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The Ubiquitin-Conjugating Enzyme Rad6 (Ubc2) Is Required for Silencing in *Saccharomyces cerevisiae*

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It has been previously shown that genes transcribed by RNA polymerase II (RNAP II) are subject to position effect variegation when located near yeast telomeres. This telomere position effect requires a number of gene products that are also required for silencing at the *HML* and *HMR* loci. Here, we show that a null mutation of the DNA repair gene *RAD6* reduces silencing of the *HM* loci and lowers the mating efficiency of *MAT*a strains. Likewise, *rad6*-Δ reduces silencing of the telomere-located RNAP II-transcribed genes *URA3* and *ADE2*. We also show that the RNAP III-transcribed tyrosyl tRNA gene, *SUP4-o*, is subject to position effect variegation when located near a telomere and that this silencing requires the *RAD6* and *SIR* genes. Neither of the two known Rad6 binding factors, Rad18 and Ubr1, is required for telomeric silencing. Since Ubr1 is the recognition component of the N-end rule-dependent protein degradation pathway, this suggests that N-end rule-dependent protein degradation is not involved in telomeric silencing. Telomeric silencing requires the amino terminus of Rad6. Two *rad6* point mutations, *rad6(C88A)* and *rad6(C88S)*, which are defective in ubiquitin-conjugating activity fail to complement the silencing defect, indicating that the ubiquitin-conjugating activity of *RAD6* is essential for full telomeric silencing.

Telomeres are generally organized into heterochromatin in a wide range of organisms, including mammals, insects, and plants (30). Although there is no direct cytological evidence to support the presence of heterochromatin in *Saccharomyces cerevisiae*, the finding that transcription of genes near yeast telomeres is reversibly repressed suggests the existence of heterochromatin at yeast telomeres (11). A similar position effect is also exhibited by the *HM* loci, where both RNA polymerase II (RNAP II)- and RNAP III-transcribed genes have been shown to be affected (17, 20). Several regulatory factors are shared by the *HM* loci and telomeres, including Sir2, Sir3, Sir4, Nat1, Ard1, and histones H3 and H4 (1, 40, 57). The involvement of the amino termini of histones H3 and H4 in both processes and the inaccessibility of both the *HM* and the telomeric domains to restriction enzymes in isolated nuclei (29) and to ectopically expressed bacterial *dam* methylase in vivo (12, 49) support the notion that telomeres and *HM* loci nucleate a closed chromatin state that confers transcriptional repression. Furthermore, the terminal tracts of $C_{1-3}A$ repeats are protected in a telosome particle, which is larger than a nucleosome, while the subtelomeric repeats X and Y′ are assembled in normal nucleosomes (60). Telomeric repeats ($C_{1-3}A$) contain a high density of Rap1 binding sites. Rap1 is thought to recruit Sir3 and Sir4 to telomeres through its carboxyl-terminal domain (14). The complex nucleated by Rap1, Sir3, Sir4, and histones has been detected by coimmunoprecipitation. Sir3 is known to spread from telomeres into adjacent regions (15, 38), and the extent of telomeric silencing is dependent upon the *SIR3* gene dosage (38). This indicates that telomeric silencing may be sensitive to the intracellular concentration of silencing factors.

One of the yeast protein degradation pathways involves *RAD6*-mediated ubiquitination (8, 52). The Rad6 protein has been shown to ubiquitinate histones H2A, H2B, and H3 in vitro (13, 53). Mutations in *RAD6* affect DNA repair, damage-induced mutagenesis, and sporulation (36). We previously showed that mutations in *RAD6* also alter the preferential integration of Ty1 elements into the 5′ ends of genes (18, 28, 35). Here, we report that the ubiquitin-conjugating activity of Rad6 is required for full silencing at the *HM* loci and at telomeres. We further show that this effect on silencing requires neither the N-end rule pathway nor the DNA repair pathway, since neither *ubr1*-Δ nor *rad18*-Δ affects silencing. These results identify a novel role of *RAD6* in modulating chromatin structure.

MATERIALS AND METHODS

Plasmid constructions. The wild-type *RAD6* and *rad6* mutant alleles used in this study, *rad6(C88A)*, *rad6(C88S)*, *rad6-153*, and $rad6_{\Delta 1-9}$, were described previously (53–55, 59). Each *RAD6* allele was cloned into either the *CENVI HIS3* plasmid pRS413 (46) or the 2μm *TRP1 ADC1* promoter plasmid pSCW231 to yield plasmids pHH1, pHH3, pHH4, pHH10, pHH11, and pHH12 (Table 1). The *Cla*I-*Bam*HI fragment from the *RAD6* plasmid pR67 (the *Cla*I site in the 3′ noncoding region of *RAD6* is mutated) was inserted into *Cla*I-*Bam*HI-digested pRS413 to create pHH1. The *Eco*RI fragment of pR67 was used to replace the *Eco*RI fragment of pR628 to make pHH10. *Eco*RI fragments from plasmids pR647, pR642, pR628, and pR661 were used to replace the *Eco*RI fragment in pHH1, yielding plasmids pHH3, pHH4, pHH11, and pHH12, respectively.

To create a *UBR1* deletion-generating plasmid, the *Bam*HI-*Pst*I *LEU2* fragment from pJJ283 (21) was inserted into *Bam*HI-*Pst*I-digested pSOB29 (4), yielding plasmid pHU1. The 3.7-kb *ubr1::LEU2* fragment was cut out with *Dra*I and *Hin*dIII.

Plasmid pAK5S+ was constructed to place the tyrosyl tRNA ochre suppressor gene, *SUP4-o*, near a telomere without any other markers and without direct selection for *SUP4-o* expression. A *Sal*I-*Not*I fragment of pAK5S+ contains *ADH4* disrupted with *SUP4-o*, an 81-bp telomeric sequence (TG_{1-3}), and *URA3* (*adh4a-SUP4-o*-(TG_{1-3})-*URA3-adh4c*). The following steps were taken to con-

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TABLE 1. *RAD6* plasmids used in this study

Plasmid	Description	Reference
pR67	*RAD6 CENIV URA3*	54
pR647	*rad6(C88A) CENIV URA3*	54
pR642	*rad6(C88S) CENIV URA3*	55
pHH1	*RAD6 CENVI HIS3*	This study
pHH3	*rad6(C88A) CENVI HIS3*	This study
pHH4	*rad6(C88S) CENVI HIS3*	This study
pHH11	*rad6-153 CENVI HIS3*	This study
pHH12	$rad6_{\Delta 1-9}$ *CENVI HIS3*	This study
pHH10	*RAD6* 2μm *TRP1*	This study
pR628	*rad6-153* 2μm *TRP1*	53
pR661	$rad6_{\Delta 1-9}$ 2μm *TRP1*	59

struct pAK5S+. pAK1 was made by two-step PCR cloning of a multiple-cloning site into pADH4UCA-IV (11) (primer 1, 5′-AGATCTGGCT CGAGGGTCGC GAGACGGATC CCCCCTGCCA CCACA; primer 2, a T7 primer; primer 3, 5′-TCGCGACCCT CGAGCCAGAT CTGGGTAATA ACTGATATAA TTA AATTG; primer 4, 5′-GCAGTACTCT GCGGGTGTAT ACAGA). pAK3 was constructed by removing the *URA3* gene from pAK1 by digesting with *Hin*dIII and *Bgl*II, blunting, adding a phosphorylated *Hin*dIII linker, digesting with *Hin*dIII, and ligating. To construct pAK4, which replaces most of the *ADH4* gene and its promoter with *URA3* to create an *ADH4-URA3* cassette, the following steps were taken. pVZADH4 (2) was digested with *Eco*RI, blunted, and religated to eliminate the *Eco*RI site, forming pVZADH4(−RI). pVZURA3 was created by cloning a *Hin*dIII/*Sma*I fragment containing *URA3* (11) into pVZ1 (38) digested with *Sac*I and *Xba*I. pVZURA3 was then digested with *Not*I and *Sal*I, and the *URA3* fragment was ligated into pVZADH4(−RI) cut with *Hpa*I and *Hin*dIII to create pAK4. pAK4 was then digested with *Eco*RI and *Not*I, and the *URA3-adh4* fragment was ligated into pAK3 digested with *Eco*RI and *Not*I to form pAK5. Finally, pAK5S+ was constructed by cloning an *Eco*RI fragment containing *SUP4-o* from pYC226 (44) into the *Hin*dIII site of pAK5, such that the orientation of transcription would be toward the telomeric TG sequence.

To construct a vector that could place *SUP4-o* at the nontelomeric *ADH4* locus without other markers, an *Eco*RI fragment from pYC226 containing *SUP4-o* was ligated into pVZADH4(−RI) digested with *Hin*dIII and *Hpa*I. The resulting plasmid, containing *adh4::SUP4-o*, was digested with *Sal*I and *Not*I and ligated into pRS306 (*URA3* YIp) digested with *Sal*I and *Not*I (46) to form pRS306-adh4::SUP4(+).

Standard DNA manipulation procedures were followed.

Culture media and strains. Standard media were used to grow yeast (45) and bacteria (43). Nutritional markers and silencing were scored on the basis of growth on synthetic complete media, each lacking a specific component (e.g., Ura [−Ura medium]). Complete medium was used as a control. The medium used for determining resistance to 5-fluoro-orotic acid (FOA) was −Ura medium containing 1 g of FOA per liter and 12 mg of uracil per liter (+FOA medium). Growth on +FOA medium indicates inactivation of the *URA3* gene (5). *S. cerevisiae* was grown at 30°C, and liquid cultures were agitated at 200 rpm during incubation. All liquid-culture experiments were carried out with mid-log-phase cells. For viability experiments, both wild-type *RAD6* and *rad6*-Δ strains were grown in yeast extract-peptone-dextrose complete medium (YPD) supplemented with 40 mg of uracil per liter. Cell dilutions were spread on plates containing complete, −Ura, and +FOA media. For *rad6*-Δ strains, up to 2×10^7 cells were spread on +FOA medium plates. The plates were incubated at 30°C for 4 days.

S. cerevisiae was transformed by using the lithium acetate procedure (19). The yeast strains used are listed in Table 2. Strains L1503, L1642, L1643, L1671, and L1673 were obtained by transforming strains UCC1021, UCC3537, UCC4543, UCC3511, and UCC3515, respectively, with the *rad6* deletion-generating plasmid pJJ105 (*rad6::LEU2*) cut with *Hin*dIII and *Bam*HI. Both standard genetic complementation tests and PCR were used to confirm the presence of each of the deletions. Strains L1654 and L1660 were obtained by transforming UCC1021 and UCC4543 with plasmid pJJ202 (*rad18::LEU2*) cut with *Hin*dIII and *Bam*HI. Standard genetic complementation was used to confirm the presence of genomic deletions of *RAD18*. Strains L1657 and L1661 were obtained by transforming UCC1021 and UCC4543 with pHU1 (*ubr1::LEU2*) cut with *Dra*I and *Hin*dIII. Southern blot analysis was used to confirm the presence of genomic deletions of *UBR1*.

Strain UCC4541 was constructed by allelic replacement by the integration-excision method. pRS306-adh4::SUP4(+) was cut with *Bam*HI and integrated into YPH499 (46). Ura^+ transformants were plated on +FOA media in order to select for recombination events that excised the *URA3* gene from the *adh4* locus, leaving the *adh4::SUP4-o* allele. Strain UCC4543 was constructed in a two-step process. The *Sal*I-*Not*I fragment from pAK5S+ containing *adh4a-SUP4-o*-(TG_{1-3})-*URA3-adh4c* was transformed into YPH499. Ura^+ transformants were plated on +FOA media to select for chromosome breakage events that caused the loss of the *URA3* gene and created a new telomere by using the 81 bp of the TG_{1-3} sequence as a telomeric seed. This resulted in a strain that contained *SUP4-o* next to a telomere, without any known promoter in the vicinity. To disrupt *SIR2*, plasmid pJR531 (24) digested with *Sph*I and *Eco*RV was used to transform UCC4543, with selection for His^+ transformants. To disrupt *SIR3*, plasmid pΔSIR3HIS3 (1) was digested with *Eco*RI and used to transform UCC4543 as described above. To disrupt *SIR4*, plasmid pRS4.2 (24) was digested with *Pvu*II and used to transform UCC4543 as described above. All of the above-mentioned strains were confirmed by Southern blot hybridization.

Scoring telomeric silencing. Silencing of the telomere-located *URA3* gene was scored on the basis of growth on −Ura medium and +FOA medium plates. The strains were grown in YPD to an optical density at 600 nm of about 0.8 to 1.0. The cells were harvested, washed, and resuspended in distilled H_2O at a concentration of about 2×10^8/ml. Five microliters of 10-fold serial dilutions were spotted on plates containing complete, −Ura, and +FOA media, which were then incubated at 30°C for 3 days. Since growth on the −Ura medium plates was essentially identical to growth on the complete medium plates, the former are not shown in the figures. For *ADE2* red- and white-sector experiments, strains were grown in YPD with 40 mg of adenine per liter, spread onto YPD plates, and incubated at 30°C for 3 and 5 days (wild-type *RAD6* and *rad6*-Δ strains, respectively), until the colonies were of approximately equal sizes. The plates were stored at 4°C for 1 or 2 weeks to allow development of the red color before pictures were taken. *SUP4-o* expression was scored on the basis of both colony color on YPD and growth on −Ade medium.

Quantitative mating assay. Quantitative matings were done as described previously (51), with slight modifications. Two pairs of isogenic strains, LP2752-4B–L1249 and UCC3537-L1642, were used. Equal numbers of cells of *MAT***a** and *MAT*α strains were mixed, placed on nitrocellulose filters, and incubated at 30°C

TABLE 2. Yeast strains used in this study

Strain	Description	Reference
UCC1021	*MAT***a** *ura3-52 lys2-801:dam$^+$:LYS2 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 URA3-TEL-VII-L*	12
L1503	UCC1021 *rad6::LEU2*	This study
L1654	UCC1021 *rad18::LEU2*	This study
L1657	UCC1021 *ubr1::LEU2*	This study
UCC3537	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 URA3-TEL-VII-L ADE2-TEL-V-R*	48
L1642	UCC3537 *rad6::LEU2*	This study
UCC4541	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 adh4::SUP4-o*	This study
UCC4543	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 SUP4-o-TEL-VII-L*	This study
UCC4545	UCC4543 *sir2::HIS3*	This study
UCC4546	UCC4543 *sir3::HIS3*	This study
UCC4547	UCC4543 *sir4::HIS3*	This study
L1643	UCC4543 *rad6::LEU2*	This study
UCC3511	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 hmr::URA3*	48
L1671	UCC3511 *rad6::LEU2*	This study
UCC3515	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 hml::URA3*	47
L1673	UCC3515 *rad6::LEU2*	This study
LP2752-4B	*MAT*α *his4-260,39::pBR313 his4-864,1176 lys1-1 ura3-52*	32
L1249	LP2752-4B *rad6::ura3*	35

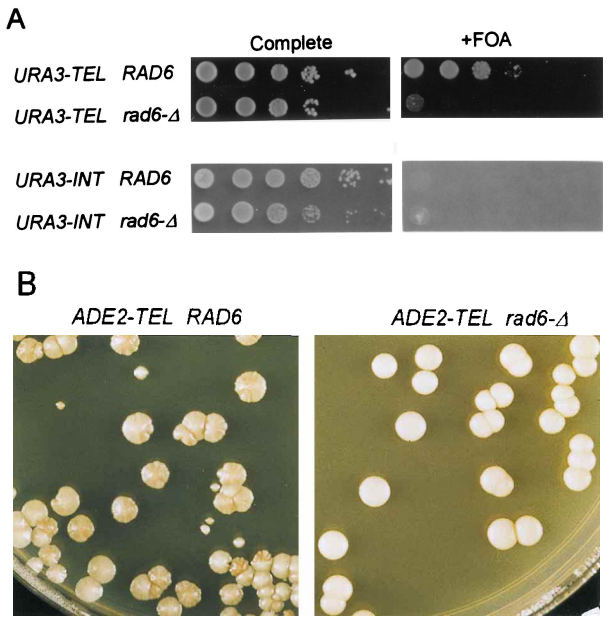

FIG. 1. Silencing of telomere-linked *URA3* and *ADE2* genes requires *RAD6*. (A) The expression of telomere VII-L-proximal *URA3* (*URA3-TEL*) and of *URA3* located at the normal internal chromosomal position (*URA3-INT*), with *RAD6* present or deleted, respectively, was assayed on +FOA medium plates. Equivalent aliquots of 10-fold serial dilutions were placed on complete, −Ura, and +FOA medium plates (growth on −Ura medium plates was identical to the growth on complete medium plates and is not shown). (B) Telomere V-R-proximal *ADE2* (*ADE2-TEL*) expression was assayed by colony color sectoring in wild-type *RAD6* and *rad6*-Δ strains on YPD.

for 6 h on the surface of YPD. The cells were then resuspended, and the numbers of haploids and diploids were determined by serial plating on −Trp medium and −Lys medium plates. Sonication was not required since the cells were not clumpy. The *MAT*α haploids and diploids grew on −Trp medium, while only the diploids grew on −Lys medium. The *MAT***a** haploids did not grow on either medium. Mating efficiencies were calculated according to the following formula: diploids/(diploids + haploid *MAT*α cells).

Primer extension analysis. Total tRNAs were isolated by phenol extraction as described previously (42). A modified primer extension method (7) that used avian myeloblastosis virus reverse transcriptase (U.S. Biochemical) was employed. The primer used for the extension reaction, 5′-GTCGAACGCC CGAT CTCAAG-3′, is complementary to the conserved loop downstream of the tyrosyl tRNA anticodon. The primer was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase according to the procedure provided by U.S. Biochemical. The primer extension reaction was performed in the presence of 0.2 mM (each) dATP, dGTP, dTTP, and ddCTP at 42°C for 40 to 45 min. The extension product was separated by gel electrophoresis in 20% polyacrylamide gels, visualized by autoradiography, and quantitated by a PhosphorImager (Molecular Dynamics).

RESULTS

Deletion of *RAD6* reduces the silencing of telomeric RNAP II-transcribed genes *URA3* and *ADE2*. Yeast strain UCC1021 carries the *ura3-52* allele at the normal *URA3* chromosomal position, and an additional wild-type *URA3* gene is inserted into the left end of chromosome VII. The presence of the wild-type *URA3* gene allows the strain to grow on −Ura medium. However, because of its proximity to a telomere, the *URA3* gene is silenced in a significant fraction of the cells. This repression can be detected by growth on medium containing the toxic uracil analog FOA (+FOA medium) (5, 11). Four independent deletion derivatives of *RAD6* were made in strain UCC1021, and the levels of telomeric silencing in the wild-type *RAD6* and *rad6*-Δ derivatives were compared. A large reduction ($>10^4$) in survival rates on +FOA medium was observed, indicating that telomeric silencing in *rad6*-Δ strains was lost (Fig. 1A; Table 3). Furthermore, the viability of the *rad6*-Δ derivatives on −Ura medium was increased to about 85.5%, compared with about 25.5% for the isogenic wild-type *RAD6* strain (Table 3). This indicates that deletion of the DNA repair gene *RAD6* reduces the silencing of a telomere-proximal *URA3* gene.

In order to check if the effect of *rad6*-Δ on the telomeric silencing of *URA3* is gene specific, another telomere-proximal RNAP II-transcribed gene, *ADE2*, was examined. UCC3537 is a wild-type *RAD6* strain carrying an ochre mutation, *ade2-101*, at the normal *ADE2* chromosomal location and a wild-type *ADE2* allele near a telomere. This strain is phenotypically Ade$^+$, but it gives rise to red- and white-sectored colonies on YPD due to the telomeric silencing of *ADE2*. Four independent *rad6* deletions failed to exhibit any red sectors (Fig. 1B). This strain also contains a copy of *URA3* near telomere VII-L. It is known that genes near two different telomeres may or may not be simultaneously silenced in the same cell (38). Here we found that *rad6* deletions reduced the silencing of *URA3* (data not shown) as well as of *ADE2*, which indicates that *rad6*-Δ reduces silencing at the ends of two different chromosomes in the same cell. Hence, the telomeric silencing of both RNAP II-transcribed genes, *URA3* and *ADE2*, requires *RAD6*. We conclude that *RAD6* is essential for the general telomeric silencing of RNAP II-transcribed genes.

Telomeres exhibit *SIR*-dependent silencing of an RNAP III-transcribed gene, *SUP4-o*. To determine if telomeric silencing is specific for RNAP II-transcribed genes or if, like the silencing at *HM* loci, it can act on RNAP III-transcribed genes as well, we placed the tRNA gene *SUP4-o* next to the telomere of the left arm of chromosome VII. *SUP4-o* is an ochre suppressor, and its expression can suppress the ochre mutation of the *ade2-101* allele present in the cell. Cells that lack Ade2 cannot grow on −Ade media and display a red-colony phenotype on YPD. Therefore, by monitoring colony color and growth on −Ade medium, the expression of *SUP4-o* could be observed.

As Fig. 2 shows, when *SUP4-o* is placed at a nonsilenced locus, it is expressed and consequently suppresses the *ade2-101* ochre allele, resulting in improved growth on −Ade medium. However, when *SUP4-o* is placed near a telomere, its expression is reduced to the point where it can no longer suppress *ade2-101*. This telomeric repression requires the presence of the *SIR2*, *SIR3*, and *SIR4* gene products, since deletion of any one of these genes eliminates the telomeric silencing of *SUP4-o*. The mechanism by which telomeres silence tRNA genes is similar to that of the telomeric silencing of RNAP II-transcribed genes, since *SIR* genes are required for both processes.

Silencing of *SUP4-o* is reduced by deletion of *RAD6*. In order to rule out the possibility that the effects of *rad6*-Δ on silencing were mediated by general changes in the transcription levels of RNAP II-transcribed genes, we investigated the importance of

TABLE 3. Viabilities of wild-type *RAD6* and isogenic *rad6*-Δ strains on −Ura and +FOA media[a]

Strain	Genotype	% Viability on:[b]	
		−Ura medium	+FOA medium
UCC1021	*RAD6 URA3-TEL*	25.5 ± 0.5	75.5 ± 21.0
L1503	*rad6*-Δ *URA3-TEL*	85.5 ± 12.5	0.0014 ± 0.0006

[a] UCC1021 and L1503 were grown in YPD, washed, and plated on complete, −Ura, and +FOA media as described in Materials and Methods. The number of CFU on complete media was considered to be 100%.

[b] Values are means ± standard deviations.

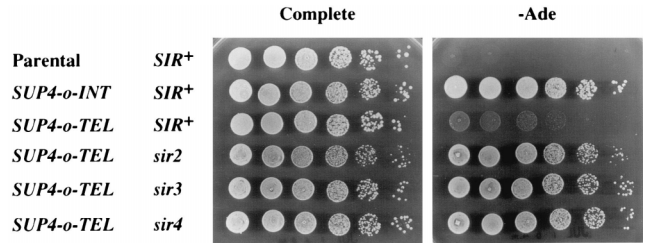

FIG. 2. *SUP4-o*, an RNAP III-transcribed tRNA gene, is silenced by a telomere in a *SIR*-dependent manner. *SUP4-o* was placed near a telomere (*SUP4-o-TEL* [strain UCC4543]) or at a nontelomeric locus (*SUP4-o-INT* [strain UCC4541]) in a strain containing the *ade2-101* ochre allele. The expression of *SUP4-o* was determined on the basis of the ability of the strain to grow on −Ade media. The parental strain (YPH499) failed to grow in the absence of adenine, whereas UCC4541, with a nontelomeric *SUP4-o*, grew well on such media. When *SUP4-o* was near a telomere, its ability to suppress *ade2-101* was greatly reduced. This silencing was alleviated by the disruption of the silencing gene *SIR2*, *SIR3*, or *SIR4*.

RAD6 for the expression of a telomeric RNAP III-transcribed gene. Seven independent deletions of *RAD6* were made in a strain with a telomeric *SUP4-o* gene. All the deletion derivatives gained the abilities to grow on −Ade medium and to form white colonies on YPD, indicating that the silencing of telomeric *SUP4-o* was reduced (Fig. 3A).

A modified primer extension analysis (7) was performed to check the transcription level of telomeric *SUP4-o* in both *RAD6* wild-type and isogenic-deletion strains (Fig. 3B). As shown in Fig. 3C, *SUP4-o* bears a G-to-U change in the anticodon of $tRNA^{Tyr}$. When ddCTP instead of dCTP is added to the reverse transcription reaction mixture, *SUP4-o* tRNA gives rise to a larger product than does wild-type $tRNA^{Tyr}$. The effect of deleting *RAD6* on the transcription level of telomere-located *SUP4-o* was measured by using the wild-type $tRNA^{Tyr}$ level as an internal loading control. PhosphorImager scans of the 20% acrylamide–7 M urea gel electrophoresis gel indicates that the level of *SUP4-o* tRNA is about 60 to 80% higher in the *rad6*-Δ strain (Fig. 3B, lane 2) than it is in the parental *RAD6* strain (lane 1).

RAD6* is required for silencing at mating-type loci.** Since several regulatory factors function in silencing at both telomeres and *HM* loci, we tested whether *RAD6* is also required for silencing at the *HM* loci. We attempted to detect this effect by comparing the mating efficiencies of *RAD6* and *rad6*-Δ strains. A quantitative mating assay was performed with two pairs of isogenic *RAD6* and *rad6*-Δ strains (Table 4). When both mating partners were *rad6*-Δ, the mating efficiency was reduced sevenfold relative to the mating efficiency of the isogenic *RAD6* wild-type strains. Interestingly, when the *MATa** strain was *RAD6* wild type, there was no effect on the mating efficiency regardless of the *RAD6* allele in the *MAT*α strain. In contrast, the mating efficiency was significantly reduced when only the *MAT***a** strain was *rad6*-Δ. These results indicate that the deletion of *RAD6* preferentially affects mating in *MAT***a** strains over mating in *MAT*α strains.

We also determined the effect of *rad6*-Δ on *HM* loci by deleting *RAD6* in strain UCC3515, containing *URA3* in *HML*. Silencing of *hml::URA3* in five independent *rad6*-Δ derivatives of UCC3515 was reduced about 10- to 100-fold (Fig. 4). This indicates that *RAD6* is required for silencing at the *HML* locus. Three independent deletions of *RAD6* in a strain containing *TRP1* at *HMR* remained unable to grow on −Trp medium (data not shown). However, in strain UCC3511 (48), in which the *HMR* silencing is weakened by the insertion of the *URA3* gene between the E and I elements, the deletion of *RAD6* (5 independent transformants) reduced silencing about 10^4-fold

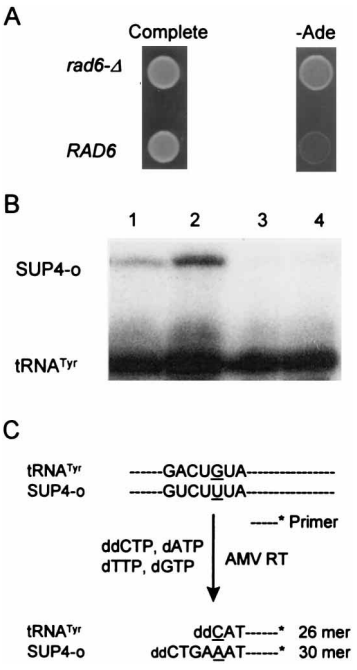

FIG. 3. Silencing of *SUP4-o* located near a telomere requires *RAD6*. (A) Expression of telomere VII-L-located *SUP4-o* was assayed by growth on −Ade media in the presence or absence of *RAD6*. (B) Analysis of *SUP4-o* tRNA transcript level by primer extension. Total cellular tRNA was reverse transcribed in the presence of ddCTP. Lane 1, *RAD6 SUP4-o-TEL*; lane 2, *rad6*-Δ *SUP4-o-TEL*; lane 3, *RAD6*; lane 4, *rad6*-Δ. The wild-type $tRNA^{Tyr}$ is an internal loading control. Positions of the wild-type $tRNA^{Tyr}$ and *SUP4-o* extension products are shown. (C) Schematic of primer extension analysis. The nucleotide sequences of wild-type $tRNA^{Tyr}$ and *SUP4-o* in the vicinity of the anticodon, the labeled 20-mer primer, and the primer extension products are shown. The different nucleotides in the anticodon of *SUP4-o* and $tRNA^{Tyr}$ are underlined. AMV RT, avian myeloblastosis virus reverse transcriptase.

(Fig. 4), indicating that *RAD6* is involved in silencing at *HMR*. These data show that silencing at both *HM* loci requires *RAD6.*

The ubiquitin-conjugating activity of *RAD6* is required for silencing. Cysteine 88, the only cysteine residue in Rad6, is essential for the thioester linkage between Rad6 and ubiquitin. Rad6(C88A), in which cysteine 88 has been changed to alanine, cannot form a thioester conjugate with ubiquitin, whereas Rad6(C88S), with a change of this cysteine to serine, can form a conjugate of ubiquitin but is unable to transfer the ubiquitin to target proteins. Both *rad6(C88A)* and *rad6(C88S)* completely block all *RAD6* functions and resemble *rad6* null mutations (54, 55). Centromeric plasmids carrying the *rad6(C88A)* and *rad6(C88S)* alleles were introduced into *rad6*-Δ strains L1643 (data not shown) and L1503 (Fig. 5). While the wild-type

TABLE 4. Mating efficiencies of *RAD6* and *rad6*-Δ strains[a]

RAD6 allele in:		Mating efficiency[b]
*MAT*α strain	*MAT***a** strain	
RAD6	*RAD6*	0.35 ± 0.07
RAD6	*rad6*-Δ	0.09 ± 0.04
rad6-Δ	*RAD6*	0.36 ± 0.16
rad6-Δ	*rad6*-Δ	0.05 ± 0.02

[a] Strains and mating efficiency assay are described in Materials and Methods. Mating efficiency was calculated as follows: diploids/(diploids + haploid *MAT*α tester). Each experiment was repeated five times. The significance of the differences was verified by the *t* test with Microsoft Excel 5.0 software.

[b] Values are means ± standard deviations.

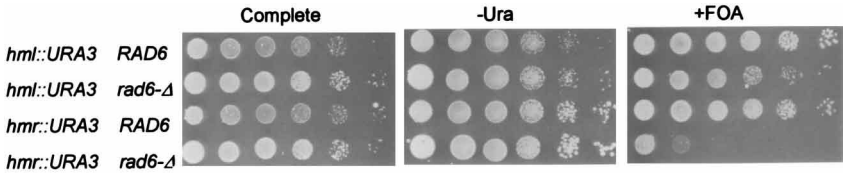

FIG. 4. Silencing of *HM*-linked *URA3* is reduced by the *rad6*-Δ mutation. The silencing of *hml::URA3* and *hmr::URA3* in *RAD6* wild-type and *rad6*-Δ strains was assayed by growth on −Ura and +FOA media.

RAD6 restored silencing, *rad6(C88A)* and *rad6(C88S)* failed to restore silencing. Thus, the ubiquitin-conjugating activity of Rad6 is important for silencing.

Rad6, a 172-amino-acid protein, contains a highly conserved amino terminus and a nonconserved acidic carboxyl terminus (39, 59). The $rad6_{\Delta 1-9}$ mutation produces a protein lacking the first nine residues of Rad6, and the *rad6-153* mutation produces a protein lacking the last 19 residues of the acidic tail. Neither centromeric nor multiple-copy plasmids carrying $rad6_{\Delta 1-9}$ restored silencing, while plasmids carrying *rad6-153* did (data not shown). In addition, overexpression of *RAD6* had no effect on telomeric silencing (data not shown).

***RAD18* and *UBR1* are not required for telomeric silencing.** We deleted *RAD18* in strains containing *URA3* (seven independent transformants of strain UCC1021 [Fig. 6]), *ADE2* (eight independent transformants of strain UCC3537 [data not shown]), and *SUP4-o* (six independent transformants of strain UCC4543 [data not shown]) near telomeres. Silencing was not reduced in any of these cases. *UBR1* was also deleted in strains containing *URA3* (two independent transformants of UCC1021 [Fig. 6]), and *SUP4-o* (four independent transformants of UCC4543 [data not shown]) near telomeres. Telomeric silencing was not affected in *ubr1*-Δ strains either. Thus, neither of the two known Rad6-binding proteins is important for silencing.

DISCUSSION

The data reported here demonstrate that silencing at telomeres and *HM* loci requires *RAD6*. The ubiquitin-conjugating activity of Rad6 was shown to be essential for silencing, since *rad6(C88A)* and *rad6(C88S)*, which encode proteins that are defective in ubiquitin-conjugating activity, fail to restore silencing. One indication of the loss of telomeric silencing is the expression of a telomerically located *URA3* gene which causes sensitivity to +FOA medium. Since *rad6*-Δ cells grow more poorly than wild-type cells, it is possible that the deletion of *RAD6* makes cells sensitive to +FOA medium without activating the telomeric *URA3*. However, consistent with the reduction-of-silencing hypothesis, the viability of L1503 (*rad6*-Δ, *URA3-TEL*) on −Ura medium was higher than that of the *RAD6* parent strain, UCC1021. The reduction of silencing of telomere-located *ADE2* and *SUP4-o* demonstrated again that *RAD6* is required for general telomeric silencing. Furthermore, deletion of *RAD6* preferentially affects transcription of genes at silent loci, since it causes an increase in the transcription of the *SUP4-o* tyrosyl tRNA gene located near telomere VII-L relative to the transcription of wild-type tyrosyl tRNA genes not present at telomeres.

RAD6 was also demonstrated to be required for silencing at the *HM* loci. Silencing at *HML* and at a weakened *HMR* is reduced in *rad6*-Δ strains. The mating efficiency of *MAT***a** strains, but not of *MAT*α strains, is reduced upon deletion of *RAD6*, indicating that silencing at *HML* is more easily disrupted by *rad6*-Δ than silencing at *HMR*. Sterility has been shown to increase in old yeast mother cells, and the aging process in general has been proposed to be related to the breakdown of silencing (23, 50). Indeed, we found that *RAD6* null mutations decreased the mean life span from 23 to 7 generations (18). However, it was previously shown that *rad6*-Δ increases the doubling time and the percentage of unseparated cells with the nucleus in the isthmus in a growing population (26). This suggests that the shortened life span in *rad6*-Δ cells may be caused by defects in cell growth.

Several lines of evidence suggest that DNA repair and recombination are related to chromatin structure and silencing. Deletions of chromatin assembly factors (*CAC1*, *CAC2*, and *CAC3*) cause sensitivity to UV and reduce telomeric silencing (9, 22, 37). Mutations in *SIR2* cause an increase in the level of recombination in ribosomal DNA (10), and *sir3* mutants exhibit an elevated rate of mitotic recombination (34). Sir3 interacts with Rad7, and the repair of UV lesions in the absence of *RAD7* activates the expression of telomerically silent genes (33). We have previously speculated that the effect of *rad6*-Δ on the Ty1 transposition rate (35) and target site selection (28) arises from alterations in chromatin structure. This notion is consistent with our current finding that *rad6*-Δ reduces silencing and the recent finding that *RAD6* is involved in transposition and transcription of Ty1 elements inserted in the tandem array of rRNA genes (6). However, despite extensive efforts, we have not been able to detect differences in *RAD6* and *rad6*-Δ strains with regard to the nucleosome patterns of a TRP1ARS1 plasmid or the accessibility to restriction enzymes (18).

N-end rule-mediated protein degradation (58) requires an interaction between the extremely conserved amino terminus of Rad6 and Ubr1, an E3 enzyme (59) which recognizes the specific N-terminal residue of the target protein. The $rad6_{\Delta 1-9}$ mutant lacking the amino terminus is defective in sporulation and shows some UV sensitivity. However, a strain with $rad6_{\Delta 1-9}$ differs from a strain with a complete deletion of *RAD6* because it is much more resistant to UV and is not deficient in UV-induced mutagenesis (59). Here, we show that the $rad6_{\Delta 1-9}$ mutation is defective in silencing. However, since a null mutation of *UBR1* has no effect on telomeric silencing, N-end rule-mediated degradation is not required. The amino terminus of Rad6 may have an additional role in interactions with components of the silencing machinery.

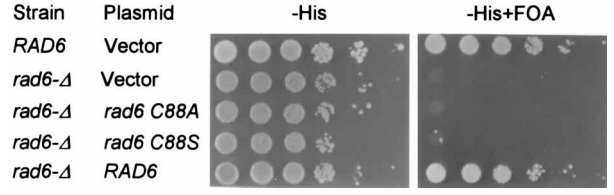

FIG. 5. The ubiquitin-conjugating activity of *RAD6* is required for silencing. Strains (*RAD6* and *rad6*-Δ) containing *URA3* near telomere VII-L were transformed with *RAD6* or mutant *rad6* alleles on pRS413-based *CEN HIS3* plasmids. The silencing of *URA3* was assayed on +FOA medium plates.

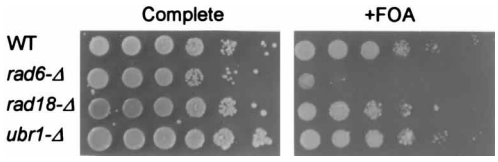

FIG. 6. *RAD18* and *UBR1* are not required for telomeric silencing. The expression of *URA3* near telomere VII-L was assayed as described in the legend to Fig. 1. Equivalent aliquots of 10-fold serial dilutions were spotted on complete and +FOA medium plates. Wild-type (WT) and *rad6*-Δ strains were used as controls.

Rad6 directly interacts with Rad18, another key factor in the *RAD6* DNA repair pathway, and this interaction may target Rad6 to DNA damage sites via its ability to bind single-stranded DNA (3). Rad6 forms separate complexes with Rad18 and Ubr1, apparently using distinct domains for the independent interactions (3). The amino terminus of Rad6 is not required for interaction with Rad18 (3). The acidic carboxyl-terminal domain of Rad6 may also be dispensable for interaction with Rad18, since the Rad6 homologs from *Schizosaccharomyces pombe* and humans, homologs which contain no acidic domains, still coprecipitate with yeast Rad18 (3). We found that *RAD18* is not required for telomeric silencing. Furthermore, our finding that the nonconserved Rad6 carboxyl tail is not required for telomeric silencing in *S. cerevisiae* leaves open the possibility that homologs of Rad6 could also be involved in maintaining the chromatin structure in higher eukaryotes. Indeed, in mice the inactivation of *mHR6B*, a homolog of *RAD6*, causes male infertility, primarily due to the defective progression through the elongation and condensation phases of spermatid development (41).

One hypothesis to explain our results proposes that Rad6 mediates degradation of some negative silencing regulators in a Ubr1-independent manner. It is already known that transcription factors can antagonize silencing, since the transcription factor Ppr1 has been shown to be required for the expression of telomere-located *URA3*. The deletion of *PPR1* enhances the silencing of *URA3*, and the overexpression of *PPR1* alleviates silencing (2). At least one transcription factor, Gcn4, has been shown to be regulated by the Rad6 and Ubc3 ubiquitination pathways (25).

The effect of *rad6*-Δ on silencing may result from a disruption of the normal cell cycle progression, since *rad6*-Δ causes a growth defect. It is known that mutations in some cell cycle transcriptional regulator genes (*GAL11*, *SWI4*, and *MBP1*) and cyclin genes (*CLN3*, *CLB5*, and *CLB2*) can suppress silencing defects in *rap1*^{s} *hmr*Δ*A* strains (27, 56).

Another way to explain our result is to propose that the ubiquitination of certain silencing factors is essential for silencing. This hypothesis is consistent with recent evidence that the deletion of Ubp3, a deubiquitinating enzyme, can improve silencing in yeast (31) and that mutations in a putative deubiquitinating enzyme enhance position effect variegation in *Drosophila melanogaster* (16).

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