The Rts1 Regulatory Subunit of PP2A Phosphatase Controls Expression of the HO Endonuclease via Localization of the Ace2 Transcription Factor*§

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Background: The yeast HO gene is tightly repressed and expressed only in mother cells.

Results: Mutation of the PP2A Rts1 phosphatase results in decreased HO expression due to altered localization of the Ace2 transcription factor.

Conclusion: Altered Ace2 localization results in inappropriate Ash1 repressor expression in mothers.

Significance: Changes in transcription factor phosphorylation can affect localization and cause inappropriate expression of a repressor.

The RTS1 gene encodes a subunit of the PP2A phosphatase that regulates cell cycle progression. Ace2 and Swi5 are cell cycle-regulated transcription factors, and we recently showed that phosphorylation of Ace2 and Swi5 is altered in an rts1 mutant. Here we examine expression of Ace2 and Swi5 target genes and find that an rts1 mutation markedly reduces expression of the HO gene. The decreased HO expression in an rts1 mutant is significantly restored by an additional ace2 mutation, a surprising result because HO is normally activated by Swi5 but not by Ace2. Ace2 normally accumulates only in daughter cells, and only activates transcription in daughters. However, in an rts1 mutant, Ace2 is present in both mother and daughter cells. One of the genes activated by Ace2 is ASH1, a protein that normally accumulates mostly in daughter cells; Ash1 is a transcriptional repressor, and it blocks HO expression in daughters. We show that in the rts1 mutant, Ace2 accumulation in mother cells results in Ash1 expression in mothers, and the Ash1 can now repress HO expression in mothers.

The yeast HO gene is under complex regulation, and has been an important model system for the study of transcriptional regulation (1). The HO gene encodes an endonuclease that initiates mating type interconversion by cleaving at the MAT locus. HO is cell cycle-regulated, with expression occurring in late G1, after START. HO gene activation requires multiple activators and coactivators recruited in sequence during the cell cycle, and these factors induce waves of nucleosome eviction along the promoter as the cell cycle progresses. Inappropriate expression of an endonuclease could be toxic to cells, and the chromatin structure at HO ensures tight transcriptional repression.

The Ash1 protein contributes to the repressive character of HO chromatin (2). The Ash1 DNA-binding protein recruits the Rpd3(L) histone deacetylase complex to HO to facilitate transcriptional repression. Ash1 is cell cycle-regulated, and the protein is present at the promoter only transiently during the cell cycle. ASH1 mRNA is expressed in late M phase, and the mRNA is transported to the bud tip in daughter cells (3). Consequently, most of the Ash1 protein is present in daughter cells, resulting in repression of HO expression in daughter cells. HO is normally expressed only in mother cells, but is expressed in both mother and daughter cells in an ash1 mutant (4, 5). Transcriptional activation of the ASH1 gene requires either of two transcription factors active in M and early G2 phases, Swi5 or Ace2 (5). Swi5 and Ace2 have identical DNA-binding domains and recognize the same DNA sequence, but they activate different genes (6, 7). Some target genes, such as EGT2 and ASH1, are known as Ace2-or Swi5 genes because they can be activated by either factor; expression is sharply reduced only in the ace2 swi5 double mutant (7). Other target genes are only activated by Ace2, or only by Swi5. One of these Swi5-only target genes is HO.

The Ace2 transcription factor is regulated in multiple ways. There is cell cycle regulation both for transcription of the gene and for nuclear accumulation of the protein (6, 8). In G2 phase, the Ace2 protein is sequestered in the cytoplasm due to phosphorylated residues within the nuclear localization sequence (NLS), and this masks the NLS (9). The Cdc14 phosphatase is released from sequestration at anaphase (10), and it can then dephosphorylate Ace2, allowing it to enter the nucleus (11).

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‡ This article contains supplemental Movies S1–S3.

§ The abbreviations used are: NLS, nuclear localization sequence; NES, nuclear export sequence.

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An rts1 Deletion Affects Ace2 Localization and HO Expression

TABLE 1
Strains used in this study

| Fig. | Strains                                                                 |
|------|------------------------------------------------------------------------|
| Fig.1| DY150: MATa ade2 can1 his3 leu2 trp1 can1 ura3                        |
|      | DY3925: MATa swi5::TRP1 ade2 can1 his3 leu2 trp1 can1 ura3            |
|      | DY1993: MATa ace2::HIS3 ade2 can1 his3 leu2 trp1 can1 ura3            |
|      | DY4653: MATa ace2::HIS3 swi5::TRP1 ade2 can1 his3 leu2 trp1 can1 ura3 |
|      | DY15088: MATa rts1::KanMX ade2 can1 his3 leu2 trp1 can1 ura3         |
|      | DY15724: MATa rts1::KanMX swi5::TRP1 ade2 can1 his3 leu2 trp1 can1 ura3|
|      | DY15728: MATa rts1::KanMX ace2::HIS3 ade2 can1 his3 leu2 trp1 can1 ura3|
|      | DY15732: MATa rts1::KanMX ace2::HIS3 swi5::TRP1 ade2 can1 his3 leu2 trp1 can1 ura3|

However, Ace2 also has a nuclear export sequence (NES) (11, 12), which prevents Ace2 from accumulating in mother cell nuclei. In daughter cells, the NES is inactivated by the Cbk1 kinase, and Ace2 persists in the nucleus for a short period of the cell cycle (13). Thus, Ace2 only accumulates in daughter cell nuclei, and Ace2 target genes are only expressed in daughters (11, 14).

The ASH1 gene is transcribed in two pulses in M and early G1 phases. ASH1 is first activated by Swi5, as Swi5 enters the nucleus slightly before Ace2 (8). ASH1 is transcribed in both mothers and daughters (14), but the ASH1 mRNA is transported to the daughter cell (3). Slightly later in the cell cycle, beginning at the time of cytokinesis, Ace2 accumulates in daughter cell nuclei (13) and activates ASH1 expression. Most of this expression occurs after the completion of cytokinesis, so this mRNA is exclusively in daughters.

Rts1 is a subunit of the PP2A phosphatase (15). Disruption of the RTS1 gene affects cell cycle progression and expression of G1 cyclin genes (16). We recently used proteome-wide mass spectrometry to identify proteins whose phosphorylation is regulated by PP2A-rrts1, and we found that phosphorylation of both Swi5 and Ace2 are affected in an rts1 mutant (17). In this study, we examine the effect of an rts1 mutation on expression of genes activated by Swi5 and Ace2. Although there is little effect on most Swi5 and Ace2 target genes, we find that expression of HO is markedly reduced. Our analysis indicates that the rts1 mutation, which alters Ace2 phosphorylation (17), also affects localization of Ace2. Ace2 now spends more time in the mother cell nucleus, where it can activate ASH1 transcription, and this Ash1 blocks HO expression.

EXPERIMENTAL PROCEDURES

All of the yeast strains are in the W303 background, and they are listed in Table 1. Standard methods were used for strain constructions. The ace2, ash1, rts1, and swi5 mutations are all gene deletions. RNA levels were measured by quantitative RT-PCR as described, using primers listed in Ref. 7. RNA values were normalized to an RPR1 internal control, using primers F2430 (CACCTATGGGCGGGTTATCAG) and F2431 (CTT-AGGCCGAACCTCCGTA). ChIPs were performed as described (7) using the 4A6 monoclonal antibody to the Myc epitope (Millipore) and antibody-coated magnetic beads (Pan Mouse IgG beads, Invitrogen). Each ChIP sample was first normalized to an input DNA sample and then to the ChIP signal for a CDC2 control region. ChIP primers for HO were F2093 (AATGCTGGAGCAAAATTTCAATCAG) and F2094 (GGAGCCCCTCAGACATTG). ChIP primers for CDC2 were F2093 (GAAGAAGAG-GTTGGCCTTATTAC) and F2094 (CCTGTAACAGCAGCCATTTCT).

Single cell measurements of HO-GFP were performed as described (18). Briefly, we grew yeast under an agar pad on a heated microscope and used an automated program to acquire phase and fluorescent images every 4 min for 8 h. Multiple movies of independent colonies can be recorded in a single experiment. One movie is included as supplemental Movie S1. We then used a MATLAB program to quantitate the fluorescence intensity of each cell, which was plotted as a function of time. In addition to HO-GFP, all the strains also contain Myo1-mCherry as a cell cycle marker. The “Peak expression level per cell cycle” was calculated as the difference between the peak and the trough of GFP intensity within each cell cycle.

The Ace2-GFP fusion uses a yeast enhanced GFP and was integrated at the ACE2 locus, expressed under the control of the native ACE2 promoter. For Ace2-GFP measurements, cells were grown in synthetic defined media containing 20 µg/ml adenine overnight at room temperature, and Ace2-GFP localization was visualized by live-cell imaging using a Leica TCS SP5 inverted confocal microscope (Leica Microsystems, PlanApo 63×). Time-lapse experiments were imaged in 35-mm glass-bottom culture dishes (P35-1.5-10-C; MatTek) coated with 10 mg ml⁻¹ concanavalin A (Sigma-Aldrich) and
An rts1 Mutation Reduces HO Expression—A mutation affecting the Rts1 subunit of the PP2A phosphatase results in increased phosphorylation of both the Ace2 and the Swi5 transcription factors (17). This change in phosphorylation could affect the activity of these transcription factors, and we therefore examined expression of target genes known to be activated by Ace2 and Swi5 (7). Some target genes, such as EGT2 and ASH1, are known as Ace2-or-Swi5 genes because they can be activated by either factor; an rts1 mutation has only modest

An rts1 mutant does not affect expression of most Ace2 and Swi5 target genes. RNA was prepared from cells in mid-log phase, and specific mRNAs were measured by quantitative RT-PCR. Error bars indicate the standard deviation of two biological replicates. a, target genes activated by Ace2 or Swi5. b, Ace2-only target genes. c, Swi5-only target genes. d, HO expression in WT, rts1, ash1, and rts1 ash1 strains.

FIGURE 1.

An rts1 Deletion Affects Ace2 Localization and HO Expression

immersed in 3 ml of synthetic defined medium lacking tryptophan. Images were acquired at 3-min intervals in eight z-series with a step size of 0.5 μm. Images were analyzed with ImageJ (National Institutes of Health) and Adobe Photoshop.

The Ash1-GFP fusion uses an enhanced GFP and was integrated at the ASH1 locus, expressed under the control of the native ASH1 promoter. For Ash1-GFP measurements, cells were grown in YP medium with 2% galactose and 2% raffinose until an optical density of 0.5. Cells were then transferred to YP medium with 2% raffinose to arrest cells at G2/M. Then 2% galactose was added to release cells from the arrest, and images were acquired at 40 min following release using an Olympus BX51 fluorescence/differential interference contrast microscope and a MagnaFire SP S99810 camera. Images were analyzed with Adobe Photoshop.

RESULTS

An rts1 Deletion Affects Ace2 Localization and HO Expression
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FIGURE 2. An ash1 mutation suppresses the rts1 defect in HO expression in both mother and daughter cells. Upper, histograms show the amplitude of GFP expression in single mother and daughter cells for rts1 and ash1 rts1 strains, as described (18). For each panel, two independent time-lapse measurements were carried out, each containing two micro-colonies of the same strain. One movie of the ASH1 rts1 HO-GFP strain is in supplemental Movie S1. Lower, the table lists the number of cells counted, the percentage in which HO-GFP was active, and the relative level. Expression levels were normalized so that the average expression level in wild type mother cells is 1. The data for ASH1 RTS1 and ash1 RTS1 strains are from Ref. 18. NA, not available.

|                | Counted | % on | Level     | Counted | % on | Level     |
|----------------|---------|------|-----------|---------|------|-----------|
| mothers        |         |      |           |         |      |           |
| ASH1 RTS1      | 154     | 98% ± 1 | 1.00 ± 0.02 | 131     | 2% ± 1 | NA        |
| ash1 RTS1      | 127     | 98% ± 1 | 1.00 ± 0.02 | 62      | 94% ± 3 | 1.11 ± 0.06 |
| ASH1 rts1      | 97      | 33% ± 5 | 0.50 ± 0.04 | 98      | 5% ± 2  | 0.42 ± 0.07 |
| ash1 rts1      | 39      | 100% ± 0 | 0.70 ± 0.04 | 55      | 100% ± 0 | 0.93 ± 0.04 |
| daughters      |         |      |           |         |      |           |

effects on expression of Ace2-or-Swi5 genes (Fig. 1a). Similarly, in the rts1 mutant, few changes are seen in expression of CTS1 or DSE2, Ace2-only genes (Fig. 1b). For two Swi5-only genes, PIR1 and PST1, we see increased expression in the ACE2 swi5 rts1 strain as compared with the ACE2 swi5 RTS1 control, and also in the ace2 swi5 rts1 strain as compared with the ace2 swi5 RTS1 strain (Fig. 1c). This is particularly striking for PST1 where expression in the ace2 swi5 rts1 strain is at 50% of wild type, as compared with 7% for ace2 swi5 RTS1. It is surprising that these genes are expressed in the absence of both the Ace2 and the Swi5 activators, and we interpret the results as meaning that some other activator is now more capable at these promoters in the absence of the PP2A Rts1 phosphatase.

The results with another Swi5-only target gene, HO, are quite different. HO expression is markedly reduced to 18–27% of wild type in the rts1 mutant (Fig. 1, c and d). This decreased expression is unique to HO as the two other Swi5-only genes, PIR1 and PST1, show expression of 89 and 128%, respectively, in the rts1 mutant. HO is expressed in an ace2 SWI5 rts1 mutant (41%), but at a lower level than in the ace2 SWI5 RTS1 strain (87%). Importantly, Swi5 is still required for HO activation in the rts1 mutant. What is special about HO that causes it to be expressed at lower levels in an rts1 mutant? Additionally, why is the repression partially relieved in an ace2 rts1 double mutant?

The HO Expression Defect in an rts1 Mutant Is Suppressed by an ash1 Mutation—One unique feature of HO regulation is that it is asymmetrically expressed, with expression in daughter cells strongly repressed by Ash1 (4, 5) (see also Fig. 2). We therefore examined HO expression in the rts1 ash1 double mutant and found that the ash1 mutation suppresses the defect in HO expression caused by rts1 (Fig. 1d). This result raised the question of whether the additional HO expression in rts1 ash1 cells is solely from expression in daughter cells, or whether there is also an increase in HO expression in mother cells. To address this question, we used an HO-GFP reporter, analyzing expression using single cell time-lapse fluorescence microscopy, as described (18). With this method the fluorescence intensity emanating from individual cells is quantitated, and this provides a measure of promoter activity in single mother and daughter cells. Single cell analysis shows that the rts1 mutation reduces the fraction of mother cells that express HO, from 98% of wild type mother cells to 33% of rts1 mothers (Fig. 2). Addi-
An rts1 Deletion Affects Ace2 Localization and HO Expression

The observation that the repression of Because Ace2 activates suggests that Ace2 contributes in some way to the repression.

**Mutant is partly relieved by an mutant, allowing Ash1 in mother cells to block change in Ace2 localization, thereby reducing expression. Ace2 normally shows significant accumulation only in daughter cells, with a very transient appearance in mothers (11, 14). It is possible that the increased phosphorylation of Ace2 by the Cbk1 kinase causes increased nuclear retention of Ace2, and thus increased Ace2 activity, in both mother and daughter nuclei. The prolonged nuclear localization of Ace2 in rts1 mother cells could also be caused by the cell cycle delay seen in rts1 mutants because Ace2 represses transcription of G1 cyclins (16, 17).

**Increased Ash1 Localization in rts1 Mothers Requires Ace2**—We next evaluated Ash1 localization using Ash1-GFP fusions. The Ash1-GFP signal was weaker in the three mutant strains as compared with wild type, and in many cells, the signal was too weak to observe. To enrich for cells in which the Ash1 signal was visible, we made pGAL::CDC20 versions of these strains, allowing for G$_S$/M arrest and synchronous release by withdrawal and reintroduction of galactose, respectively. Cells were arrested and released, and at 40 min after release, many photographs were taken at random positions throughout the slide. The photographs were examined subsequently, and each mother-daughter pair of cells was scored for Ash1 localization as daughter only, both mother and daughter, or mother only. Selected images are shown in Fig. 4a, and quantitation is shown in Fig. 4b. For the mutant strains (particularly the ace2 rts1 strain), fewer cells were able to be scored due to an extremely weak signal.

In wild type cells, Ash1-GFP fluorescence was seen nearly exclusively in daughter cells; among mother-daughter pairs, 93% showed daughter-only fluorescence, 7% showed mother and daughter fluorescence, and none showed a signal only in mothers. (Fig. 4b). These results are consistent with previous quantitation (4). There was decreased fluorescence signal in the ace2 mutant as compared with wild type, and quantitation of
An rts1 Deletion Affects Ace2 Localization and HO Expression

**FIGURE 4. Ash1 accumulation in mother cells in an rts1 mutant.** Cells with the Ash1-GFP reporter were arrested at G$_2$/M, and samples were examined by fluorescence microscopy at 40 min following release from the arrest. a, representative images. b, individual mother-daughter cell pairs were examined and classified as having daughter-only expression (D only), mother and daughter expression (M + D), or mother-only expression (M only). Ash1-GFP signals were extremely weak in the ace2 rts1 strain, and most cells had no visible signal. c, ChIP experiments were performed with wild type and rts1 cells containing Ash1-Myc. Control ChIP experiments were performed with untagged wild type and mutant strains. Ash1 binding to HO is significantly increased in an rts1 mutant. Error bars in ChIP assays reflect the standard deviation of ChIP assays from two biological replicates.

localization showed a slight increase in mother cell fluorescence. The rts1 mutant was notable because Ash1-GFP showed equivalent localization in mothers and daughters. The Ash1-GFP fluorescence signal was weaker in rts1 cells than in wild type, and the Ash1-GFP signal was barely detectable in the ace2 rts1 double mutants. Ash1-GFP could not be detected at all for most of the ace2 rts1 cells. Although the localization pattern could be discerned for some of the ace2 rts1 cells, the quantitation of these ace2 rts1 cells should be interpreted cautiously.

In summary, in rts1 mutant cells, Ash1 is present in both mother and daughter cells, as opposed to the pattern of exclusively daughter cell localization seen in wild type. Notably, the fluorescence data suggest that Ace2 is required to see strong Ash1-GFP signals in mother cells in the rts1 mutant. This agrees with the RNA expression data, in which the HO repression in an rts1 mutant is at least partly dependent upon Ace2.

**Increased Ash1 Binding to HO in an rts1 Mutant**—Increased Ash1 accumulation in mother cells should result in more Ash1 bound to promoters in mother cells. To address this question, we performed ChIP experiments to measure Ash1 binding to HO (Fig. 4c). In the wild type strain, Ash1 binding is at 1.6, as compared with the wild type strain. Importantly, Ash1 binding is much higher, at 2.8, in the rts1 mutant. Thus, the rts1 mutation results in increased Ash1 binding to HO.

**DISCUSSION**

The Rts1 subunit of PP2A phosphatase has important roles in regulating cell cycle progression (16), and we have shown that an rts1 mutation results in increased phosphorylation of two cell cycle-regulated transcription factors, Ace2 and Swi5 (17). Here we show that expression of only one of the target genes activated by Ace2 or Swi5, HO, is reduced in an rts1 mutant. Normally, HO is expressed only in mother cells because the Ash1 repressor accumulates preferentially in daughter cells. In contrast, Ash1 accumulation is equivalent in mother and daughter cells in an rts1 mutant. The presence of Ash1 in mothers is sufficient to reduce HO expression.

HO is activated by the Swi5 transcription factor, but not by Ace2 (6). Phosphorylation of Swi5 is affected by an rts1 mutation (17), and one possibility is that this altered phosphorylation affects the function of Swi5 as a transcriptional activator, leading to reduced HO expression. However the rts1 mutation does not affect expression of PIR1 and PST1, suggesting that Swi5 continues to be a robust activator. The major distinction is that HO is strongly repressed by Ash1, whereas PIR1 and PST1 are not. Ace2 and Swi5 contain the same DNA-binding domain and recognize the same DNA sequence, but they activate different target genes (7). Although Ace2 can bind to the HO promoter in vitro (6), chromatin immunoprecipitation experiments show that Ace2 does not bind to HO in vivo (7). Nonetheless, Ace2 is essential for the loss of HO expression in the rts1 mutant (Fig. 1c). Ace2 normally accumulates only in daughter cell nuclei (14), but in an rts1 strain, Ace2 is present in mother cell nuclei. This Ace2 present in mother cells activates transcription of ASH1; as cytokinesis has occurred, the ASH1 mRNA cannot be exported to daughter cells, and the Ash1 protein produced represses HO in mother cells.

Ace2 localization is regulated by both nuclear import and export sequences, but the details of how it accumulates exclusively in daughter cells remains an important question (19). The Cbk1 kinase phosphorylates Ace2 at multiple positions, and it is believed that one of these phosphorylation events inactivates the NES. The dominant ACE2 mutations that suppress a cbk1 mutation are present within the NES, and these mutant Ace2 proteins show some accumulation in mother cells (11, 20). Additionally, Cbk1 accumulates preferentially in wild type daughter cells (21). These results suggest that the presence of Cbk1 specifically in daughters is important for the daughter-only pattern of Ace2 accumulation.

How does an rts1 mutation allow Ace2 accumulation in mother cells? One possibility is that in the absence of the PP2A$_{\text{Rts1}}$ phosphatase, the hyperphosphorylation of Ace2 by the Cbk1 kinase changes its localization pattern. Alternatively, Ace2 localization could be controlled by other proteins whose
activity is controlled by dephosphorylation by Rts1. We note that Lre1 has recently shown to be an inhibitor of the Cbk1 kinase that is required for nuclear entry by Ace2 (22). Lre1 is phosphorylated, and it is a substrate of the Cdk1 kinase and the Cdc14 phosphatase (22). Lre1 phosphorylation is also regulated by the Rts1 phosphatase, either directly or indirectly, as an rts1 mutation affects Lre1 phosphorylation (17). Thus, one possible explanation is that an rts1 mutation results in increased Lre1 phosphorylation, and hyperphosphorylated Lre1 no longer inhibits Cbk1. Cbk1 could be hyperactive in the rts1 mutant, perhaps facilitating accumulation of Ace2 in mother cell nuclei. Further studies will be needed to test this hypothesis.

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