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The E2 Ubiquitin Conjugase Rad6 Is Required for the ArgR/Mcm1 Repression of *ARG1* Transcription

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Transcription of the *Saccharomyces cerevisiae ARG1* gene is under the control of both positive and negative elements. Activation of the gene in minimal medium is induced by Gcn4. Repression occurs in the presence of arginine and requires the ArgR/Mcm1 complex that binds to two upstream arginine control (ARC) elements. With the recent finding that the E2 ubiquitin conjugase Rad6 modifies histone H2B, we examined the role of Rad6 in the regulation of *ARG1* transcription. We find that Rad6 is required for repression of *ARG1* in rich medium, with expression increased ~10-fold in a *rad6* null background. Chromatin immunoprecipitation analysis indicates increased binding of TATA-binding protein in the absence of Rad6. The active-site cysteine of Rad6 is required for repression, implicating ubiquitination in the process. The effects of Rad6 at *ARG1* involve two components. In one of these, histone H2B is the likely target for ubiquitination by Rad6, since a strain expressing histone H2B with the principal ubiquitination site converted from lysine to arginine shows a fivefold relief of repression. The second component requires Ubr1 and thus likely the pathway of N-end rule degradation. Through the analysis of promoter constructs with ARC deleted and an *arg80 rad6* double mutant, we show that Rad6 repression is mediated through the ArgR/Mcm1 complex. In addition, analysis of an *ada2 rad6* deletion strain indicated that the SAGA acetyltransferase complex and Rad6 act in the same pathway to repress *ARG1* in rich medium.

In response to the role of Rad6/Ubc2 as an E2 ubiquitin conjugase, mutations in its gene affect multiple cellular processes. Rad6 acts with Rad18 in pathways of DNA repair (3–5, 46) and with the E3 ubiquitin ligase Ubr1 in the pathway leading to the degradation of multiubiquitinated protein substrates via the 26S proteasome (22, 48, 69). Independently of Rad18 and Ubr1, Rad6 is required for transcriptional silencing at telomeres and the *HM* loci (34). The ability of Rad6 to ubiquitinate histones H2A, H2B, and H3 in vitro (29, 30, 39) and H2B in vivo (59) has led to the suggestion that its ability to regulate gene expression results from changes in chromatin structure. This idea is supported by findings that disruption of *rad6* results in changes in the sites of integration of retrotransposons (47, 56) and that a strain with a K123R mutation in the principal ubiquitination site of histone H2B has the same sporulation defect as a strain with *rad6* deleted (59).

The *ARG1* promoter provides a valuable system to study the role of factors involved in the activation and repression of transcription. *ARG1* encodes argininosuccinate synthetase, which is required in a pathway that also includes *ARG2*, *ARG5,6*, *ARG8*, *ARG3*, and *ARG4* for the biosynthesis of arginine. Transcription of this group of genes is subject to general amino acid control mediated by the activator protein Gcn4 (14, 20, 32). *ARG1*, as well as *ARG5,6*, *ARG8*, and *ARG3*, is also subject to repression by arginine (10, 14, 16, 20, 38, 50). Arginine repression requires a DNA binding complex of ArgR proteins, Arg80/ArgRI and Arg81/ArgRII, as well as Mcm1 (1, 8, 23–26, 51, 52, 57, 58). Interestingly, this same ArgR/Mcm1 complex is required for induction of *CAR1* and *CAR2*, which are required for arginine catabolism (43, 52, 67). For both activation and repression, the ArgR/Mcm1 complex binds to upstream arginine control (ARC) elements (10, 17, 21, 24, 51). *ARG1* contains two ARC elements, centered at −185 and −225 relative to the start site of transcription, that contribute to the arginine-specific repression.

The genomewide analysis of Holstege et al. (33) has shown that *ARG1* regulation requires the histone acetyltransferase Gcn5. When grown in rich medium, cells with *gcn5* deleted had an ~8-fold increase in expression of *ARG1* (33). We have recently found that repression of *ARG1* in rich medium requires multiple components of the SAGA acetyltransferase complex and that this repression correlates with increased acetylation of histone H3 (58a). In the present work, we show that repression of *ARG1* in rich medium also requires Rad6. Repression by Rad6 depends upon the active-site cysteine. Rad6-dependent repression is mediated by the ArgR/Mcm1 complex and acts through a pathway common to the SAGA components. Histone H2B is a likely target of Rad6 ubiquitination, since a K123R mutation that abolishes the principal ubiquitination site in H2B acts in the same pathway to regulate expression of *ARG1*. Unlike silencing at telomeres and the *HM* loci (34), a component of Rad6 repression at *ARG1* also involves the E3 ubiquitin ligase Ubr1.

MATERIALS AND METHODS

Yeast strains. All yeast strains were derivatives of BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) or BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) (71) and were purchased from Research Genetics. They include BY4282 (*ada2Δ0*), BY4425 (*rad6Δ0*), BY618 (*arg80Δ0*), BY15787 (*rad18Δ0*), BY14814 (*ubr1Δ0*), BY249 (*gcn4Δ0*), BY13026 (*htb2Δ0*), and BY16148 (*ubp3Δ0*). *URA3* disruptions of

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rad6 were made using a disruption allele synthesized by PCR with the oligonucleotides 5′-AAGATTATTTTTAGGCAGACAGAGACTAAAAGATAAAGCGTC ATGAAGCTTTTCAATTCA-3′ and 5′-ATATCGGCTCGGCATTCATCATTA AGATTCTTTTGATTTTTCTCACCGAGATTCCCGGGTAATA-3′. This allele was integrated into BY249, BY618, BY14814, and BY4282 to generate the double-deletion strains CY1214 (*gcn4 rad6*), CY1251 (*arg80 rad6*), CY1304 (*ubr1 rad6*), and CY1215 (*ada2 rad6*), respectively. Strains CY1272 and CY1256 are derivatives of the *htb2Δ0* strain (BY13026) which contain *HIS3* insertions directly downstream of the *htb1* allele. They were constructed by double-strand gene replacement in BY13026 using *Eco*RI-*Xba*I-digested CB1469 (wild-type *HTB1*) and CB1474 ($HTB1_{K123R}$; see below). The strains were verified by PCR using oligonucleotides flanking the relevant alleles.

DNA constructs. LacZ reporter constructs were cloned as *his3-lacZ* fusions into the *LEU2* centromeric plasmid YCp87 (11). The *ARG1* promoter constructs contain promoter sequences from −614 (*ARG1-lacZ*) or −302 ($ARG1_{\Delta ABF1}$-*lacZ*), relative to the transcriptional start site and 213 bp of coding region fused to the first *Hin*dIII of *HIS3* (Ricci et al., submitted). $ARG1$-$lacZ_{\Delta ARC}$ is a derivative of *ARG1-lacZ* in which the upstream ARC elements spanning nucleotides −175 to −197 and −214 to −239 have been replaced by *Sal*I and *Bam*HI restriction enzyme sites, respectively, using PCR-based mutagenesis strategies (58a).

The construction of C-terminally hemagglutinin (HA)-tagged TATA-binding protein (TBP) and its insertion into YCplac33 is described elsewhere (58a). The *HIS3* centromeric plasmids expressing wild-type Rad6 (HH1) and Rad6-C88A (HH4) were generously provided by Susan Liebman (34).

The *HTB1* integrating allele was constructed by PCR using oligonucleotides 5′-TTGAATTCTAAAAGAATTGGAATAAAAGTAC-3′ and 5′-GCTCTAGAG AATTGGCCTTAGTAGTGG-3′ and cloned as an *Eco*RI-to-*Xba*I fragment into pTZ18. The insert contains a unique *Bam*HI site into which was inserted a 1.8-kb fragment that contains *HIS3* to give CB1469. CB1474 with K123R was engineered by site-directed mutagenesis using CB1469 as the template and oligonucleotide 5′-GG TACTAGAGCTGTTACCAGGTACTCTTCCTCTACTC-3′ and its complement (68).

β-Galactosidase assays. For the analysis of *ARG1-lacZ* fusion reporters, saturated cultures grown in minimal medium were inoculated at a 1/100 dilution into yeast-peptone-dextrose (YPD) or minimal medium (supplemented with the required amino acids) and grown at 30°C to an A_{600} of 1.0 to 1.5. Equal stability of the plasmids in the strains was verified by cell counts on rich- and minimal-medium plates. The cells were pelleted, washed in *lacZ* buffer, and concentrated 5- to 10-fold. β-Galactosidase activity was determined using *O*-nitrophenyl-β-D-galactosidase (ONPG) as a substrate, as described by Ausubel et al. (2), and standardizing to cell density.

RNA analyses. Saturated cultures were grown in minimal medium and diluted 1/100 in YPD. The cells were grown at 30°C to an A_{600} of ~1.3 (~10^7 cells/ml; 10-ml total volume), and RNA was extracted by the hot acidic phenol method as described by Ausubel et al. (2). For Northern analysis, 12 μg of total RNA was separated by agarose-formaldehyde gel electrophoresis and probed with ^{32}P-labeled DNA fragments for *ARG1* and *ACT1* as described by Skerjanc et al. (63). The *ARG1* and *ACT1* probes were each ~700 bp long and were constructed by PCR using oligonucleotides 5′-GTTGGGTACCTCTTTGGCAA-3′ and 5′-GCCCAGAATGATG ACGTTACCC-3′ for *ARG1* and 5′-ACGAATTCAGAGTTGCCCCAGAAGAA C-3′ and 5′-CCCGGATCCACATTTGTTGGAAGGTA-3′ for *ACT1.* Following 16 h of hybridization at 42°C, the blots were washed five times for 5 min each time at 42°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate (SDS) and for 15 min at 65°C in 0.1× SSC–0.2% SDS. The blots were exposed to film for visualization, and densitometry was carried out by PhosphorImager analysis (ImageQuant version 1.11; Molecular Dynamics). Background was subtracted, and the *ACT1* intensity was used to correct for loading. Primer extension analysis with 20 ng of *ARG1* primer 5′-CCTTGGCGGCATCGA AATCTTC-3′ end labeled with [^{32}P]ATP and 25 μg of total RNA was performed as described by Martens and Brandl (49).

Chromatin immunoprecipitation (ChIP). Chromatin was prepared as described by Hecht and Grunstein (31) with the following modifications. One hundred fifty milliliters of cells grown to an A_{600} of ~1.5 was treated with 1% formaldehyde for 20 min at room temperature with occasional swirling. The cells were pelleted, washed twice in phosphate-buffered saline (140 mM NaCl, 2.5 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5), and then suspended in 1.2 ml of ice-cold lysis buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% [vol/vol] Triton X-100, 0.1% [wt/vol] sodium deoxycholate with protease inhibitors [1 mM phenylmethylsulfonylfluoride; 1 mM benzamidine; 0.5 mg of *N*-tosyl-L-phenylalanine chloromethylketone/ml; 0.1 mg of aprotinin/ml in 10 mM HEPES-KOH, pH 8.0; 1.0 μg of leupeptin/ml; and 1.0 μg of pepstatin/ml]). The suspension was aliquoted into 400-μl volumes in 1.5-ml microcentrifuge tubes containing equal volumes of 0.5-nm-diameter glass beads (31). Cross-linked chromatin was isolated, pooled, and fragmented (31), and the chromatin solution (400 μl) was incubated with 15 μl of ascites fluid derived from the 12CA5 cell line for 4 h at 4°C. Protein G-Sepharose (Pharmacia Biotech, Inc.) was added for an additional hour, followed by 5-min washes as follows: once in lysis buffer containing 0.5 M NaCl; once in a solution of 10 mM Tris-HCl (pH 8.0), 0.25 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 1.0 mM EDTA; and once in 10 mM Tris-HCl (pH 8.0)–2 mM EDTA (TE). The immunoprecipitated material was eluted from the beads by heating them at 65°C for 30 min in 100 μl of TE containing 1% SDS, followed by centrifugation at 10,000 × *g*. Cross-links were reversed by incubation at 65°C for 12 h. DNA was extracted with phenol-chloroform and then chloroform and precipitated in 3 volumes of ethanol containing 20 μg of glycogen, 0.1 volume of 5 M LiCl, and 50 mM Tris-HCl (pH 8.0) at −20°C. The DNA was pelleted, washed with 70% ethanol, and resuspended in TE. The precipitated DNA was analyzed by quantitative PCR using the *ARG1* primers 5′ ATACTATTGAGACAGTGCCAG T-3′ and 5′-ACGGCTCTCCAGTCATTTATG-3′ and the *ACT1* primers 5′ CATTCTTCCTTATCGGATCCTCA-3′ and 5′GGAAGGAAGAATACAAGA GAGAGG-3′. The linear range for each primer pair was determined using decreasing amounts of template. Approximately 1/50 of the precipitated DNA and 1/3,000 of the total DNA were used in a 50-μl volume containing 50 pmol of primers, 0.2 mM deoxynucleoside triphosphates, 1× reaction buffer (Promega, Inc.), 1.5 mM $MgCl_2$, 1 mg of glycogen/ml, and 2 U of *Taq* polymerase. The cycling program was 2 min at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72 °C, and a final extension at 72°C for 5 min. The products were analyzed on a 6% native polyacrylamide gel, stained with ethidium bromide, and photographed under UV on a gel documentation system (Alpha Innotech Corp.).

RESULTS

Rad6 represses ARG1 transcription. The yeast *ARG1* promoter is subject to complex mechanisms of activation and repression. Our recent work has indicated that a component of this regulation requires chromatin modification mediated by the SAGA histone acetyltransferase complex (58a). Since the E2 ubiquitin-conjugating enzyme Rad6 has been implicated in transcriptional repression (34) and histone modification (59), we chose to examine its potential role in the repression of *ARG1* transcription. A centromeric plasmid expressing a *lacZ* translational fusion containing *ARG1* sequences from −614 to +213 (Fig. 1A; *ARG1-lacZ*) was introduced into the wild-type yeast strain BY4741 and the isogenic *rad6* deletion strain, BY4425. Saturated cultures were grown in minimal medium and then diluted in rich medium (YPD). As shown in Fig. 1B, expression of *ARG1-lacZ* was increased 9.3-fold in the strain lacking *RAD6*, indicating that Rad6 acts to repress *ARG1* expression in rich medium. A similar analysis was performed after cells were grown in minimal medium. As the result of Gcn4 activation and a loss of arginine repression, expression of *ARG1-lacZ* increased when BY4741 (*RAD6*) was grown in minimal medium compared to growth in YPD. However, in minimal medium the increase in expression in the *rad6* deletion background was <1.7-fold, indicating that repression by Rad6 occurs predominately in rich medium.

To verify that the increase in *ARG1-lacZ* expression was the result of an increase in RNA levels, Northern analysis was performed. BY4741 (*RAD6*) and BY4425 (*rad6Δ0*) strains were grown in rich medium. RNA was isolated from exponentially growing cells, separated by electrophoresis, and probed by Northern blotting with DNA fragments from *ARG1* and *ACT1* (Fig. 2). Under conditions in which *ACT1* mRNA levels were unchanged, the level of *ARG1* mRNA increased 10.5-fold (as determined from densitometry of three experiments). Primer extension analysis indicated that disruption of *RAD6*

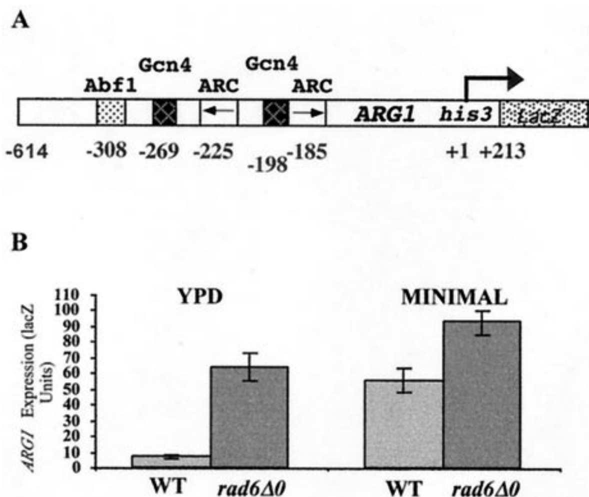

FIG. 1. Rad6 is required for the repression the *ARG1* promoter in rich medium. (A) *ARG1-lacZ* reporter construct. *ARG1* sequences from −640 to +213 were cloned as a *Bam*HI-*Hin*dIII fragment into YCp87 to generate a *his3-lacZ* translational fusion on a *LEU2* centromeric plasmid. Previously mapped regulatory sites (19) are shown as follows: ARC elements (centered at −175 and −214 relative to the principal transcriptional start site [16]), A TATA elements at −73 is not shown. Gcn4 binding sites (−195 and −265), and the Abf1 consensus sequence (−302). (B) β-Galactosidase analysis of *ARG1-lacZ* expression in wild-type (WT) and *rad6* deletion backgrounds. Yeast strains BY4741 (wild type) and BY4425 (*rad6Δ0*) containing YCp87-*ARG1-lacZ* were grown to saturation in minimal medium and then diluted 1/100 in YPD medium or minimal medium (2% glucose supplemented with the required amino acids). The cells were harvested at an A_{600} of ~1.5, and β-galactosidase activity was determined using ONPG as a substrate. Activities were standardized to cell density. The error bars represent the standard error of the mean for two experiments performed in triplicate.

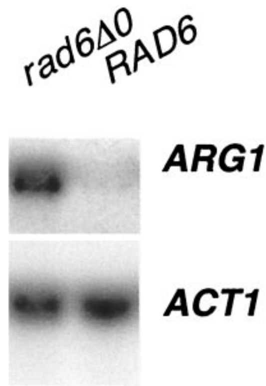

FIG. 2. Northern analysis of *ARG1* in a *rad6* disruption strain. Yeast strains BY4741 (wild type) and BY4425 (*rad6Δ0*) were grown to saturation in minimal medium and then diluted 1/100 in YPD. The cells were harvested, and total RNA was isolated. Twelve micrograms of total RNA was separated by electrophoresis on a 1.0% agarose formaldehyde gel and probed with ^{32}P-labeled DNAs specific for *ARG1* and *ACT1*. The blot was visualized by autoradiography and quantitated by PhosphorImager analysis (ImageQuant 1.11). The blot shown is representative of analyses performed in triplicate.

did not result in changes to the principal mRNA start site for *ARG1* (at −72 relative to the translational start site) (reference 16 and data not shown).

We next used a ChIP assay to determine if increased binding of TBP to the promoter paralleled the enhanced expression of *ARG1*. BY4741 and BY4425 (containing *ARG1-lacZ*) were transformed with a centromeric plasmid expressing HA epitope-tagged TBP. ChIP assays were performed on these *RAD6* and *rad6* extracts using anti-HA antibody. The PCR results for the ChIP analysis (Fig. 3) indicate that there was increased binding of TBP to the *ARG1* promoter in the absence of Rad6. Densitometry of three independent experiments indicated that this increase was ~2.4-fold. By comparison, the *ACT1* promoter showed no change in the binding of TBP.

Ubiquitin-conjugating activity of Rad6 is required for repression of *ARG1* expression. Ubiquitin conjugation by Rad6 involves the covalent attachment of ubiquitin to C88 of Rad6 through a thioester linkage (64). To determine if Rad6 represses the expression of *ARG1* through a mechanism that requires ubiquitination, centromeric plasmids expressing wild-type Rad6 and Rad6 with a C88A mutation (kindly provided by Susan Liebman) were introduced into BY4425 (*rad6Δ0*) containing *ARG1-lacZ*. β-Galactosidase activity was determined after growth in rich medium. As shown in Fig. 4A, Rad6-C88A was unable to complement the null allele, suggesting that ubiquitination mediated by Rad6 is required for the repression of *ARG1*.

If ubiquitin conjugation by Rad6 is required for repression of *ARG1*, then enhanced ubiquitination would be expected to lead to hyperrepression. The ubiquitin protease Ubp3 could act reciprocally with Rad6, removing ubiquitin moieties conjugated by Rad6. Such a role for *UBP3* in the regulation of

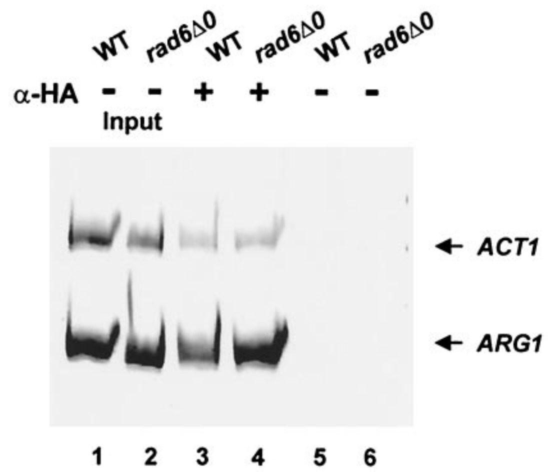

FIG. 3. Rad6 inhibits the binding of TBP to the *ARG1* promoter. BY4741 (wild type [WT]) and BY4425 (*rad6Δ0*) containing HA epitope-tagged TBP were grown in 150 ml of YPD medium and cross-linked with 1% formaldehyde followed by mock immunoprecipitation with no antibody (−; lanes 1 and 2) or immunoprecipitation with anti-HA (α-HA) antibody (+; lanes 3 and 4). Immunoprecipitated DNA and input DNA (lanes 5 and 6) were analyzed by PCR using primers specific for *ARG1* and the *ACT1* promoter. Linear ranges for PCR were determined by serial dilution. The purified extended products were analyzed on 6% native polyacrylamide gels and stained with ethidium bromide. The data are representative of three independent whole-cell extracts and ChIP assays.

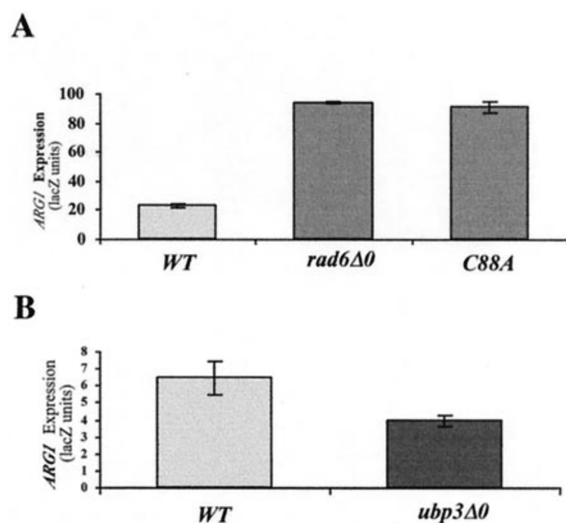

FIG. 4. (A) Ubiquitin conjugase activity of Rad6 is required for its repression of *ARG1* expression. BY4425 (*rad6Δ0*) containing YCp87-*ARG1-lacZ* and either no plasmid (*rad6Δ0*), a *HIS3* centromeric plasmid expressing wild-type Rad6 (HH1; WT), or Rad6-C88A (HH4; C88A) (35) was grown to saturation in minimal medium and then diluted 1/100 in YPD medium. The cells were harvested at an A_{600} of ~1.5, and β-galactosidase activity was determined using ONPG as a substrate. Activities were standardized to cell density. The error bars represent the standard error of the mean of three samples. (B) Deletion of *UBP3* results in reduced expression of *ARG1* in rich medium. Yeast strains BY4741 and BY16148 (*ubp3Δ0*) containing *ARG1-lacZ* were grown, and β-galactosidase activity was determined as described above ($n = 4$; $P = 0.04$). The error bars represent the standard error of the mean.

transcription is suggested by the findings that its deletion leads to hyperrepression at telomeres and *HML* (53) and that it is essential for growth in the absence of the transcription elongation factor TFIIS (18). We thus analyzed the expression of *ARG1-lacZ* in the *ubp3* deletion strain, BY16148, after the growth of cells in YPD medium. As shown in Fig. 4B, deletion of *ubp3* resulted in a decrease in expression of *ARG1-lacZ* to a level 60% of that seen in the wild-type background ($P = 0.04$). Although the effects of *ubp3* disruption may be indirect, this result is consistent with a role for ubiquitination in the regulation of *ARG1*.

K123R mutations within histone H2B result in increased expression of *ARG1*. Histone H2B is a target for ubiquitination by Rad6 in vivo and in vitro (29, 30, 39, 59). Modification of histone H2B by Rad6 has been predicted to alter chromatin structure in a way that could influence transcription. To test this possibility, an allele of *HTB1* containing a K123R mutation at the site of ubiquitination was introduced by gene replacement as the only cellular copy of histone H2B (yeast strain CY1272). As previously observed for the K123R mutation (59), CY1272 showed a reduced rate of growth in rich and minimal media (not shown). *ARG1-lacZ* expression was determined in this strain as well as the isogenic strain containing an integration of wild-type histone H2B (CY1256). As shown in Fig. 5A, $H2B_{K123R}$ resulted in a 4.6-fold increase in expression of *ARG1*. Since this was less than the 8.5-fold increase seen upon disruption of *RAD6*, to determine if mutations in *htb1* and *rad6* were acting through the same pathway, *ARG1-lacZ* expression was also determined in CY1284, which contains $htb_{K\text{-}R}$ in the *rad6* disruption background. *ARG1-lacZ* expression in CY1284 was increased 7.5-fold compared to the wild

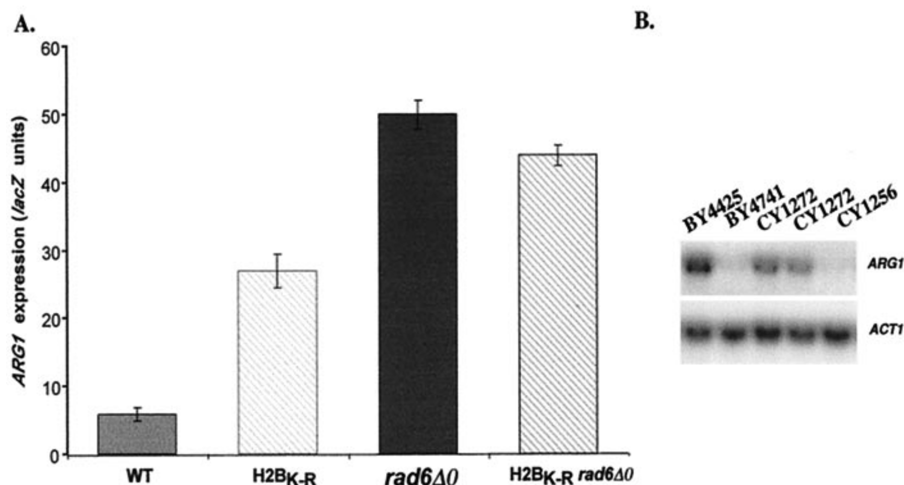

FIG. 5. A K123R mutation within histone H2B results in relief of repression of the *ARG1* promoter. (A) An allele of *HTB1* containing a K123R mutation at the site of ubiquitination was introduced by gene replacement as the only cellular copy of histone H2B (yeast strain CY1272; $H2B_{K\text{-}R}$). The isogenic strain CY1256 (WT) expressing wild-type H2B was similarly constructed. Yeast strain CY1284 ($H2B_{K\text{-}R}$ *rad6Δ0*) was constructed by disrupting *rad6* in the CY1272 background. These strains, as well as BY4425 (*rad6Δ0*), all containing YCp87-*ARG1-lacZ*, were grown to saturation in minimal medium and then diluted 1/100 in YPD medium. The cells were harvested at an A_{600} of ~1.5, and β-galactosidase activity was determined using ONPG as a substrate. Activities were standardized to cell density. The error bars represent the standard error of the mean of four samples. (B) Northern analysis. Yeast strains BY4425 (*rad6Δ0*), CY1256 (*htb2Δ0*), CY1272 ($H2B_{K\text{-}R}$ *htb2Δ0*), and BY4741 (wild type) were grown to saturation in minimal medium and then diluted 1/100 in YPD. The cells were harvested, and total RNA was isolated. Twelve micrograms of total RNA was separated by electrophoresis on a 1.0% agarose formaldehyde gel and probed with ^{32}P-labeled DNAs specific for *ARG1* and *ACT1*. The blot was visualized by autoradiography and quantitated by PhosphorImager analysis (ImageQuant 1.11). The blot is representative of analyses performed in triplicate. Two independent RNA preparations from CY1272 are shown.

TABLE 1. *ARG1-lacZ* expression in *rad6*, *rad18*, and *ubr1* deletion strains

Strain	YPD medium		Minimal medium	
	ARG1-lacZ expression	Null/WT ratio	*ARG1-lacZ* expression	Null/WT ratio
rad6Δ0	80	12	149	1.7
rad18Δ0	8.2	1.2	95	1.1
ubr1Δ0	21	3.1	154	1.7
ubr1Δ0 rad6Δ0	83	12	142	1.6
WT[a]	6.7		90	

[a] WT, wild type.

type (CY1256). The nonadditive effects of the double deletion indicate that *HTB* and *RAD6* are acting in the same pathway to regulate *ARG1*.

Northern analysis was performed to ensure that the effect of $htb_{K\text{-}R}$ on expression in rich medium was at the level of *ARG1* mRNA. As shown in Fig. 5B, $htb_{K\text{-}R}$ resulted in an increase in *ARG1* mRNA levels (compare CY1272 to CY1256). This increase was 5.5-fold, as determined by densitometry of four experiments, compared to 10.5-fold upon disruption of *RAD6* (for *RAD6*, compare BY4425 to BY4741). Together, these results suggest that ubiquitination of histone H2B by Rad6 plays a significant role in the repression of *ARG1* in rich medium.

Ubr1 is required for a component of the Rad6-mediated repression. The result described above indicated that approximately 5-fold of the 10-fold increase in *ARG1* expression seen in the absence of *rad6* was linked to histone H2B. Rad6 also interacts with Ubr1 and Rad18 for the multiubiquitination of amino-end rule proteolytic substrates and in mediating postreplication repair, respectively (3). To examine whether either of these interactions could account for the remaining Rad6-dependent repression of *ARG1*, expression of *ARG1-lacZ* was determined in BY15787 (*rad18Δ0*) and BY14814 (*ubr1Δ0*). As shown in Table 1, deletion of *rad18* did not result in increased expression of *ARG1-lacZ* when cells were grown in YPD or in minimal medium. Disruption of *ubr1* resulted in an ~3-fold increase in expression of *ARG1-lacZ* when cells were grown in YPD and 2-fold for cultures grown in minimal medium. Rad6 and Ubr1 are acting in the same pathway to regulate *ARG1*, since the *rad6 ubr1* double mutant showed the same level of expression as the disruption of *rad6* alone. A component (~25%) of the Rad6-dependent repression of *ARG1* that is seen in rich medium is thus linked to Ubr1 and potentially proteolysis. This component can account for all of the Rad6-dependent repression seen in minimal medium.

We performed a Northern analysis to verify that Ubr1 was affecting the level of *ARG1* mRNA in rich medium. As shown in Fig. 6, disruption of *ubr1* resulted in an increase of *ARG1* mRNA (threefold, as determined by densitometric analysis of two experiments), in contrast to an *ACT1* control, which was unaffected.

Repression by Rad6 at the ARG1 promoter requires components of arginine control. The *ARG1* promoter has been extensively analyzed to identify *cis*-acting elements that are involved in both the activation and repression of transcription (15, 17). As shown in Fig. 1A, the *ARG1* promoter contains an upstream binding site for Abf1, two sites for Gcn4, and two sites for interaction of the ArgR/Mcm1 complex (ARC elements). Rad6 may repress transcription by influencing these *trans*-acting factors, or it could be recruited by these factors. To analyze the roles of Abf1 and the ArgR/Mcm1 proteins, $ARG1_{\Delta ABF1}$-*lacZ* and $ARG1_{\Delta ARC}$-*lacZ*, which lacked the Abf1 binding site and ARC elements, respectively, were engineered. While deletion of the Abf1 binding site ($ARG1_{\Delta ABF1}$) resulted in a 2-fold decrease in the expression of the *ARG1* promoter when cells were grown in rich medium, the Abf1 binding site was not required for the Rad6 effect, since β-galactosidase activity is elevated 12-fold in the *rad6* deletion background (Fig. 6). As expected for loss of the ARC elements, expression of the $ARG_{\Delta ARC}$ promoter increased relative to the intact promoter when cells were grown in rich medium. Interestingly, only a 1.8-fold increase in β-galactosidase activity was seen when this allele was present in the *rad6* deletion strain. This was significantly less than the ~12-fold increase that would be expected if Rad6 acted fully independently of the ARC elements, thus supporting the view that the ARC elements play a role in repression by Rad6. The 1.8-fold increase in expression of the $ARG_{\Delta ARC}$ promoter in the *rad6* deletion background can be attributed to the Ubr1 component of its repression, since twofold of the total threefold effect seen upon deletion of *ubr1* is independent of the ARC elements (Fig. 7, compare *ARG1* and Δ*arc* promoters in the wild-type and *ubr1Δ0* strains).

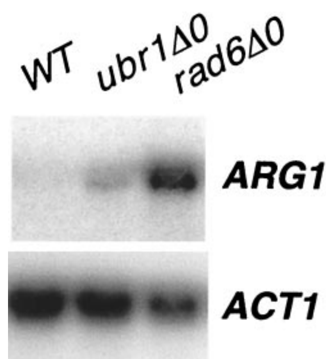

FIG. 6. ARG1 expression in a *UBR1* deletion strain . Yeast strains BY4741 (wild type [WT]), BY4425 (*rad6Δ0*), and BY14814 (*ubr1Δ0*) were grown to saturation in minimal medium and then diluted 1/100 in YPD. The cells were harvested, and total RNA was isolated. Twelve micrograms of total RNA was separated by electrophoresis on a 1.0% agarose formaldehyde gel and probed with ^{32}P-labeled DNAs specific for *ARG1* and *ACT1*. The blot was visualized by autoradiography and quantitated by PhosphorImager analysis (ImageQuant 1.11). The blot shown is representative of three experiments.

To verify the potential role of the ARC elements and to examine the roles of the two consensus binding sites for Gcn4, we assayed the expression of *ARG1-lacZ* in *arg80 rad6* and *gcn4 rad6* double-deletion backgrounds (Table 2). Disruption of *gcn4* resulted in a decrease in expression of *ARG1-lacZ* relative to the wild type; however, deletion of *rad6* in the *gcn4* background resulted in a 10.6-fold increase in β-galactosidase activity, indicating that Gcn4 is not required for Rad6-dependent repression. Disruption of *arg80* alone resulted in a significant increase in *ARG1* expression compared to the wild type. Similar to the result with $ARG_{\Delta ARC}$-*lacZ*, deletion of *rad6* in

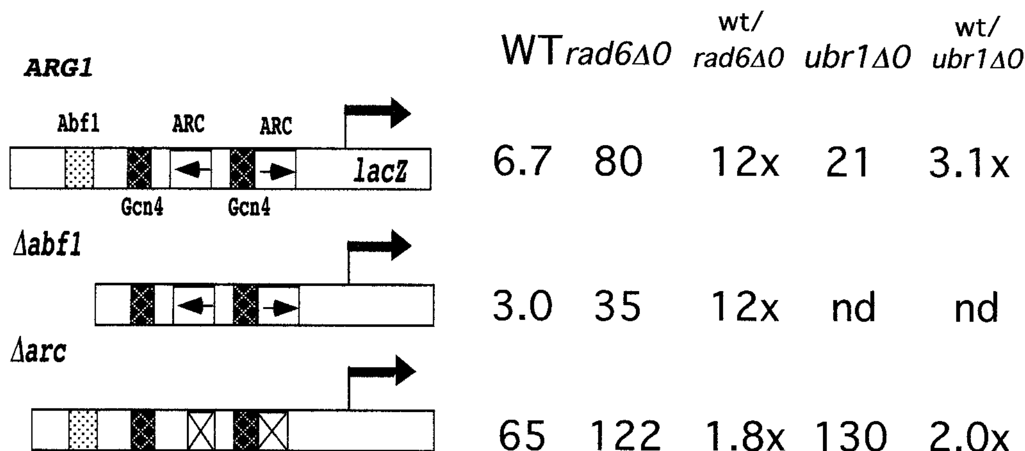

FIG. 7. Recruitment of Rad6 to the promoter requires components of the ArgR/Mcm1 complex. Saturated cultures of BY4425 (*rad6Δ0*), BY14814 (*ubr1Δ0*), and BY4741 (wild type [WT]) containing YCp87-*ARG1-lacZ*, YCp87-*ARG1*$_{\Delta ABF1}$-*lacZ*, or YCp87-*ARG1*$_{\Delta ARC}$-*lacZ* were grown in minimal medium and then diluted 1/100 in YPD. The cultures were grown to an A_{600} of 1.0 to 1.5. β-Galactosidase activity was determined using ONPG as the substrate, standardizing to cell density. *lacZ* units represent the averages of the mean for an experiment performed in triplicate with a standard error of the mean under 10%. The ratio of *lacZ* expression in the null strain versus the wild-type (wt) strain is shown for each *lacZ* fusion construct. nd, not determined.

this *arg80* deletion background resulted in only a 60% increase in expression. The finding that the fold increases in *ARG1* expression as a result of deleting *rad6* and *arg80* were not additive suggests that the arginine control proteins are necessary for Rad6-mediated repression.

Rad6 and Ada2 act through related pathways to repress *ARG1* transcription in rich medium. We have recently found that components of the SAGA coregulatory complex, including Gcn5, Ada2, and Ngg1/Ada3, are required for the repression of the *ARG1* promoter in rich medium (58a). To determine if Rad6 and Ada-dependent repression of *ARG1* are acting through a common pathway, we examined expression of *ARG1-lacZ* in an *ada2 rad6* double-deletion strain (Table 3). In this experiment, deletion of *rad6* resulted in an 11-fold increase in β-galactosidase activity when cells were grown in rich medium. This was compared with an ~5-fold increase upon disruption of *ada2* (BY4282). Expression of *ARG1-lacZ* in the double deletion (*ada2 rad6*; CY1215) was comparable (8.6-fold) to that found for the *rad6* disruption. The nonadditive effects of *ada2* and *rad6* disruptions suggest that Rad6 and components of Ada/SAGA are acting through a shared pathway to regulate *ARG1* expression.

TABLE 2. *RAD6* repression of *ARG1-lacZ* in *arg80* and *gcn4* deletion strains

Strain	*ARG1* expression (*lacZ* units)	*rad6Δ0*/*RAD6* ratio
rad6Δ0	53	
		6.2
Wild type	8.5	
arg80Δ0 rad6Δ0	135	
		1.6
arg80Δ0	86	
gcn4Δ0 rad6Δ0	35	
		10.6
gcn4Δ0	3.3	

DISCUSSION

There are a number of documented links between gene regulation and ubiquitination. Indeed, histones were the first proteins found to be ubiquitinated (27, 70), and this ubiquitination has been correlated with increased transcription (6, 19, 54). Ubiquitination of other targets has also been shown to influence transcription. These include the activation of NF-κB (40), the turnover of p53 (62), and the regulated turnover of the hypoxia-inducible factor 1α transcription factor by a ubiquitin ligase complex that includes the von Hippel-Lindau tumor suppressor protein (reviewed in reference 41). Notably, in some cases (for example, NF-κB and histones), ubiquitination does not lead directly to protein turnover. In yeast, the related hect-domain E3 ubiquitin ligases, Rsp5 and Tom1, have been implicated in transcriptional regulation. Rsp5 was identified as a suppressor of Spt3 (cited in reference 35), is required for activation by human steroid receptors in yeast (37), and modifies the carboxyl-terminal domain of the largest subunit of RNA polymerase II (7, 13, 36). Deletion of *tom1* leads to transcriptional changes at the *GAL10* and *ADH2* promoters similar to those associated with components of the Ada complex (60). Also in agreement with the idea that targeted ubiquitination at a promoter can result in changes in transcription are the findings that UreB1, which contains a C-terminal hect-domain, was initially identified through its DNA binding activ-

TABLE 3. *ARG1-lacZ* expression in *rad6* and *ada2* deletion strains

Strain	*ARG1* expression (*lacZ* units)	Deletion/WT ratio
WT[a]	7.9	1
rad6Δ0	87	11
ada2Δ0	38	4.8
ada2Δ0 rad6Δ0	68	8.6

[a] WT, wild type.

ity (28), and that the TBP-associated factor $TAF_{II}250$ possesses ubiquitin-activating and -conjugating activities (55). Furthermore, Salghetti et al. (61) have recently determined that the ubiquitn ligase Met30 is required for transcriptional activation by a LexA-VP16 fusion in yeast. Interestingly, a direct role for ubiquitination in the process was suggested by the fact that transcriptional activation was restored upon fusing ubiquitin to the amino terminus of LexA-VP16.

Rad6 is required for transcriptional repression of *ARG1*. Previous work had shown that Rad6 was involved in transcriptional repression at telomeres and the *HM* loci (34). This suggested that Rad6 might play a more general role in gene regulation. We looked for possible effects of Rad6 on *ARG1* expression because *ARG1* is subject to distinct activation and repression mechanisms in which there is a marked requirement for gene-specific transcription factors and coactivators (15, 17, 33, 44). Analysis of *ARG1-lacZ* fusions revealed that Rad6 was required for the repression of *ARG1* in rich medium. *ARG1-lacZ* expression was increased ~10-fold when a *rad6*-disrupted strain was grown in YPD medium. Initially, we had concerns that a component of the increase in β-galactosidase levels in *rad6* strains might result from reduced turnover of the reporter protein because of the involvement of Rad6 in the proteosomal degradation of proteins (22, 48, 69). However, Northern analysis confirmed that the increase was due to alterations in mRNA levels.

Histone H2B as a target for Rad6 in transcriptional regulation. The work of Robzyk et al. (59) has shown that ubiquitinated histone H2B is not found in strains with *rad6* disrupted and that a lysine-to-arginine change at the principal site of ubiquitination (K123R) within H2B confers defects in mitotic cell growth and meiosis similar to those caused by disruption of *rad6*. We have now shown that histone H2B is a likely target for Rad6 at the *ARG1* promoter. A K123R mutation in H2B results in elevated levels of *ARG1* expression. Through the analysis of a strain carrying $htb_{K\text{-}R}$ in the *rad6* disruption background, we confirmed that Rad6 and histone H2B were acting in the same pathway to regulate expression of *ARG1*. Ubiquitination of histone H2B provides an obvious potential mechanism for Rad6 regulation of *ARG1* expression through the modification of chromatin structure. How ubiquitination of H2B affects nucleosomal structure and function is unclear, but it is not likely to be related to direct turnover of histones.

As well as Rad6, the SAGA component proteins (including Ada2, Ngg1/Ada3, Gcn5, Spt7, and Spt8) are required for the full repression of *ARG1* in rich medium (33, 58a). Double disruptions indicate that Rad6 and Ada2 are acting through a common pathway. Furthermore, disruption of *gcn5* and *rad6* both result in increased promoter binding of TBP, and repression by Rad6 and Ada2 require the ArgR/Mcm1 complex (58a). It is attractive to propose that SAGA and Rad6 are acting together to create a repressive chromatin structure or that one of the nucleosome modifications acts as the signaling event allowing the second chromatin modification to occur. This model is consistent with the finding that the effect of a K123R mutation within histone H2B on the expression of *ARG1* closely parallels the effect of disrupting *ada2*. While our experiments do not address the order of events that facilitate repression, the ability of gene-specific regulators to interact with SAGA (9, 12, 45, 65, 66) suggests that targeting of SAGA by ArgR/Mcm1 and the resulting histone acetylation may initiate the process. Rad6 may then recognize acetylated nucleosomes and ubiquitinate histone H2B. Transcriptional repression could result from the rotational or translational repositioning of nucleosomes after their modification such that recruitment of the basal transcriptional machinery is sterically inhibited. We cannot exclude alternative models in which the initial ubiquitination of histone H2B facilitates the binding of the ArgR/Mcm1 complex or the preferential acetylation of nucleosomes by SAGA, although these mechanism do not provide an obvious means for promoter targeting.

The *ARG1* promoter is subject to activation through the general control pathway involving Gcn4 and arginine repression involving the ArgR/Mcm1 regulatory complex. Kornitzer et al. (42) have shown that Gcn4 is subject to turnover by the ubiquitin pathway. This raised the possibility that the increase in *ARG1* expression seen in the absence of Rad6 was due to increased levels of Gcn4-dependent activation. However, this is not the case, since increased *ARG1* expression was observed in a strain with *gcn4* deleted. Together with the finding that Rad6-dependent repression requires the ArgR/Mcm1 complex, this result implies that deletion of Rad6 leads to derepression of *ARG1*, not to enhanced activation. Based upon findings that expression of the ArgR/Mcm1-activated gene *CAR1* is not stimulated in the absence of Rad6 (data not shown), an alternative view that Rad6 is required to convert ArgR/Mcm1 from an activator to a repressor seems unlikely.

A component of the Rad6-dependent repression of *ARG1* is independent of histone H2B but requires the E3 ubiquitin ligase Ubr1. This repression is apparent whether cells are grown in rich or minimal medium. The involvement of Ubr1 (and Rad6) in N-end rule proteolysis suggests that the Ubr1 component of *ARG1* regulation requires protein turnover. Our analyses to map the elements required for Ubr1-dependent repression do not clearly define a potential target for proteolysis that can account for the full effect; however, the increase in expression is reduced from approximately threefold to twofold upon removal of the ARC elements and in minimal medium, suggesting that turnover of ArgR/Mcm1 may partially contribute.

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