Molecular Genetic Analysis of the Yeast Repressor Rfx1/Crt1 Reveals a Novel Two-Step Regulatory Mechanism
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In Saccharomyces cerevisiae, the repressor Crt1 and the global corepressor Ssn6-Tup1 repress the DNA damage-inducible ribonucleotide reductase (RNR) genes. Initiation of DNA damage signals causes the release of Crt1 and Ssn6-Tup1 from the promoter, coactivator recruitment, and derepression of transcription, indicating that Crt1 plays a crucial role in the switch between gene repression and activation. Here we have mapped the functional domains of Crt1 and identified two independent repression domains and a region required for gene activation. The N terminus of Crt1 is the major repression domain, it directly binds to the Ssn6-Tup1 complex, and its repression activities are dependent upon Ssn6-Tup1 and histone deacetylases (HDACs). In addition, we identified a C-terminal repression domain, which is independent of Ssn6-Tup1 and HDACs and functions at native genes in vivo. Furthermore, we show that TFIID and SWI/SNF bind to a region within the N terminus of Crt1, overlapping with but distinct from the Ssn6-Tup1 binding and repression domain, suggesting that Crt1 may have activator functions. Crt1 mutants were constructed to dissect its activator and repressor functions. All of the mutants were competent for repression of the DNA damage-inducible genes, but a majority were “derepression-defective” mutants. Further characterization of these mutants indicated that they are capable of receiving DNA damage signals and releasing the Ssn6-Tup1 complex from the promoter but are selectively impaired for TFIID and SWI/SNF recruitment. These results imply a two-step activation model of the DNA damage-inducible genes and that Crt1 functions as a signal-dependent dual-transcription Activator and repressor that acts in a transient manner.

Transcription induction of repair genes upon DNA damage is conserved in all organisms examined, from Escherichia coli to human beings (11, 50). Among these genes are those encoding the subunits of enzyme ribonucleotide reductase (RNR), which catalyzes the rate limit step in deoxyribonucleotide triphosphate synthesis. In the yeast Saccharomyces cerevisiae, the RNR2, -3, and -4 genes are predominantly regulated by a transcriptional repression mechanism through the DNA damage response elements, or X boxes, which are recognized by the sequence-specific DNA binding protein Rfx1/Crt1 (20). The X boxes are also found in the promoter of HUG1. The function of HUG1 is not known, but it shows genetic interactions with genes in the DNA damage checkpoint pathway (1). The repression of the Crt1 gene by its own product suggests that a negative feedback pathway is important for the reestablishment of the repression state after the elimination of DNA damage (20).

The Ssn6-Tup1 corepressor is crucial for repression of the DNA damage-inducible genes. The corepressor is recruited to the target promoters by the N-terminal (1–240) region of Crt1 and is released together with the repressor upon DNA damage (20, 23, 48, 49). Ssn6-Tup1 is a yeast global corepressor regulating genes controlled by distinct cellular pathways (for a review, see reference 38). Multiple mechanisms can be utilized in Ssn6-Tup1 function, including (i) nucleosome positioning through histone tail binding (6, 9, 10), (ii) histone deacetylase (HDAC) recruitment (2, 8, 44, 47), and (iii) direct interference with activators or transcription machineries (15, 17, 19, 27). Both the Ssn6-Tup1 recruitment and histone deacetylation are localized to the upstream repression sequences (URS), which contains the binding sites for Crt1 (7, 48). A repressive nucleosome array over the RNR3 promoter is dependent upon Ssn6-Tup1 and Crt1 (24). Deletion of Crt1, Ssn6, or TUP1 or inducing the cell with the DNA-damaging agent methyl methan sulfate (MMS) causes the disruption of the nucleosome array and gene activation, suggesting the critical role of chromatin structure in RNR3 gene regulation (24). Work from our lab also showed that the Ssn6-Tup1-dependent nucleosome positioning at RNR3 requires the collaboration of the ISW2 nucleosome remodeling spacing complex, and the loss of nucleosome positioning upon DNA damage requires the SWI-SNF chromatin remodeling complex (37, 48), indicating that its regulation requires a balance between nucleosome positioning and remodeling.

Crt1 belongs to the winged-helix family of DNA binding proteins, characterized by their unique “winged-helix” DNA binding domain with a separate and independent dimerization domain (12, 14). Its homologues in higher eukaryotes are generally referred to as RFX proteins. In contrast to the human RFX proteins, which are known to be involved in both the activation and repression of transcription (21, 22, 36), Crt1 was initially isolated as a repressor and was shown to dissociate from the target promoter upon induction, arguing against a role in activation (20). However, Crt1 was later found to interact with TFIID, which generally acts as a coactivator (23), suggesting that it may have activator functions at DNA damage-inducible genes or other genes in vivo. In addition, the
corepressor Ssn6-Tup1 has recently been shown to function as a coactivator at some target promoters (28, 29). Thus, the activation of the DNA damage-inducible genes might require transient activation functions of either Crt1 or Ssn6-Tup1.

Here we describe the characterization of the repression and activation functions of Crt1. We demonstrate that Crt1 contains two distinct repression domains and a region within the N terminus that is required for activation. Targeted mutagenesis of Crt1 was conducted to identify mutants that disrupt its activation functions while preserving repression activities. All of the mutants, when reintroduced into a crt1-null strain, are capable of repressing DNA damage-inducible genes, recruiting Ssn6-Tup1 to the URS, and establishing a nucleosomal array over RNR3. Significantly, derepression of transcription was specifically blocked in most mutants, which thus are “derepression defective.” Chromatin immunoprecipitation assays suggest that these mutants are blocked after corepressor release but at the coactivator recruitment step. These results imply a Crt1-dependent two-step activation model for DNA damage-inducible genes and suggest that Crt1 can function as a transcription activator, analogous to its mammalian homologues.

MATERIALS AND METHODS

In vitro mutagenesis and strain construction. The wild-type CRT1 gene (~798 to +2986) was cloned by amplification of yeast genomic DNA by PCR, digested with restriction enzymes ApaI and EagI, which cut at the 5’ and 3’ ends, respectively, and inserted into the same sites in the pRS404 plasmid (4). There is an EcoRI site at +516 (corresponding to amino acid residue 172) of the CRT1 open reading frame. Crt1 mutants were constructed by in vitro mutagenesis as follows. The Δ162–172 mutant plasmid was constructed by replacing the ApaI/EcoRI fragment in the wild-type plasmid with a digested PCR product corresponding to ~798 to +486 of the CRT1 locus. The Δ172–182, Δ172–202, and Δ172–220 mutants were constructed by replacing the EcoRI/Agal insertion with PCR products corresponding to ~546 to +2986, ~606 to +2986, and ~660 to +2986 of the CRT1 locus, respectively. The Δ181–200 and Δ203–220 mutants were constructed by oligonucleotide site-directed mutagenesis. The pRS404-CRT1 wild-type and mutant derivatives were then digested with StuI, which cut at ~522 of CRT1, and integrated into a strain deleted of the coding sequence of CRT1 (ΔCrt1::KanMX). YJR851. The integration and copy number were confirmed by PCR and Southern blotting.

The C-terminus mutations, Δ644–811 and Δ709–811, were constructed by inserting a stop codon by homologous recombination as described previously (25). Deletion of SNS6, TUP1, and HDACs was carried out by one-step replacement using PCR-generated cassettes (4). A complete list of strains is found in Table 1. Primer sequences and details of the constructs are available upon request.

RNA isolation and Northern blot. RNA isolation was carried out as previously described (42). Ten to fifteen milliliters of yeast cells grown in YPAD (1% yeast extract, 2% peptone, 20 μg/ml adenine sulfate, 2% dextrose), treated or untreated with 0.03% MMS, was harvested by centrifugation, washed with cold STE buffer (10 mM Tris-Cl [pH 7.5], 10 mM EDTA, 500 mM NaCl). RNA was released with bead beating in the presence of 150 μL phenol-chloroform, extracted once more with phenol-chloroform, precipitated with ethanol, and dissolved in diethyl pyrocarboxylic benzamide-treated water. Twenty micrograms of RNA was separated on 1% formaldehyde agarose gels and transferred to a nylon membrane (Amersham-Pharmacia) by capillary blotting. After UV cross-linking and a 4-h prehybridization at 65°C, radiolabeled gene specific probes were added and incubated overnight.

β-Galactosidase assay. β-Galactosidase assays were carried out as described in a previous publication (32). In brief, the LexA-Crt1 fusion proteins were expressed from pE2G20. The reporter plasmid pK101 contains LacZ (β-galacto-

sidosiase) under the control of a minimal GAL1 promoter in which four LexA operators were inserted upstream of the TATA box (5). The LexA and reporter plasmids were cotransformed into yeast strains and selected on proper synthetic dropout (SD) medium supplemented with dextrose (2%). Three to six colonies from each transformation were picked, inoculated to 5 ml liquid SD-raffinose (3%), and incubated at 30°C with shaking until saturation. The liquid cultures were then resseeded into 5 ml fresh SD-raffinose liquid medium, grown to log phase (optical density at 600 nm [OD600] of 0.5 to 1.0), collected by centrifuga-

tion, and washed with cold STE (10 mM Tris-Cl [pH 7.4], 100 mM NaCl, 1 mM EDTA). The cell pellets were resuspended in 250 μl of beads breaking buffer (100 mM Tris-Cl [pH 8.0], 1 mM dithiothreitol, 20% glycerol), cell lysates were prepared by vortexing in the presence of glass beads, and β-galactosidase activity was analyzed. For the β-galactosidase assays with the Δrdp3 Δhda1 Δsot2 mutant (YJR475) (ΔUR4), a derivative of pK101 containing a TRP1 marker was used. GST pull-down assay. Full-length or fragments of CRT1 was amplified by PCR and cloned into pGEX3 or pRET3aGST (a gift from Song Tan). The glutathione

TABLE 1. List of strains used in this study

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using polyclonal antiserum against TATA-binding protein (TBP), TAF1, TAF6, or monoclonal 9E10 antibody (Covance) to detect nine-Myc-tagged Snf2. The Snf6/Tup1 interaction assays were performed as follows. The 35S-labeled Snf6 and Tup1 proteins were produced using an in vitro transcription/translation rabbit reticulocyte system in the presence of [35S]methionine (Promega). Twenty-five micrograms of GST-Crt1 (or mutant derivatives) was incubated with 20 µl of cotranslated Snf6 and Tup1 in 80 µl of binding buffer (20 mM HEPES-KOH, pH 7.5), 150 mM potassium acetate, 1 mM EDTA, 1 mM dithiothreitol, 5% (vol/vol) glycerol, and 0.01% NP-40. After a 60-min incubation at 4°C, the beads were collected by low-speed centrifugation and washed four times for 10 min each with 500 µl of binding buffer. The bound proteins were eluted in SDS-PAGE loading buffer, separated by SDS-PAGE, stained, treated with En3Hance (Dupont-NEN), dried, and exposed to X-ray film.

**Nucleus isolation.** Nuclei isolation was carried out essentially as described previously (24, 35). In brief, 1 liter of cells were grown in YPAD rich medium to an OD600 of around 1.0, harvested, and digested with Zymolyase T100 (Seikagaku). Spheroblasts were lysed by homogenization, and the nuclei were isolated and washed by differential centrifugation. The nuclei were resuspended in digestion buffer, accordingly to the size of the nuclei pellet, and digested by 0, 2, 4, and 8 units/ml of micrococcal nuclease (MNaSe) (Worthington) for 10 min at 37°C. The digestion was stopped by the addition of EDTA, and the DNA was purified by RNase A and proteinase K treatment and phenol chloroform-isooamyl alcohol extraction. The purified DNA then was digested by PstI, electrophoresed on agarose gels, and detected by Southern blotting using a 200-bp probe specific for one end of the PstI fragment (24). Naked DNA was treated the same, except that the MNaSe digestion was after the purification of the DNA from the nuclei and less enzyme was used.

**ChIP.** The chromatin immune precipitation (ChIP) assay was performed as described previously, with minor changes (18, 49). A 50-ml culture of cells were grown in YPAD medium to an OD600 of 0.5 to 1.0 (the induced cells were treated at an OD600 of 0.7 with 0.03% MMS and incubated for 2 h before harvest). Then, the cells were cross-linked with 1% (vol/vol) formaldehyde at room temperature for 15 min. The formaldehyde was quenched by the addition of glycine to 125 mM and shaking for 15 min at room temperature. After washing, cells were then broken by vortexing with glass beads, and the lysate was sonicated. The lysates were then clarified by centrifugation, and 200 µl of anti-TBP, -TAF1, or -Snf2 polyclonal antiserum, or monoclonal 9E10 antibody (Covance) to detect nine-Myc-tagged Snf2. The immunoprecipitated DNA was amplified using primers flanking the core promoter/TATA box was used (amplifying from ~480 to ~236). For TBP, TAF1, and Snf2 recruitment, a primer pair flanking the core promoter/TATA box was used (amplifying from ~179 to ~80). The PCR products were loaded into a 2% agarose gel, stained with ethidium bromide, scanned with the Typhoon system (Molecular Dynamics), and quantified using ImageQuant software.

**RESULTS**

**Crt1 has Snf6-Tup1-independent repression activities.** The DNA damage-inducible genes RNR3 and HUG1 are repressed through the combined actions of Crt1 and the Snf6-Tup1 corepressor complex. However, both genes are derepressed to a higher level in a Δcrt1 mutant than in either a Δsnf6 or Δtup1 mutant (1, 24, 51) (Fig. 1). This is strikingly obvious for HUG1 (Fig. 1). Crt1 can bind to either Tup1 or Snf6 in vitro (20, 24), so the residual repression activity in the single corepressor mutants could be due to redundancy. A number of lines of evidence suggest that Snf6 and Tup1 can function independently. It is known that both Snf6 and Tup1 can bind to histone deacetylases individually (8, 44). Also, deleting SSN6 or TUP1 has distinct affects on the repression and chromatin structures at certain loci (6, 45). To rule out redundancy, we analyzed the expression of RNR3 and HUG1 in a double Δsnf6 Δtup1 mutant. Figure 1 shows that the double mutant had a level of derepression similar to that of the single mutants, indicating that SSN6 and TUP1 do not contribute individual, redundant repression functions at these two genes. This is in agreement with our studies showing that deleting SSN6 or TUP1 individually has identical effects on the chromatin structure of RNR3 and those of another group, showing that the recruitment of Tup1 to promoters requires Snf6 (7, 24).

**FIG. 1.** Snf6-Tup1-independent repression by Crt1. (A) The levels of RNR3 and HUG1 expression in wild-type cells and Δsnf6, Δtup1, and Δcrt1 mutants were analyzed by Northern blotting. Small cellular RNA (scR1), transcribed by RNA polymerase III, was used as the loading control. The RNR3 and HUG1 hybridization signals were quantified, normalized to scR1, and expressed relative to the signal from untreated wild-type cells, which was arbitrarily set as 1.0. (B) Expression of RNR3 in wild-type and corepressor mutants before and after treatment with 0.03% MMS for 2 h. (C) Chromatin immunoprecipitation assay using antibodies raised against the N terminus of Crt1 and Tup1 proteins were produced using an in vitro transcription/translation system. The bound proteins were then clarified by centrifugation, and 200 µl of diluted anti-Tup1p polyclonal antibody (1/200). The immune complexes were isolated with 25 µl of protein A-Sepharose CL-4B beads (Amersham-Pharmacia) and washed extensively, and the DNA was eluted from the beads. The cross-links were reversed by incubating the samples at 65°C overnight. After purification, the precipitated and input DNA was analyzed by semiquantitative PCR. For Crt1 and Tup1 cross-linking, a primer pair flanking X boxes in the upstream regulatory sequence (URS) was used (amplifying from ~480 to ~236). For TBP, TAF1, and Snf2 recruitment, a primer pair flanking the core promoter/TATA box was used (amplifying from ~179 to ~80). The PCR products were loaded into a 2% agarose gel, stained with ethidium bromide, scanned with the Typhoon system (Molecular Dynamics), and quantified using ImageQuant software.
Next, we used epistasis analysis to rule out two additional explanations for the phenotypic differences between CRT1 and corepressor mutants. \(\Delta ssn6\) and \(\Delta ssn6\) mutants display a variety of phenotypes, including slow growth and temperature sensitivity (41, 46), suggesting that the reduced transcription could be due to reduced cell vitality. In addition, recent reports suggest that Tup1 plays a positive role in transcription of salt- and galactose-induced genes (28, 29), and it is possible that Tup1 plays a similar role at \(\beta\)-galactosidase gene from \(E.\ coli\) under the control of LexA binding sites and a minimal \(GAL1\) promoter. \(\beta\)-Galactosidase activities were measured from cell extracts and shown on the top panels. Data are also presented in the bottom panel as the level of repression compared to results with LexA alone, which was set at 1.0. Mapping of the N-terminal (A) and C-terminal (B) repression domains.

thermore, we detected a reproducible ~1.7-fold increase in CRT1 cross-linking in the corepressor mutants. More importantly, we found that the level of CRT1 cross-linking is reduced about fivefold in wild-type cells but only ~1.5-fold in the corepressor mutants and that the level of cross-linking in the corepressor mutants after MMS treatment was equal to that in untreated wild-type cells. Thus, the failure to observe significant derepression of \(RNR3\) in the corepressor mutants after MMS treatment results from the persistence of CRT1 at the URS.

Identification of two repression domains in CRT1. Our laboratory revealed that the N-terminal 240 amino acids (1 to 240) of CRT1 contain a strong repression domain and interact with Sn6-Tup1 in vitro (23). To gain more insight into the function of CRT1, we further mapped this domain by analyzing the ability of LexA-CRT1 fusion proteins to repress a LacZ reporter construct weakly, but unlike the N terminus of CRT1, it did not bind to Sn6-Tup1 in vitro (23), thus suggesting that the C terminus may contain Sn6-Tup1-independent repression activity. Given that LexA-CRT1(319–585) showed no repression activity in the same assay, we directed our studies towards the
The N- and C-terminal domains repress via distinct mechanisms. The mechanisms of the two Crt1 repression domains were examined by conducting the repression assay with corepressor mutants. Figure 3A shows that the ability of LexA-Crt1(1–240) to repress transcription was severely compromised in Δssn6, Δtup1, and Δssn6 Δtup1 cells compared to wild-type cells. This is consistent with GST pull-down data showing that the 1–240 region binds Snf6-Tup1 in vitro (23; also see below) and suggests that the vast majority of the repression activity of this region is mediated through the corepressor complex. In contrast, LexA-Crt1(595–811) repressed transcription to similar levels in mutants and in wild-type cells, arguing that the C-terminal repression domain is not dependent upon the corepressor complex, again consistent with our observations that the C terminus of Crt1 does not bind to Snf6-Tup1 in vitro. As reported previously, fusing full-length Crt1 to LexA repressed transcription about 20- to 25-fold, about half as well as LexA-Crt1(1–240) (23). The cause of this is unknown. Nonetheless, the ability of full-length Crt1 to repress transcription was partially compromised in the corepressor mutants, and interestingly, the magnitude of its repression in corepressor mutants is similar to that of the LexA-Crt1(595–811) derivative. This result might be expected, given that the C-terminal repression domain functions independently of Snf6-Tup1, and suggests it can repress the reporter construct within the context of full-length Crt1.

Snf6-Tup1 binds to HDACs, and deletion of multiple HDAC genes causes partial derepression of a number of Snf6-Tup1 target genes, including RNR3 and HUG1 (8, 44, 47, 49). This suggests that Snf6-Tup1 represses genes by recruiting HDACs to promoters. Thus, we tested if HDACs are required for the function of the N- and C-terminal repression domains of Crt1. Even though the C-terminal repression domain of Crt1 functions independent of Snf6-Tup1, it may repress transcription by directly recruiting HDACs. First, we examined the ability of LexA-Crt1 derivatives to repress transcription in a Δrp3d mutant, since Rpd3 binds to Snf6-Tup1 (8, 44). Deleting RPD3 increased the activity of the reporter gene even when LexA alone was expressed. We found that the repression activity (repression over that of LexA) of the N-terminal domain was reduced about threefold in Δrp3d cells compared to that in wild-type cells (Fig. 3B). A significant level of repression was observed, however, and the ability of the N terminus to repress was more strongly effected in corepressor mutants than the Δrp3d mutant (compare Fig. 3A and 3B). Deleting RPD3 weakly affected the ability of LexA-Crt1(1–811) to repress and had no significant effect on the activity of the C-terminal repression domain.

The inability of a single Δrp3d mutation to fully compromise repression could result from redundancy among the HDAC genes. In many cases, deletion of multiple HDAC genes is required to observe significant levels of derepression of Snf6-Tup1-regulated genes (8, 44; V. M. Sharma and J. C. Reese, unpublished data). Thus, we examined repression in strains containing deletions in multiple HDAC genes. Hda1 is reported to interact with Tup1 in vitro, although this is controversial (8, 44), and thus, we extended our analysis to strains containing a Δhda1 mutation. Surprisingly, deleting HDA1 had no detectable affect on the ability of Crt1, or any of its derivatives, to repress in this assay (Fig. 3C). Further, deleting HDA1 did not decrease the level of repression in a Δrp3d background: the level of repression was very similar in the Δrp3d and Δrp3d/hda1 mutants. This was unexpected, given that deletion of HDA1 caused increases in acetylation of histones at RNR3 and weak derepression of DNA damage-inducible genes (49; V. M. Sharma and J. C. Reese, unpublished data). The inability of the Δhda1 mutation to reduce repression by the N-terminal domain might be due to the fact that Δhda1 mutants show increased acetylation in only histone H3 and H2B in vivo, whereas deletion of RPD3 caused increases in all four histones (39). Finally, we examined a triple mutant (Δrp3d/Δhost1/Δhost2) that was shown to cause partial derepression of Tup1-regulated genes (7) and found that derepression by the N-terminal domain was significantly reduced, but a measurable level of repression was still observed. In all HDAC mutants, the level of repression by the LexA-Crt1 derivatives was much less than that observed in the Δssn6 and Δtup1 mutants (compare Fig. 3A with 3C). This can be explained by the ability of Snf6-Tup1 to repress by interfering with the mediator or affecting the positioning of nucleosomes over the promoter (for a review, see references 33 and 38). However, since we have not exhausted all combinations of HDAC mutations, it is unclear if this is the case.

The C terminus of Crt1(709–811) is a bona fide repression domain in vivo. The N-terminal repression domain of Crt1 functions through Snf6-Tup1 (Fig. 3A), but the mechanism of the C-terminal domain is not clear. The uncertainty of the mechanism of the C-terminal repression domain, and the fact that it was identified using an artificial assay system, prompted us to verify that it functions as a repression domain in vivo at native target genes. To do so, we have constructed Crt1 mutants containing truncations within its C terminus by introducing a stop codon by homologous recombination at its natural chromosomal locus (25). CRT1 mutants crt1(Δ709–811) and crt1(Δ644–811) were isolated and analyzed. Deletion of amino acids 644 to 811 caused a very severe repression defect in RNR3 and HUG1, and the level of mRNA was close to that of MMS-treated cells or a Δcord1 mutant (Fig. 4A and Fig. 1). The complete loss of repression by this mutant is not consistent with the C terminus playing a lesser role in repression, as predicted from LexA-Crt1 reporter assays (Fig. 2A), suggesting that a trivial defect explains this result (see below). On the other hand, the crt1(Δ709–811) mutation caused partial dere-
pression, about 10-fold, of RNR3 and HUG1 in the absence of DNA damage, and further derepression was observed when these cells were treated with MMS (Fig. 4A). This observation is consistent with the weaker repression activity of the C-terminal domain in the reporter assay. Western blotting of extracts prepared from these cells revealed that the crt1(Δ644–811) and crt1(Δ709–811) mutants accumulate at lower and higher levels than wild-type Crt1, respectively (Fig. 4B). The higher level of the crt1(Δ709–811) mutant protein may be caused by partial derepression of CRT1 transcription, since CRT1 represses its own expression as part of a negative feedback loop (20). The lower level of the crt1(Δ644–811) mutant protein suggests that it might be unstable.

Since these deletions are within the C terminus, which may play a role in DNA binding and/or dimerization (22; Z. Gearhart-Hines and J. C. Reese, unpublished data), we examined the ability of these mutants to cross-link to RNR3 in vivo, using the ChIP assay. Polyclonal antiserum raised against the N terminus of Crt1(1–240) was used, so these mutations should not affect protein-antibody interactions. As reported previously (20, 24), strong cross-linking of wild-type Crt1 was detected over the RNR3 URS, and its association was reduced significantly by MMS treatment (Fig. 4C). The assay also reveals that the crt1(Δ644–811) mutant does not bind to RNR3 in vivo; thus, the repression defect results from the lack of promoter binding in vivo. In contrast, the crt1(Δ709–811) mutant cross-linked to RNR3 as well as the wild-type protein, and its cross-linking was reduced to a degree similar to that of wild-type Crt1 by MMS treatment (Fig. 4C). This indicates that the reduced repression in the crt1(Δ709–811) mutant is not due to trivial defects in DNA binding or defects in the DNA damage response pathway. Since a function of Crt1 is to recruit Tup1 to promoters, the reduced repression in the crt1(Δ709–811) mutant could be caused by reduced Tup1 recruitment, although this is not expected given that Tup1 binds to the N terminus of Crt1. So we examined the ability of the crt1(Δ709–811) mutant to recruit Tup1 to RNR3, using the ChIP assay. Figure 4D shows that Tup1 cross-linked to RNR3 in the crt1(Δ709–811) mutant as well as in cells containing wild-type Crt1. Thus, the results of Fig. 4 strongly suggest that the C terminus of Crt1 plays a role in repression in vivo and that defects in promoter recognition or Tup1 recruitment cannot explain the reduced repression activity of the crt1(Δ709–811) mutant.

If in fact the C-terminal repression domain functions independently of the N-terminal domain and the Ssn6-Tup1 complex as the LexA-reporter system implies, then a Crt1 mutant containing a deletion of both the N- and C-terminal repression domains would display a level of derepression similar to that of a Δcrt1 mutant. Unfortunately, deleting the N terminus of Crt1 produces mutants that are unable to bind to the RNR3 promoter in vivo and/or are not expressed to high levels (not shown). Thus, we used another strategy to test if the two domains function independently of each other in vivo. TUP1 or SSN6 was deleted in a crt1(Δ709–811) background, which we predicted would result in higher levels of derepression of RNR3 and HUG1 than the single mutants. Consistent with results shown in Fig. 1, deleting SSN6, TUP1 or a combination resulted in a partial derepression compared to results with MMS-treated cells (Fig. 5). Likewise, the crt1(Δ709–811) mutant displayed partial derepression. Importantly, deleting ei-

**FIG. 3.** Corepressor and HDAC dependency of the two repression domains. To test the mechanism of the two Crt1 repression domains, repression assays were carried out as described in the legend to Fig. 2 with strains containing deletions of the genes of corepressor complex and HDACs. β-Galactosidase activities are shown on the top and fold repression on the bottom of each panel. (A) Ssn6-Tup1 dependence; (B and C) HDAC dependence.
her SSN6 or TUP1 in the crt1(Δ709–811) background increased the level of derepression beyond that seen in the single corepressor or crt1(Δ709–811) mutants. The level of derepression was not as strong as in MMS-treated cells, however, which was particularly clear at HUG1. This suggests that Crt1 might have additional repression functions that lie outside of the 709–811 region and that it is Ssn6-Tup1 independent. Nonetheless, the results suggest that Crt1 has two repression domains that function through independent mechanisms, and both contribute to the repression of DNA damage-inducible genes in their natural context.

The N-terminal repression domain is distinct from that required for coactivator interactions. The interaction of Crt1 with the TFIID coactivator is both puzzling and intriguing, considering that Crt1 is considered a repressor of gene transcription and is released from the promoter when the gene is activated (20, 24; also see Fig. 4C). In our previous studies, the smallest N-terminal fragment of Crt1 identified to bind to both TFIID and Ssn6-Tup1 was the 1–240 fragment (23). With the N-terminal repression domain redefined more precisely to amino acids 1 to 130 (Fig. 2), we were interested in mapping the region required for TFIID interaction to see how closely the coactivator and corepressor interaction domains coincide. We examined the interaction of Crt1 with the Ssn6-Tup1, TFIID, and SWI/SNF complexes in GST pull-down assays (Fig. 6A). Assays were conducted using in vitro-cotranslated Ssn6-Tup1 and whole-cell extracts. Ssn6 and Tup1 were cotranslated in the same reaction mix, but it is unclear that they form an intact Ssn6-Tup1 complex; however, Ssn6 and Tup1 can bind to Crt1 individually (20; B. Li and J. C. Reese, unpublished data). In vitro-translated Ssn6 and Tup1 interacted with GST-Crt1(1–240) as previously reported, and the interac-

FIG. 4. Analysis of the C-terminal repression domain in vivo. (A) Northern blot analysis of RNR3 and HUG1 expression in C-terminal truncation mutants. Cells were treated with 0.03% MMS for 2 h where indicated. The expression levels of RNR3 and HUG1, relative to the signal from untreated wild-type cells, are indicated below each panel. (B) Western blot examining the protein level of Crt1 mutants. (C) ChIP analysis of the binding of Crt1 mutants to RNR3 URS and its response to DNA damage. Antiserum raised against Crt1(1–240) was used in the immunoprecipitation. Preimmune serum was used for IP background control. (D) ChIP analysis of Tup1 cross-linking to RNR3 URS in the crt1(Δ709–811) mutant.

FIG. 5. Epistasis analysis of the crt1(Δ709–811) and Δssn6 Δtup1 mutants. The levels of RNR3 and HUG1 mRNA were measured by Northern blotting and analyzed as described in the legend to Fig. 1. Cells were treated with 0.03% MMS for 2 h where indicated. ScR1 is a loading control.
tions. Since the region of Crt1 that interacts with corepressors and coactivators is overlapping but distinct, mutants can be made that disrupt coactivator interaction without perturbing corepressor recruitment, therefore allowing us to discriminate its repression versus activation functions. We constructed a series of crt1 mutants with internal deletions within amino acid residues 160 to 240. These mutants were made in vitro and reintroduced back into the CRT1 locus, and the expression of RNR3 and HUG1 was examined. The Northern blot presented in Fig. 7A indicates that all of the mutants were capable of repressing RNR3 and HUG1 to a level equal, or nearly equal, to that of wild-type cells. Strikingly, four out of the six mutants were unable to achieve a high level of derepression upon MMS treatment (Fig. 7A). These mutants, which will be referred to as “derepression defective” from here on, resulted from the deletion of amino acids 162 to 172, 172 to 202, 172 to 220, and 181 to 200. One of the mutants, crt1(Δ203–220), displayed a small amount of derepression and was capable of inducing RNR3 and HUG1 to higher levels than those for wild-type cells. The cause of this is unknown. All of the mutants are expressed to levels similar to those for wild-type Crt1 in cells (data not shown), which is expected given that their repression functions were intact.

Crt1 and Tup1 dissociate from the promoter upon DNA damage (20, 24, 48) (Fig. 4C and D), and therefore, derepression could be blocked if the mutants are unable to sense the damage signal and/or leave the promoter. So we next examined the release of the Crt1 mutants and the Tup1 corepressor from the promoter upon DNA damage using the ChIP assay. Since we introduced mutations within the N terminus of Crt1, polyclonal antiserum raised against residues 240 to 811 of Crt1 was used. As shown in Fig. 7B, most mutants, except for the crt1(Δ172–220) and crt1(Δ203–220) mutants, showed normal cross-linking to the RNR3 promoter in untreated cells. We speculate that the reduced immunoprecipitation of DNA in the crt1(Δ172–220) and crt1(Δ203–220) mutant samples results from reduced cross-linking efficiency rather than reduced DNA binding ability in vivo, because these mutants display normal repression activities (Fig. 7A) and recruit Tup1 to the promoter (see below). It appears that all mutants containing a deletion of amino acids 203 to 220 cross-linked less well to RNR3. Interestingly, the region between 172 and 220 contains multiple lysine residues, with four clustered lysines between 203 and 220, which could serve as good targets for formaldehyde-mediated cross-linking. Upon DNA damage, the level of cross-linking of the derepression-defective Crt1 mutants was reduced in all cases. However, there were some differences. The reduction in cross-linking of some mutants was not equal to that of wild-type Crt1, specifically the crt1(Δ162–172), crt1(Δ172–202), and crt1(Δ181–200) mutants. The cross-linking of wild-type Crt1 was reduced about fivefold, whereas the reduction in these mutants was ~two- to threefold.

Next, we examined corepressor recruitment and release by monitoring cross-linking of Tup1 to RNR3. Figure 7B shows that Tup1 is cross-linked to RNR3 in the absence of DNA damage, and treating cells with MMS resulted in a significant reduction. The ChIP assay reveals that Tup1 is cross-linked to RNR3 in all of the mutants; however, the level was slightly reduced compared to that in wild-type cells. This is unlikely to have functional consequences, since the mutants repress tran-

FIG. 6. Mapping of the corepressor and coactivator binding regions of Crt1. Crt1 fragments were expressed and purified as GST fusion proteins and immobilized on glutathione-agarose beads. (A) Binding of Crt1 fragments to in vitro-translated and 35S-labeled Snf5 and Tup1 was analyzed using the pull-down assay, and the bound fraction and 1/20 of the input were detected by autoradiography. The retention of TFIIID from whole-cell extracts was detected by Western blotting using polyclonal TAF1 and TBP antiserum. (B) Binding of SWI/SNF to the N terminus. Pull-downs were performed using extracts of cells containing the Snf2 gene tagged with nine-Myc epitopes, and the bound protein was detected using the monoclonal antibody 9E10.
The ChIP assays for Crt1 and Tup1 described above suggest that activation of \( RNR3 \) involves steps in addition to the dissociation of repressors from the promoter, and the lack of chromatin remodeling implies a defect in \( \text{SWI/SNF} \) recruitment or function. We examined the recruitment of \( \text{SWI/SNF} \) using a polyclonal antiserum to Snf2. As reported previously, \( \text{SWI/SNF} \) was recruited to the \( RNR3 \) promoter upon DNA damage in wild-type cells (37) (Fig. 10). Likewise, it was also recruited well in the \( c\Delta 172–182 \) and \( c\Delta 203–220 \) mutants, which are not derepression defective. Strikingly, no \( \text{SWI/SNF} \) recruitment was observed in any of the derepression-defective mutants (Fig. 10). On the other hand, the \( \Delta 172–220 \) derivative failed to bind to these complexes in this assay. Even though we expected to see a better correlation between the activation defects and binding, it is possible that the less-extensive mutations (smaller deletions) weaken the binding in vivo, but this cannot be detected in the pull-down assay (see below).

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defective mutants were likewise defective for TFIID recruitment, in contrast to the increase observed in the wild type and the \textit{crt1}\textsubscript{Δ172–182} and \textit{crt1}\textsubscript{Δ203–220} mutants (Fig. 10). Thus, our data show that these mutants are defective for TFIID and SWI/SNF recruitment and nucleosome remodeling, which indicates that the mutants are blocked at the coactivator recruitment step after corepressor release. Furthermore, even though we failed to detect a defect in the binding of two of the three derepression-defective mutants to TFIID and SWI/SNF in pull-down assays in vitro (Fig. 8), these mutants are defective for SWI/SNF and TFIID recruitment in vivo. Collectively, our results suggest that Crt1 participates in the activation of \textit{RNR3} via a two-step regulatory mechanism, corepressor release, and coactivator recruitment.

DISCUSSION

DNA binding proteins in yeast are usually defined as activators or repressors based upon the transcriptional phenotypes of their mutants. For example, if deleting a gene for a transcription factor causes enhanced transcription, it is defined as a repressor. Crt1 was isolated as a repressor, and it was not thought to act as an activator (20, 51). In the case of the DNA damage-inducible \textit{RNR} genes, Crt1’s role in activation was inconsistent with the current model that it is phosphorylated and released from the promoter upon gene activation and that the \textit{RNR} genes are strongly expressed in a \textit{crt1} mutant (20, 24). Furthermore, the coactivators TFIID, SWI/SNF, and Mediator are constitutively associated with the promoter of \textit{RNR3} in a \textit{Δcrt1} mutant, suggesting that it is not essential for transcription factor recruitment (37). All of the above argue that Crt1 acts only as a repressor at DNA damage-inducible genes. However, its potential to be involved in activation functions was suggested by its interaction with TFIID (23), but it was unclear if Crt1 can act as a dual activator-repressor at a specific gene or as an activator at one locus and a repressor at another.
Ssn6-Tup1 corepressor complex, in particular, the repressed chromatin structure at the promoter. It’s analogous to a locked door. The key is needed only when the door is locked, but once unlocked, it is no longer required to pass through. In a broader sense, these results suggest that defining the activities of a protein based solely upon the phenotypes of a null mutant can be misleading. A similar conclusion can be drawn from recent analysis of the Tup1 repressor (28, 29).

Crt1 is required to overcome its own repression and acts as a repressor-activator via a novel mechanism. DNA damage signals convert Crt1 from a repressor to an activator, perhaps via its phosphorylation (20). The mechanism of how transcription factors act as a signal-dependent repressor activator is best characterized for steroid hormone receptors, where unbound receptor recruits corepressors and ligand binding causes corepressor release and coactivator recruitment (for a review, see references 16 and 40). Similarly, in yeast, Ume6 acts as a repressor and activator of early meiotic genes. This mechanism involves the signal-dependent release of the Sin3-Rpd3 HDAC complex and the association of Ime1 with Ume6 to form an activator complex (3, 34, 43). Another example is Sko1, a regulator of stress-dependent genes. Hog1-dependent phosphorylation of Sko1 causes it to be converted to an activator in collaboration with the Ssn6-Tup1 repressor complex (29). Despite some similarities, we propose that the mechanism used by Crt1 is fundamentally different from these examples, because Crt1 and Tup1 disassociate from the promoter, whereas steroid receptors, Ume6, and the Sko1-Ssn6-Tup1 complex remain bound to their promoters in the activated state. Thus, Crt1 functions as an activator by a novel mechanism. Crt1 must act in a transient manner, and once it initiates activation and coactivator recruitment, it is no longer required to sustain gene expression. A model to consider is one where Crt1 recruits SWI/SNF and TFIID to the promoter after corepressor release, causing some chromatin remodeling of the TATA-containing nucleosome. In other words, Crt1 initiates the first steps in remodeling. After the TFIID complex is firmly associated with the promoter, Crt1 disassociates, SWI/SNF is retained by contacts with preinitiation complex components, and full remodeling and transcription occur. This would be consistent with our data showing that general transcription factors are necessary to recruit SWI/SNF and that inactivating TAF12 or the large subunit of RNA polymerase II (Rpb1) in a ∆crt1 background causes the loss of SWI/SNF recruitment (37). While it is clear that Crt1 is required to recruit TFIID and SWI/SNF, it remains to be seen if it does so directly.

Another possibility is that Crt1 acts as a founding transcription factor that is required for the recruitment of another activator, which in turn recruits coactivators. The delivery of the activator to its binding sites requires the N terminus of Crt1, which is disrupted in the derepression-defective mutants.

By constructing CRT1 mutants capable of recruiting and releasing Ssn6-Tup1, we demonstrate that Crt1 performs essential activation functions during the DNA damage response. The derepression-defective mutants recruit Ssn6-Tup1 and establish a repressive nucleosomal array over RNR3 in the absence of DNA damage, sense DNA damage signals, and release from the promoter but are blocked at the coactivator recruitment step. If Crt1 plays a role in activation, why do ∆crt1 mutants display constitutive transcription and coactivator recruitment (37)? We argue that disabling the repression mechanism genetically, by deleting CRT1, is not equivalent to the reversal of the repressed state caused by the physiological, signaled release of Crt1-Ssn6-Tup1 from the promoter. Since Crt1 (with Ssn6-Tup1) is required to establish repression and position nucleosomes, repression is never established in the ∆crt1 mutant. Thus, Crt1’s role in activation is masked. It is likely that the activator function of Crt1 is required to overcome the barriers to transcription established by Crt1 and the

FIG. 10. SWI/SNF and TFIID recruitment in the derepression-defective CRT1 mutants. ChIP experiments were carried out to examine SWI/SNF and TFIID recruitment to the RNR3 promoter in response to DNA damage. Bars labeled with “+” indicate signals from cells treated with 0.03% MMS for 2 h, and “−” indicates untreated cells. Polyclonal antisera against Snf2, TAF1, and TBP were used.

FIG. 11. SWI/SNF and TFIID recruitment in the derepression-defective CRT1 mutants. ChIP experiments were carried out to examine SWI/SNF and TFIID recruitment to the RNR3 promoter in response to DNA damage. Bars labeled with “+” indicate signals from cells treated with 0.03% MMS for 2 h, and “−” indicates untreated cells. Polyclonal antisera against Snf2, TAF1, and TBP were used.
placement of GR (13, 26). While our model is reminiscent of a “hit and run” model, it appears to be mechanistically different. Crt1 is released from the promoter in a Δsnf2 mutant and in the derepression-defective mutants in the absence of SWI/SNF recruitment (Fig. 7B and 10) (Z. Zhang and J. C. Reese, unpublished data); thus, SWI/SNF is not required for Crt1 release.

Functional homology between yeast Crt1 and human RFX1. Crt1 belongs to a family of conserved transcription factors containing a modified winged-helix DNA binding domain and a separate and independent dimerization domain within the C terminus (12, 14). Its mammalian homologues, the RFX factors, can function as context-dependent activators and repressors of transcription (12, 21, 22, 36). RFX1 and Crt1 display significant homology only in their DNA binding regions (12); however, even though homology is limited to the DNA binding domains, Crt1 and RFX factors carry out parallel functions. Our results suggest that Crt1 is a multifunctional protein containing at least two repression domains and has an undiscovered role in transcriptional activation. The repression domains reside in the N and C termini. Interestingly, each domain functions through a unique mechanism. The N-terminal domain, which is the dominant, binds to Ssn6-Tup1 in vitro and requires Ssn6, TUP1, and HDACs to repress transcription in the LexA reporter assay described here. Crt1 also possesses a weaker, but significant, repression activity within its C terminus. Our results do differ from a published report showing that the C terminus of Crt1 displayed no repression functions in mammalian cells when fused to the DNA binding domain of RFX1 or Gal4 (22). The apparent inconsistency between our results and those of Katan-Khaykovich et al. could be due to differences between the two reporter systems, since RFX proteins function in a context-dependent manner and/or some cofactors required for Crt1 function might be missing in mammalian cells. In this regard, it is important to point out that we verified that the C-terminal repression domain of Crt1 is required for the full repression of two natural target genes in vivo (Fig. 4A). Thus, we argue that the C terminus of Crt1 contains a bona fide repression domain that is important in its ability to act as a repressor. Interestingly, human RXF1 contains a repression function in its C terminus (22), suggesting that this function is conserved among eukaryotes. The C-terminal domain may be important in attenuating the expression of the RNR genes in the early stages of repression after DNA damage signals diminish, which will facilitate the assembly of a Crt1-Ssn6-Tup1 complex to firmly establish a full level of repression. Alternatively, the C terminus might play a greater role in the repression of other genes. Whereas a great deal of effort has been spent in analyzing how genes are activated from the repressed state, little is known about how repression is reestablished at active loci.

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REFERENCES


