subjected to repeated centrifugation until no visible pellet can be seen at the bottom of tube. An aliquot from each of the clarified lysates was saved as input control. The rest of the clarified lysates were incubated with or without affinity-purified anti-Rpb1 antibody 8WG16 (Neoclone) at 4°C overnight. Protein A-coated agarose beads (Sigma) were added to the samples and the

incubation was continued for 3 hr at 4°C. The beads were washed twice with buffer A, twice with buffer B (50 mM HEPES pH 7.5, 500 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1mM EDTA, and phosphatase and protease inhibitors), twice with buffer C (10 mM Tris-Cl pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM EDTA, and phosphatase and protease inhibitors) and twice with TE (10 mM Tris-Cl, 1mM EDTA, pH 8.0). Bead-bound chromatin fragments were eluted by incubating the beads in buffer D (50 mM Tris-Cl pH 8.0, 1% SDS and 10 mM EDTA) at 65 °C for 30 minutes. The elution was repeated one more time and the eluates were pooled. Crosslinks were reversed by incubating the eluates at 65°C overnight. The inputs and the eluates were treated with proteinase K and extracted once with phenol, once with phenol/chloroform/isoamylalcohol (25/24/1) and once with chloroform/isoamylalcohol (24/1). The DNA in the eluates was precipitated with ethanol.

The input and immunoprecipitated DNA were amplified by PCR. The pRS415 sequence of 210 bp located immediately downstream of the *GALI* sequence in the different plasmid constructs (Fig. 1A, indicated by the open arch inside of the plasmid circle) was amplified using primers 5'-GGGGGATCCACTAGTTCTAGAGC-3' and 5'-TGAGGTACCTCACTCATTAGGCACCCC-3'. To provide an internal control of Pol II loading onto a gene, a 109 bp fragment in the coding region of the *ACT1* gene was amplified using primers, 5'-CTGAGGTTGCTGCTTTGGTTATTG-3' and 5'-CTTGGTCTACCGACGATAGATGGG-3'. The PCR products were resolved on an agarose gel and stained with ethidium bromide. The band intensities on the gel were quantified using AlphaEase 4.0 software (Innotech). The signal intensities of the amplified pRS415 sequence of 210 bp were normalized to those of the *ACT1* sequence.

RESULTS

Modulation of Rad26 and Rpb9 mediated repair in the TS of the *GAL1* gene by TATA and UAS elements

The *GALI* gene is essential for yeast cells to grow in galactose media, because it encodes a galactokinase which phosphorylates galactose to galactose-1-phosphate in the first step of galactose catabolism (29,30). To examine the modulation of Rad26 and Rpb9 mediated repair by different gene elements, we created a number of plasmids bearing the *GALI* gene with different mutations or deletions. A 2 kb *GALI-10* region encompassing the shared UAS and ~700 bp coding region of each of the genes (Fig. 1A) was amplified by PCR. The *GALI* TATA mutation was achieved by changing the original sequence ATATAAA (21) to CCATGGA. The UAS mutation was achieved by changing the cognitive triplet sequences of CGG, AGG or CGC located at both ends of the four palindromic binding sites of the transcriptional activator Gal4 (Table 2) (31). The *GALI-10* fragments with normal, mutated *GALI* TATA or UAS sequences were inserted in the *Hind* III site of plasmid pRS415 (22) (Fig. 1A). The plasmid constructs were transformed into *GAL*⁺ *rad16* cells, to eliminate GGR and specifically analyze Rad26 and Rpb9 mediated repairs in the plasmid-borne *GALI* gene.

Total RNA was isolated from the cells cultured in the galactose medium to induce the *GALI* gene (29,30). Transcription driven by the plasmid-borne *GALI* promoter was analyzed by Northern blots. To distinguish transcription on the plasmid constructs from that on the genomic *GALI* gene, the pRS415 sequence located immediately downstream of the *GALI* sequence (Fig. 1A, indicated by the open arch inside of the plasmid circle) was used as a Northern blot probe. A transcript of ~ 3 kb can be seen from the normal *GALI* construct (Fig. 1B, arrow; lanes 1, 4 and 7). The transcription level in the mutated *GALI* TATA construct is much lower than that in the normal *GALI* construct (Fig. 1B. compare lanes 1 and 2, 4 and 5, and 7 and 8). Quantification of the data (not shown) suggests that mutation of the *GALI* TATA reduced the transcription level 50 – 100 fold. Essentially no transcription can be detected in the UAS

mutated construct (Fig. 1B, lanes 3, 6 and 9). These results indicate that the *GAL1* TATA is required for high levels of transcription, while the UAS is indispensable for transcription.

Total DNA was isolated from the transformed cells following UV irradiation and repair incubation in the galactose medium. To specifically analyze Rad26 and Rpb9 mediated repairs in the plasmid-borne *GAL1* fragments, the 2 kb plasmid-borne *GAL1-10* fragments (Fig. 1A) were gel purified following digestion of the total DNA with *Hind* III and incised at CPDs with excess amount of T4 endonuclease V (32). The gel purified fragments were further restricted to release the *GAL1* fragment of interest and strand specifically end labeled with [α -³²P]dATP (23,24). The labeled fragments were resolved on a DNA sequencing gel and exposed to a Phosphorimager screen.

In *rad16*, *rad16 rpb9*, *rad16 rad26* and *rad16 rad26 rpb9* cells, the repair patterns in the plasmid-borne normal *GAL1* gene (Fig. 2A – D) were very similar to those in the genomic *GAL1* gene (14,15). These results indicate that the transcription levels and repair patterns are similar in the plasmid-borne and genomic *GAL1* genes.

In *rad16* cells, where both the Rad26 and Rpb9 mediated repairs are active, and in *rad16 rpb9* cells, where only the Rad26 mediated repair is active (14,15), mutation of the *GAL1* TATA resulted in no significant repair deficiency in the TS of the *GAL1* gene (Fig. 2, compare panels A and E, and B and F). Furthermore, the mutation did not change the initiation site of repair, which is ~ 180 nucleotides upstream of the transcription start site and ~ 100 nucleotides from the UAS (Fig. 2A, B, E and F). In contrast, in *rad16 rad26* cells, where only the Rpb9 mediated repair is active (14,15), mutation of the *GAL1* TATA essentially abolished repair in the TS of the *GAL1* gene (Fig. 2, compare panels C and G). These results indicate that the initiation site and efficiency of Rad26 mediated repair in the TS of the *GAL1* gene are largely independent of the TATA element and transcription level. In contrast, the Rpb9 mediated repair is dependent on the gene element and a high level of transcription.

Repair was observed in the UAS-mutated *GAL1* gene in *rad16* and *rad16 rpb9* cells, although it was much less efficient in the transcribed region (Fig. 2H and I, marked by hatched bar on the right of the gels) of the gene. Furthermore, the UAS mutation abolished an apparent initiation site for this repair, as repair seemed to occur throughout the *GAL1-10* region analyzed (Fig. 2H and I, region marked with brace; data not shown). However, the UAS mutation essentially eliminated repair in *rad16 rad26* cells (Fig. 2J). These results suggest that the UAS and transcription may not be absolutely required for Rad26 mediated repair, but may be required for Rpb9 mediated repair. Furthermore, the UAS may determine the initiation site of the Rad26 mediated repair in the TS of the *GAL1* gene.

Modulation of Rad26 and Rpb9 mediated repair in the NTS of the *GAL1* gene by TATA and UAS elements

In all the *rad16* cells examined, no repair can be seen in the NTS of the normal *GAL1* construct (Fig. 3A, B and C). However, mutation of either TATA or UAS resulted in repair in the NTS of the *GAL1* gene in *rad16* and *rad16 rpb9* cells (Fig. 3, compare panels A, D and G, and B, E and H), but not in *rad16 rad26* cells (Fig. 3, compare panels C, F and I). These results suggest that the TATA and UAS elements and a substantial level of transcription are essential for preventing the Rad26 mediated repair from occurring in the NTS of the *GAL1* gene. However, the Rpb9 mediated repair does not operate in the NTS of the gene in all the conditions.

The role of local DNA sequences in Rad26 and Rpb9 mediated repair

The studies described above suggest that the initiation of Rad26 mediated repair may be determined by the UAS, but is independent of the TATA element. Furthermore, the efficiency

of the Rad26 mediated repair does not seem to be significantly affected by active transcription. However, a low level of transcription still occurred in the *GAL1* TATA mutated construct (Fig. 1B, lanes 2, 5 and 8). It is possible that the mutated TATA or a nearby local sequence may be able to partially compensate for the TATA element, resulting in a low level of transcription and the initiation of Rad26 mediated repair. To examine this possibility, a series of *GAL1-10* fragments, encompassing ~ 700 bp of the *GAL10* coding sequence, the UAS and different lengths of the *GAL1* sequence (Fig. 4A) were amplified by PCR. These PCR fragments were inserted in the *Hind* III site of plasmid pRS415 (Fig. 1A). Plasmid constructs bearing the different *GAL1-10* fragments were transformed into yeast cells. Total RNA was isolated from the transformed cells cultured in the galactose medium. Transcription driven by the *GAL1* gene promoter borne on the plasmid constructs was analyzed by Northern blots, using the vector sequence located immediately downstream of the *GAL1* sequence (Fig. 1A, indicated by the open arch inside of the plasmid circle) as a probe. Strong transcription can be seen when the *GAL1* gene was deleted down to +14 (relative to the transcription start site) (*GAL1Δ* +14) (Fig. 4B, lanes 4, 8 and 12). A further deletion, which left the TATA element intact but had the *GAL1* transcription start site removed (i.e. deletion down to -72, *GAL1Δ*-72), also showed strong transcription (Fig. 4B, lanes 3, 7 and 11). However, deletion down to -111 or -185, each of which removed the TATA element, caused undetectable transcription from the sequence (Fig. 4B, lanes 1, 2, 5, 6, 9 and 10).

Total DNA was isolated from the transformed cells following UV irradiation and repair incubation. The plasmid fragments encompassing the UAS, part of the *GAL1* sequence and a 740 bp pRS415 sequence immediately downstream of the *GAL1* sequence (Figs. 1A and 4A) were released by restriction, and incised at the CPDs with excess amount of T4 endonuclease V. The plasmid fragments were purified from an agarose gel and strand specifically end labeled with [α -³²P]dATP.

In *rad16* cells, where both Rad26 and Rpb9 mediated repairs are operative, and in *rad16 rpb9* cells, where only Rad26 mediated repair is active, efficient repair can be seen in the TS of all the plasmid constructs, even in those without the TATA and detectable transcription (Fig. 5A, B, D, E, G, H, J and K). In all the constructs, repair initiates ~ 100 nucleotides from the UAS (Fig. 5, marked by open arrows). Interestingly, the approximate initiation sites of the repair can be located in the *GAL1* sequence (Fig. 5, panels D, E, G, H, J and K; marked by solid lines), or the pRS415 sequences (Fig. 5, panels A and B; marked by the dotted line). These results indicate that the initiation site and efficiency of Rad26 mediated repair in the TS of the *GAL1* gene are indeed not significantly affected by the TATA sequence, or even actual transcription. Moreover, the Rad26 mediated repair event may not be significantly influenced by other local sequences (e.g., transcription start site, sequence where Rad26 mediated repair initiates and the sequence adjacent to the TATA box).

In *rad16 rad26* cells, where only the Rpb9 mediated repair is active, efficient repair can be seen in the transcribed regions of the constructs that contain the TATA element [Fig. 5I and L, see the regions above the shaded (panel I) and solid (panel L) arrows]. In contrast, little repair can be seen in the same sequences borne on the constructs that do not have the TATA element (Fig. 5C and F). These results further suggest that the Rpb9 mediated repair is largely dependent on the TATA and active transcription.

UAS does not load Pol II onto the GAL1 promoter in the absence of TATA

The experiments described above suggest that the initiation and efficiency of Rad26 mediated repair in the *GAL1* TS are determined by the UAS, but are largely independent of either TATA or actual transcription. One explanation is that the UAS may be able to load Pol II in the absence of TATA, and the loading of Pol II may be sufficient to initiate Rad26 mediated repair, regardless of actual transcription. To test this idea, we examined loading of Pol II onto the

GAL1 promoter in the plasmid constructs bearing different *GAL1-10* fragments (Fig. 4A). Yeast cells transformed with the different plasmid constructs and the empty pRS415 vector (as control) were cultured in galactose medium to late log phase, and ChIP analysis was performed using a monoclonal antibody to Rpb1, the largest subunit of Pol II (33). A 210 bp pRS415 sequence immediately downstream of the *GAL1* sequence (Fig. 1A, marked with the open arch inside of the plasmid circle) was amplified by PCR. As shown in Fig. 6, the 210 bp pRS415 sequence in the *GAL1Δ+14* was immunoprecipitated by the anti-Rpb1 antibody. In contrast, the levels of the pRS415 sequence pulled down by the antibody are similar between the *GAL1Δ-185* construct and the empty pRS415 vector (Fig. 6). These results indicate that the UAS element may not be able to recruit Pol II to the *GAL1* promoter in the absence of TATA. Furthermore, the modulation of the Rad26 mediated repair by the UAS must be by a mechanism that is independent of Pol II.

Deletion of *SPT7* dramatically compromises Rpb9-, but not Rad26-mediated repair

SAGA, a transcriptional regulatory complex, plays an important role in transcription of the *GAL1* gene (34,35). At the *GAL1* promoter, SAGA is first recruited by the Gal4 activator to the UAS. The UAS-bound SAGA then facilitates the binding of TATA binding protein to the core promoter, thereby stimulating transcription (34,35). It has been proposed that the histone acetyltransferase activity of Gcn5 in the SAGA complex, remodels local chromatin structure and subsequently facilitates binding of the TATA binding protein (36). Spt7 is one of the SAGA subunits that are essential for the recruitment of SAGA to the *GAL1* promoter (37).

It is possible that the effect of the UAS on the Rad26 and Rpb9 mediated repair is through the recruitment of the SAGA complex to the *GAL1* promoter. To test this hypothesis, we analyzed NER in the genomic *GAL1* gene in cells with the *SPT7* gene deleted. In *spt7* cells, efficient repair occurred in the whole *GAL1* fragment, including the upstream regions where TCR did not operate (Fig. 7A). The initiation site and efficiency of repair in *rad7 spt7* cells (Fig. 7B) were very similar to those in *rad16* (Fig. 2A) or *rad7* (not shown) cells, where GGR are inactive. Similar to *rad16 rad26* cells (Fig. 2C), *rad7 rad26* cells showed very efficient repair in the *GAL1* gene (not shown). However, deletion of *SPT7* essentially abolished repair in *rad7 rad26* cells (Fig. 7C). These results indicate that the recruitment of SAGA complex to the *GAL1* promoter plays little role in the Rad26 mediated repair. In contrast, this recruitment is critical for Rpb9 mediated repair.

DISCUSSION

We showed that in the TS of the *GAL1* gene, the initiation site and efficiency of Rad26 mediated repair are dependent on the UAS, but largely independent of the TATA, other local sequences or even actual transcription. Furthermore, if the transcription level is too low (e.g., if the TATA is mutated) or absent (e.g, if the TATA is deleted or the UAS is mutated), Rad26 mediated repair occurs not only in the TS, but also in the NTS. These results are surprising in view of the widely accepted notion that Rad26 is a “transcription-repair coupling factor”.

The mechanism by which the UAS determines the initiation site of Rad26 mediated repair in the TS of the *GAL1* gene remains to be determined. Pol II may not play a role in the initiation, as our ChIP analysis suggests that the UAS is unable to recruit Pol II in the absence of TATA (Fig. 6). Also, SAGA facilitated chromatin remodeling or transcription in the *GAL1* gene seems to be dispensable for initiation of the repair, as deletion of *SPT7* did not change the initiation (Fig. 7). One possible scenario is that Rad26 is directly or indirectly recruited to the initiation site of the repair, by, for example, interacting with a transcription mediator. Alternatively, Rad26 mediated repair may be intrinsically operative in nontranscribed regions (nontranscribed genes or inter-gene regions). The UAS may simply set up a boundary at about -180 nt in the *GAL1* gene. The Rad26 mediated repair may be inhibited beyond the boundary.

A question remains as to how Rad26 mediates repair in both strands of the *GAL1* gene when the TATA or UAS is mutated or deleted. It seems that the Rad26 mediated repair will occur in both strands of a gene whenever transcription is very low or absent. The *GAL1-10* genes are highly induced by galactose, but completely repressed in glucose media (29,30). We found that efficient repair, which is dependent on Rad26 but not on Rpb9 or Rad16, also occurs in both strands of the glucose repressed *GAL1-10* genes in *rad16* cells (not shown). Rad26 (38) and its human homologue CSB (39) are DNA-dependent ATPase of the SWI2/SNF2 family proteins, which are implicated in chromatin remodeling during transcription (40). *In vitro* studies have shown that CSB is able to remodel chromatin structure at the expense of ATP hydrolysis (41). It is possible that Rad26 mediates repair in both strand of a DNA sequence that is not transcribed or very scarcely transcribed, by increasing repair protein access to sites of DNA damage in chromatin. Alternatively, a very low level of non-productive form of Pol II may be gratuitously associated with and “patrols” on both strands of a DNA sequence that is not actively transcribed (e.g., in search for transcription start sites). Rad26 may mediate repair by facilitating the “patrol” process of the non-productive form of Pol II along the DNA.

It has been shown that, in two mutant Chinese hamster ovary cell lines in which the entire *APRT* promoter region has been deleted, NER is still efficient in both strands of the promoterless *APRT* gene, even though neither strand appears to be transcribed (42). Rodent cells are profoundly deficient in GGR of CPDs, typically showing efficient TCR in the TS of actively transcribed genes, but little repair in the NTS, or in nontranscribed regions of the genome (4,43,44). Therefore, a transcription-independent non-GGR mechanism may also exist in mammalian cells. It would be interesting to test if the efficient NER in the promoterless *APRT* gene is dependent on CSB, the human homologue of yeast Rad26.

In contrast to Rad26, Rpb9 seems to mediate a repair mechanism that is strictly coupled to transcription, which is conceivable in view of the fact that Rpb9 itself is a subunit of Pol II. The Rpb9 mediated repair seems to be effective only when a high level of transcription is present, as mutation of the TATA sequence, which dramatically reduces transcription, or deletion of the TATA sequence or mutation of UAS, which eliminated transcription, essentially abolishes the Rpb9 mediated repair. We also found that the Rpb9 mediated repair does not operate in the *GAL1-10* genes in glucose cultured *GAL4⁺* cells or in galactose cultured *gal4* cells (not shown).

Following analysis of NER in different genes (15,16,18,19), it became obvious that the relative contributions of Rad26 and Rpb9 to TCR are different from gene to gene. This difference could simply be due to different levels of transcription in different genes. For highly expressed genes, such as the galactose induced *GAL1-10* genes, the Rpb9 mediated TCR is very effective, making Rad26 less important for TCR (14,15). For slowly transcribed genes, such as the constitutive *RPB2* (15,18,19) or *URA3* (16) genes, the Rpb9 mediated TCR is less effective or essentially absent and Rad26 contributes much more to the overall TCR.

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The abbreviations used are

CPD	cyclobutane pyrimidine dimer
CSA	Cockayne syndrome group A
CSB	Cockayne syndrome group B
GGR	global genomic repair
NER	nucleotide excision repair
NTS	nontranscribed strand
PCR	polymerase chain reaction
TCR	transcription coupled repair
TS	transcribed strand
UAS	upstream activating sequence
UV	ultraviolet

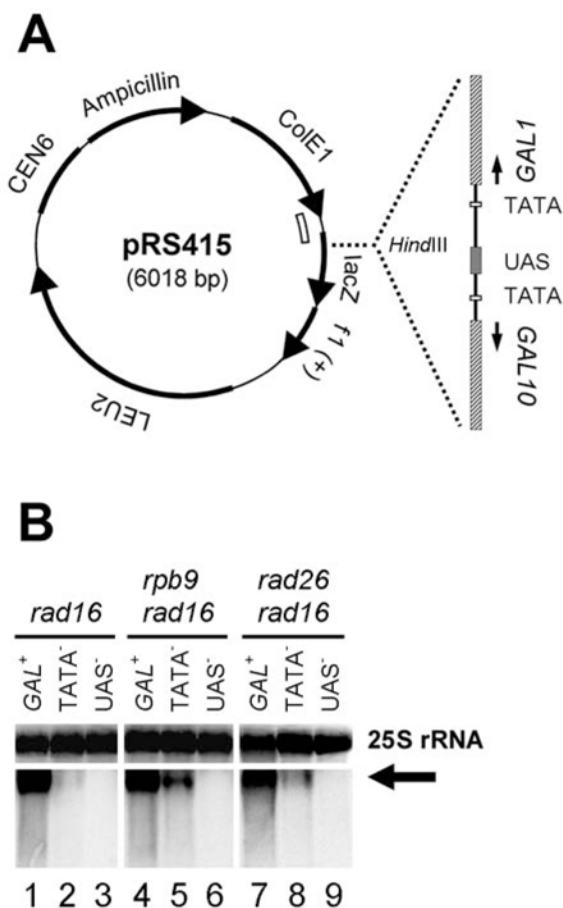


Fig 1. Transcription in plasmid-born *GALI* gene

A. Schematic showing the structure of the plasmid constructs bearing the 2 kb *GALI-10* fragments encompassing the shared UAS and ~ 700 bp of each of the genes. The open arch inside of the pRS415 circle indicates the sequence used for generating Northern blot probes. B. Northern blot showing transcription in the constructs with normal *GALI* (*GAL*⁺), mutated *GALI* TATA (*TATA*⁻) and mutated UAS (*UAS*⁻). The arrow indicates transcripts from the constructs. 25S rRNA serves as internal loading control.

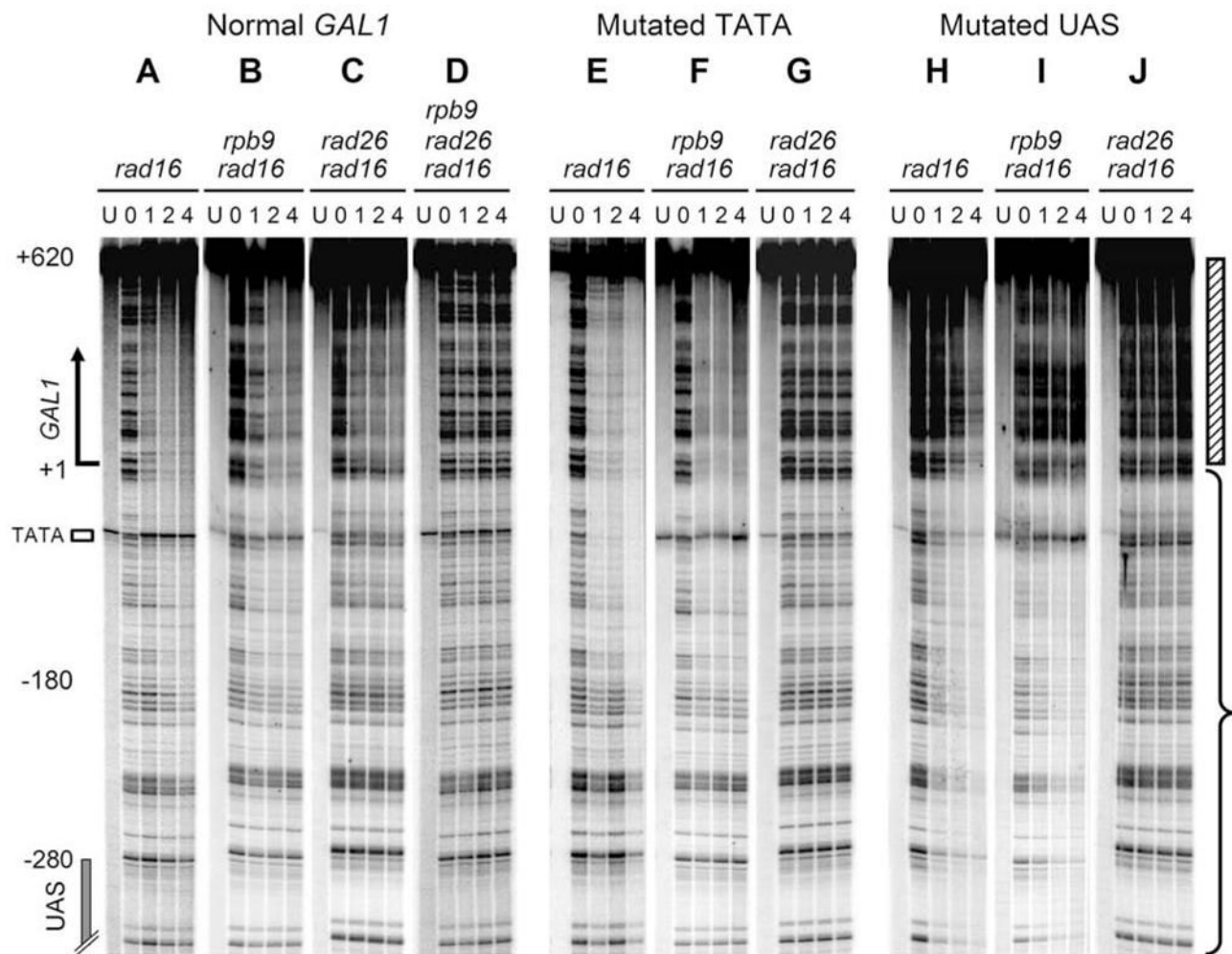


Fig 2. Rad26 and Rpb9 mediated repairs in the TS of plasmid-borne *GAL1* gene
 Lanes *U* represent unirradiated samples. Lanes *0*, *1*, *2* and *4* indicate different times (hour) of repair incubation following UV irradiation. Hatched bar and brace on the right of the gels mark the transcribed and upstream regions, respectively.

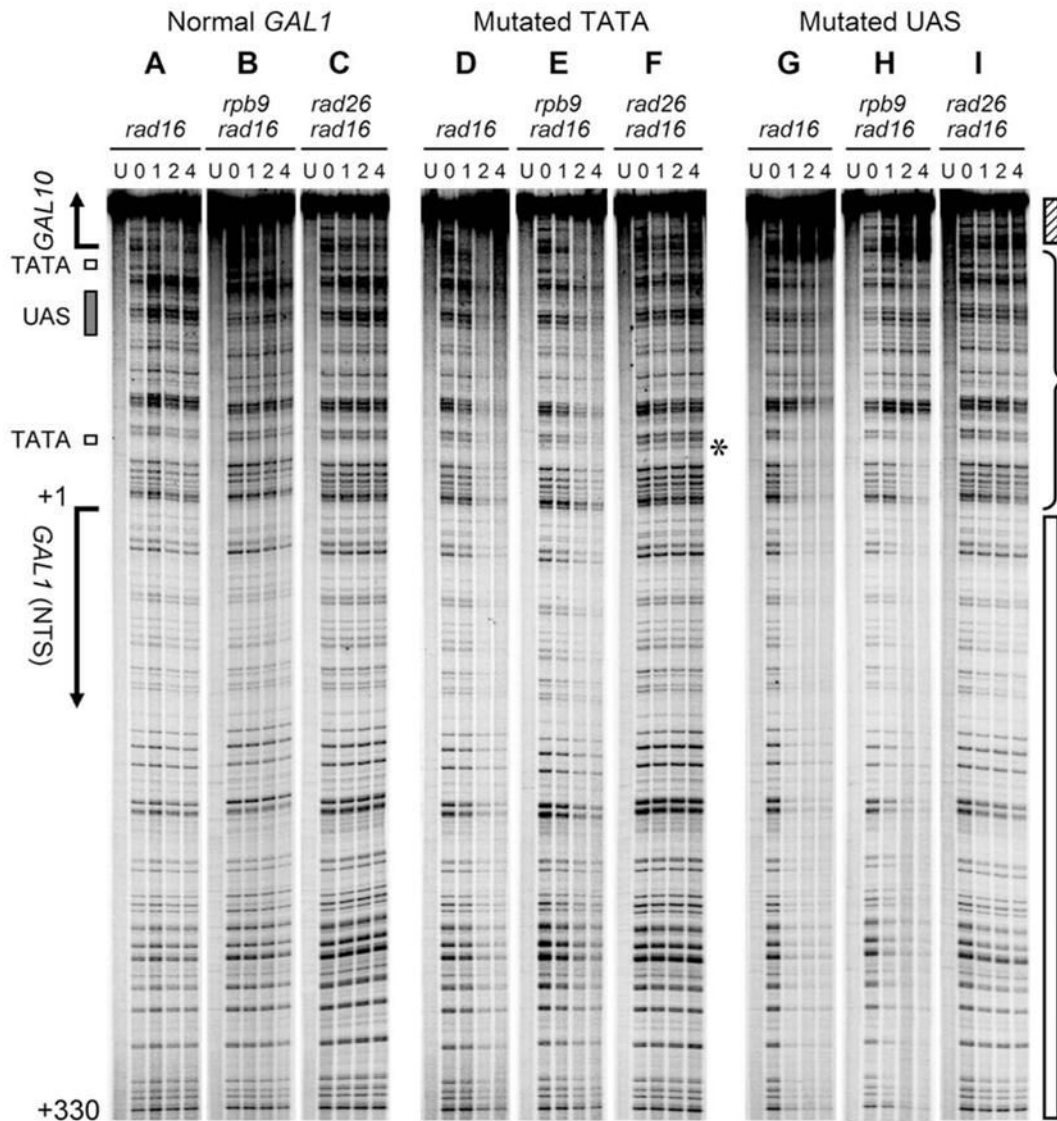


Fig 3. Rad26 and Rpb9 mediated repairs in the NTS of plasmid-borne *GAL1* gene

Lanes *U* represent unirradiated samples. Lanes *0*, *1*, *2* and *4* indicate different times (hour) of repair incubation following UV irradiation. The asterisk on the right of panel F indicates a CPD site (CC) present only in the mutated (CCATGGA) (panels D, E and F) but not in the normal (ATATAAA) *GAL1* TATA. Hatched and open bars on the right of the gels mark the transcribed regions of the *GAL10* (TS) and *GAL1* (NTS), respectively. Brace indicates the upstream region.

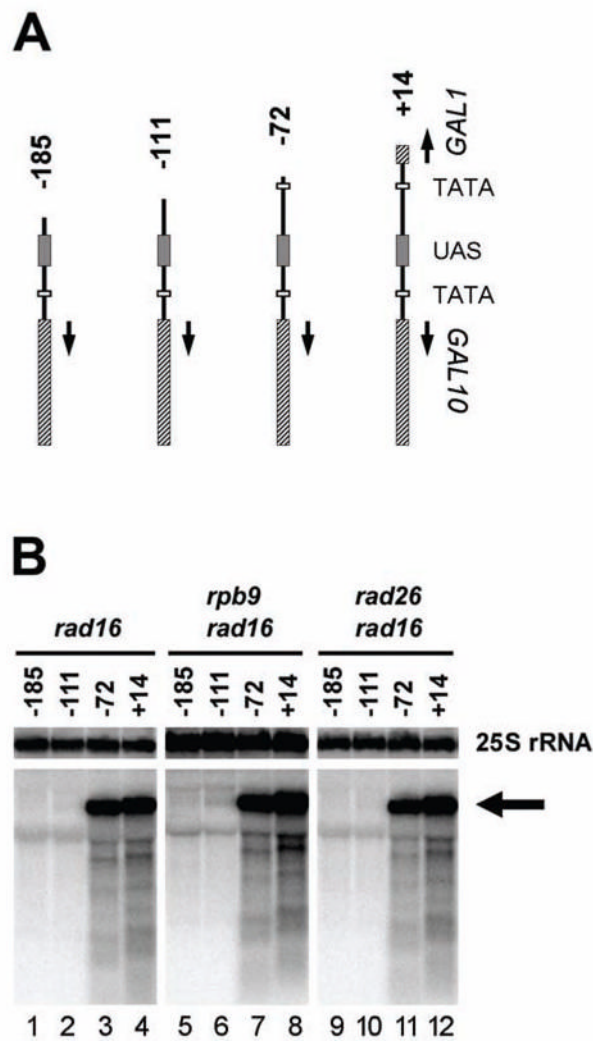


Fig 4. Transcription driven by the promoter of plasmid-born *GAL1* gene with different deletions
 A, structures of the *GAL1-10* genes with different deletions from the *GAL1* side. These *GAL1-10* fragments were inserted into the *Hind* III site of vector pRS415 (see Fig. 1A) to create different plasmid constructs. B, Northern blot showing transcription driven by the *GAL1* promoter. The arrow indicates transcripts from the constructs. The Northern blot probe was made from the pRS415 vector sequence that is immediately downstream of the inserted *GAL1-10* fragment (see Fig. 1A, marked by the open arch). 25S rRNA serves as internal loading control.

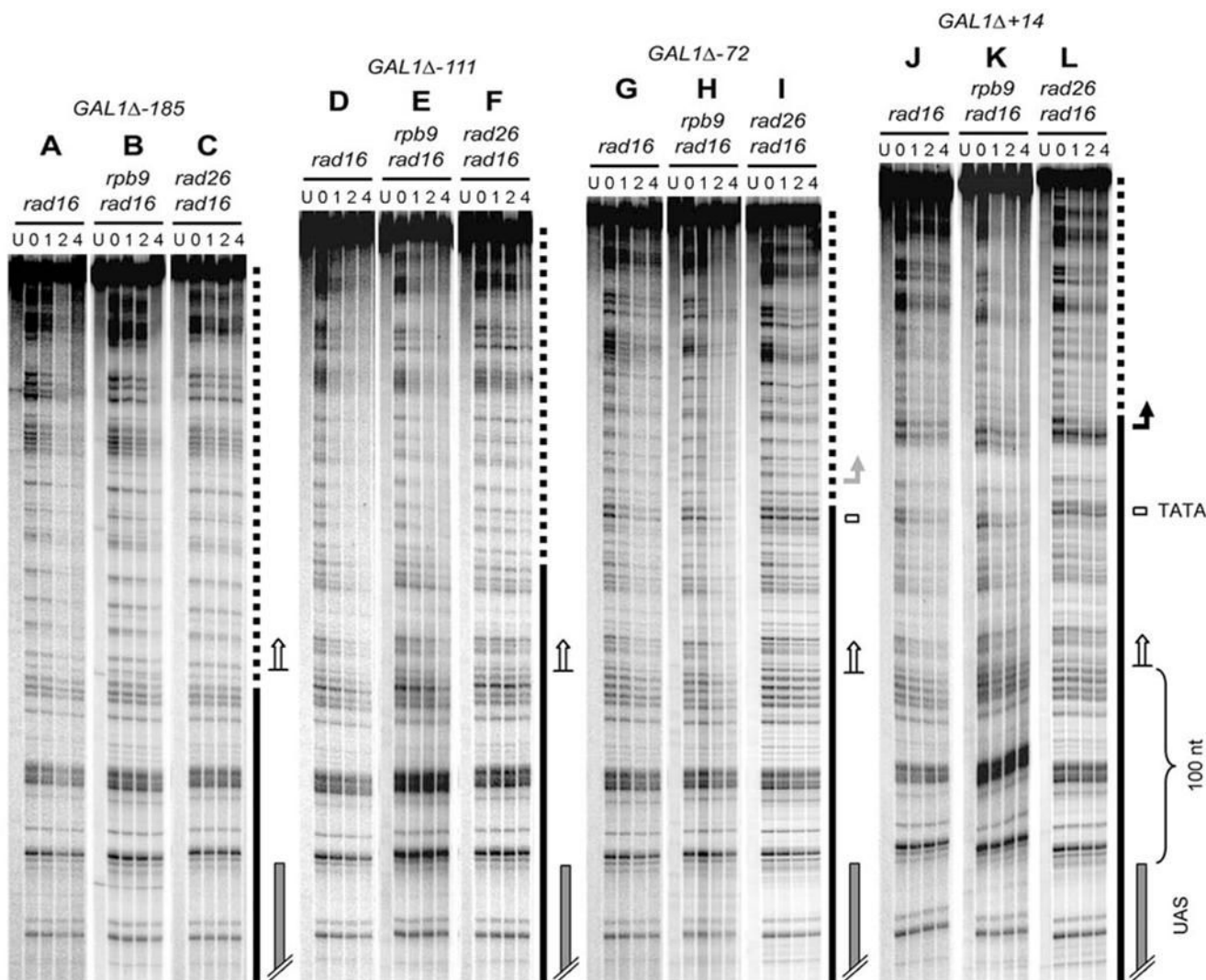


Fig 5. Rad26 and Rpb9 mediated repairs in the TS of plasmid constructs with different deletions from the *GAL1* gene

Lanes *U* represent unirradiated samples. Lanes *0*, *1*, *2* and *4* indicate different times (hour) of repair incubation. Solid and dotted lines indicate *GAL1* and vector pRS415 sequences on the constructs, respectively (see Figs. 1A and 4A). Open arrows depict the approximate initiation sites of repair. Solid bent arrow on the right of panel L indicates the *GAL1* transcription start site borne on construct *GAL1* Δ +14. Shaded bent arrow on the right of panel I marks the transcription start site driven by the *GAL1* promoter borne on construct *GAL1* Δ -72.

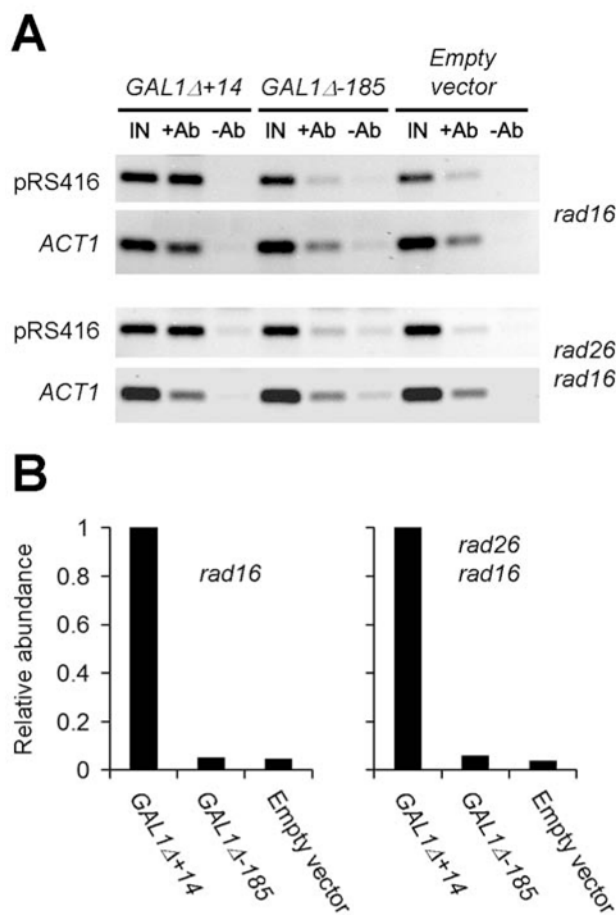


Fig 6. ChIP assay showing loading of Pol II onto a plasmid sequence by a *GAL1* promoter
 The structures of the empty pRS415 vector and the plasmid constructs *GAL1Δ+14* and *GAL1-185* are shown in Figs. 1A and 4A, respectively. The pRS415 sequence amplified by PCR was a 210 bp fragment located immediately downstream of the *GAL1* sequence (Fig. 1A, marked with the open arch inside of the plasmid circle), using template DNA isolated from CHIP input (IN), immunoprecipitated with anti-Rpb1 (+Ab), or mock immunoprecipitated (-Ab) samples. The 109 bp *ACT1* sequence was amplified under the same conditions as the 210 bp pRS415 sequence, to offer an internal control. Plots shown in panel B are relative amounts of the 210 bp pRS415 sequence immunoprecipitated with anti-Rpb1 antibody. The loading was normalized according to the signal intensities of the internal control *ACT1* fragment.

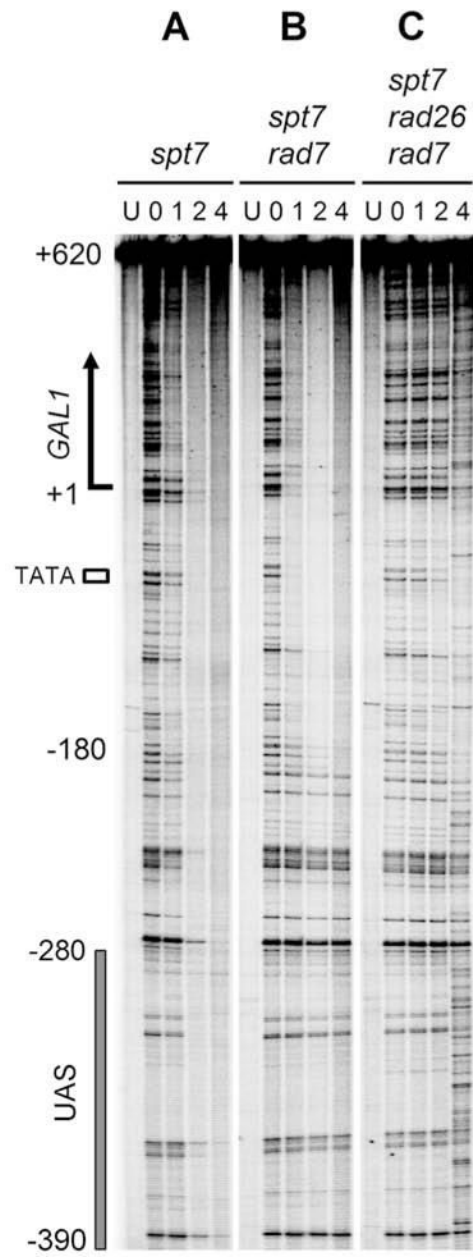


Fig 7. Repair in the TS of the genomic *GAL1* gene in *spt7* cells

Lanes *U* represent unirradiated samples. Lanes *0*, *1*, *2* and *4* indicate different times (hour) of repair incubation.

Table 1
PCR primers used to create plasmids bearing different *GALI-10* fragments

Primer	Sequence (5' → 3') ^a	Location
1	GATGTA <u>AAAGCTT</u> CTCGCGCTAC	<i>GALI0</i> , +700
2	AAACA <u>AGCTT</u> AGCCTGATCCATACCGC	<i>GALI</i> , +700
3	ACAGAT <u>CCATGG</u> ATGCAAAAAGTGCATAACCACTTT	<i>GALI</i> , TATA
4	TTGCAT <u>CCATGG</u> ATCTGTTAATAGATCAAAAATCATCGC	<i>GALI</i> , TATA
5	CTCCT <u>GCA</u> TGCGTCCTCGTCTTCACCGTTCGCGTTCCTGAAACGCAGATGTGCCTTGCGCCGCACTGCT	UAS
6	AGGAC <u>GCATGC</u> AGGAGAGTCTCCATTGGAGGGCTGTCAACCACTTGGCGGCTTCTAATC	UAS
7	TTAA <u>AGCTT</u> TTTTGTTGATACTTTTATTACATTTG	<i>GALI</i> , +14
8	TATGA <u>AGCTT</u> TTGCATTTATATATCTGTTAATAGAT	<i>GALI</i> , -72
9	ATCAA <u>AGCTT</u> CGCTGATTAATTACCCCA	<i>GALI</i> , -111
10	TAATA <u>AGCTT</u> CGTTCATTTGAAGGTTTGTGG	<i>GALI</i> , -185

^aUnderlined are native or introduced restrict sites (*Hind* III for primers 1, 2, 7 – 10, *Sph* I for primers 5 and 6, and *Sty* I for primers 3 and 4); letters in bold are introduced mutations.

Table 2Mutations in the UAS of plasmid borne *GAL1* gene^a

Gal4 sites	Normal sequence	Mutated sequence
I	<u>CGGATTAGAAGCCGCGG</u>	<u>CGGATTAGAAGCCGCCA</u>
II	<u>CGGGTGACAGCCCTCCG</u>	<u>TGGGTGACAGCCCTCCA</u>
III	<u>AGGAAGACTCTCCTCCG</u>	<u>TGGAAGACTCTCTGCA</u>
IV	<u>CGGCCGCACTGCTCCG</u>	<u>TGCCCGCACTGCTCCG</u>

^aGal4 cognitive triplet nucleotide sequences are underlined; nucleotides mutated are shown in bold.