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Regulation of conditional gene expression by coupled transcription repression and RNA degradation

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ABSTRACT

Gene expression is determined by a combination of transcriptional and post-transcriptional regulatory events that were thought to occur independently. This report demonstrates that the genes associated with the Snf3p–Rgt2p glucose-sensing pathway are regulated by interconnected transcription repression and RNA degradation. Deletion of the dsRNA-specific ribonuclease III Rnt1p increased the expression of Snf3p–Rgt2p-associated transcription factors *in vivo* and the recombinant enzyme degraded their messenger RNA *in vitro*. Surprisingly, Rnt1ps effect on gene expression *in vivo* was both RNA and promoter dependent, thus linking RNA degradation to transcription. Strikingly, deletion of *RNT1*-induced promoter-specific transcription of the glucose sensing genes even in the absence of RNA cleavage signals. Together, the results presented here support a model in which co-transcriptional RNA degradation increases the efficiency of gene repression, thereby allowing an effective cellular response to the continuous changes in nutrient concentrations.

INTRODUCTION

In higher eukaryotes, conditional mRNA degradation is believed to be generally initiated by the dsRNA-specific ribonuclease Dicer (1). Dicer cleavage generates either short interfering RNA (siRNA), or microRNA (miRNA), which trigger an RNA interference (RNAi) pathway that leads to complete degradation of the targeted mRNA (2–6). Sequence complementarity between the Dicer products and the targeted mRNA determines the site of cleavage, and confers high specificity to this RNA degradation strategy. RNAi-dependent mRNA degradation has been identified in most eukaryotes including the fission yeast *Schizosaccharomyces pombe* (7–9). *Saccharomyces cerevisiae* is among the few eukaryotes that do not express the known components of the RNAi machinery. Instead, budding yeast express only a single isoform of RNase III (Rnt1p) that is required for the maturation of both pre-rRNA and snoRNA (10,11). Recently, this enzyme was also shown to initiate the degradation of several mRNAs, including that of Mig2p, a transcription factor linked to glucose sensing and metabolism (12). This observation prompted the suggestion that Rnt1p may act as glucose-dependent gene expression regulator.

Glucose-dependent gene expression involves one of the most studied networks of transcriptionally regulated genes. In *S. cerevisiae*, glucose induces broad changes in gene expression (13–20) that are primarily triggered by two sensory pathways (Figure 1A). The first is the Snf3p–Rgt2p pathway, which directly detects glucose levels in the growth medium (17) via two glucose sensors embedded in the cell membrane called Snf3p (21,22) and Rgt2p (23). These sensors generate intracellular signals that permit the expression of glucose-transporter genes (Hxts 1–4) (19,24,25). The main target of this signalling pathway is Mth1p (26), a protein that is required for the activation of Rgt1p (27), a transcription factor that binds to the promoter of the Hxt genes and suppress their expression in the absence of glucose. In the presence of glucose, Snf3p and Rgt2p trigger the degradation of Mth1p and thus inactivate Rgt1p, thereby permitting the transcription of the Hxt genes (28,29).

The second signalling pathway senses glucose metabolism (19,30) initiated by the phosphorylation and consequent activation of the protein kinase Snf1p (31–34). In the presence of low glucose concentrations, Snf1p is dephosphorylated and becomes inactive. This allows the transcription repressor Mig1p to accumulate in the nucleus and repress the transcription of glucose metabolism genes such as the sucrose hydrolyzing enzyme Suc2p (35,36). On the other hand, at high glucose concentrations, Snf1p is active and phosphorylates Mig1p. This forces it to exit to the nucleus and to enter the cytoplasm, thus relieving the repression of Suc2p and

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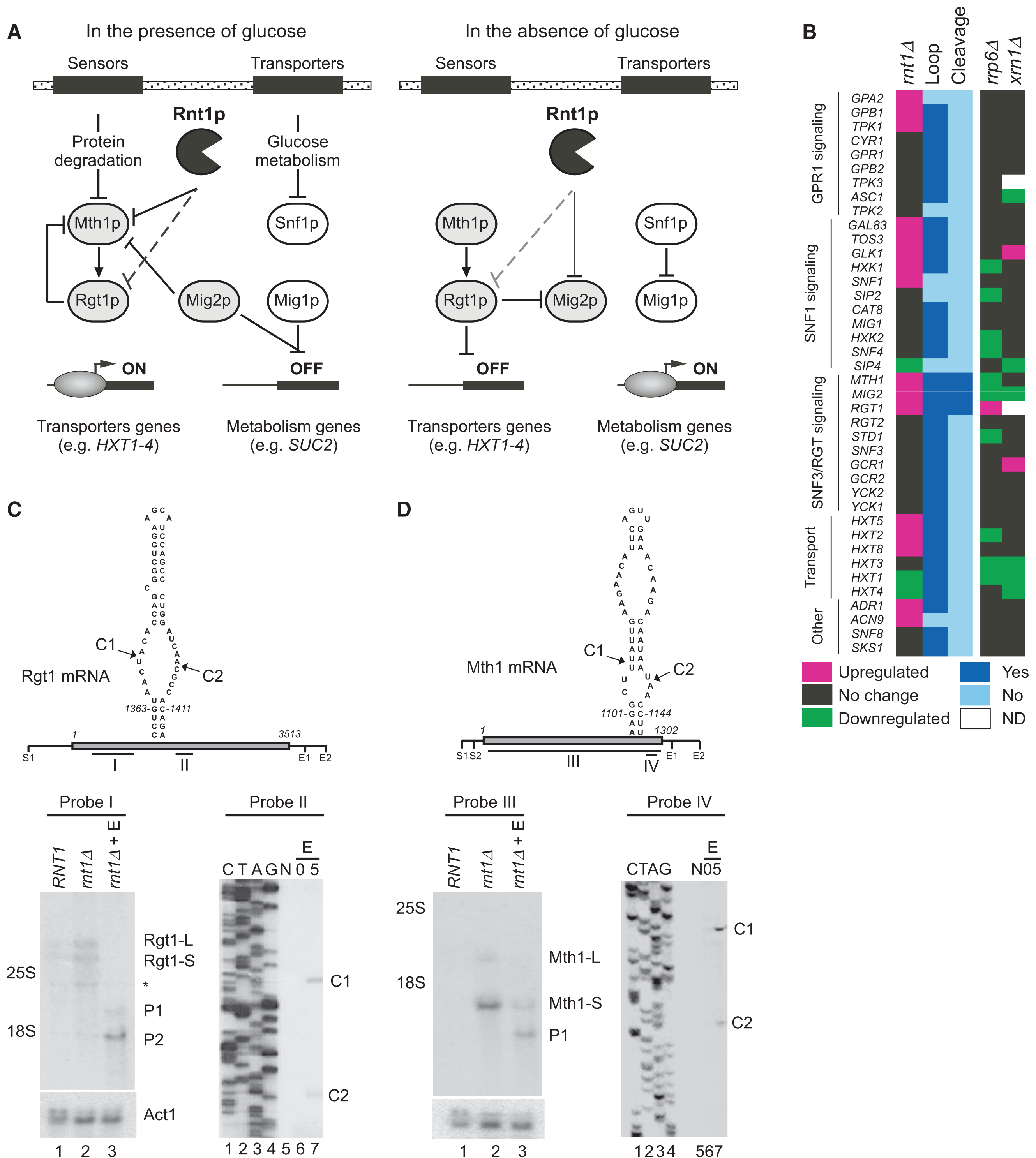

Figure 1. Yeast RNase III selectively regulates the transcription factors associated with the Snf3p–Rgt2p glucose-signalling pathway. (**A**) Schematic representation of the mechanism of glucose-dependent regulation of gene expression involving factors associated with Snf3p–Rgt2p signalling. The transcriptional state of the genes is indicated as either being activated (ON) or repressed (OFF). Arrows and bars indicate activation and inhibition, respectively. The dashed line indicates the constitutive repression of Rgt1 mRNA by Rnt1p. (**B**) Heat map representing the expression status of the genes involved in glucose signalling and transport in different mutant yeast strains. The increases and decreases in mRNA levels relative to that of the wild-type strain grown under standard laboratory conditions are indicated in magenta and green, respectively. The first column (*rnt1Δ*) indicates mRNAs that change by 1.4-fold upon the deletion of *RNT1*. The presence or the absence of Rnt1p cleavage sites (Loop), as predicted *in silico*, are indicated. The cleavage column indicates the capacity of recombinant Rnt1p to cleave the different mRNAs *in vitro*. Similarly, changes in RNA expression by ≥1.4-fold upon the deletion of *RRP6* or *XRN1* are also indicated. The genes were organized vertically according to their contributions to glucose signalling as indicated on the left. Cells where left blank when no data were available (ND). (**C** and **D**) *In vitro* cleavage of total RNA extracted from *rnt1Δ* cells. RNA was extracted from either wild-type cells (*RNT1*), or cells lacking *RNT1* (*rnt1Δ*), and subjected to northern blot analysis either directly or after incubation with recombinant Rnt1p *in vitro* (*rnt1Δ* + E). Probes specific either to the Rgt1 (**C**) or the Mth1 (**D**)

(continued)

the other glucose metabolism genes (37). The Snf3p–Rgt2p and the Snf1p-dependent pathways are linked by Mig2p (31,37–39). Mig2p, is a zinc finger repressor that is closely related to Mig1p and that regulates a distinct subset of Mig1p's targets in response to higher glucose concentrations (40). Interestingly, Mig2p is not regulated by the Snf1p pathway, but rather by the Snf3p–Rgt2p pathway, and also plays a role in the repression of glucose transporters (40). At low glucose concentrations Rgt1p represses the transcription of Mig2p and thus triggers the expression of glucose metabolism genes (39).

In both of the glucose sensing pathways described above, gene expression is mainly regulated via transcriptional activity, while RNA decay is considered a passive event that does not directly contribute to the glucose response. Nevertheless, changes in glucose concentration may selectively alter the decay rate of several mRNAs. For example, the degradation of gluconeogenesis mRNAs, such as those coding for Suc2p and Fbp1p, was shown to be accelerated in a glucose-dependent manner (41,42). This change in RNA stability appears to be specific to a particular subset of genes since other transcripts, such as the Act1 and Pgk1 mRNAs are not affected (42). The commonly accepted model for glucose-dependent RNA decay suggests that depletion of glucose induces a specific translational inhibition that sentences the mRNA for decapping and exonucleolytic degradation (41,43). In this model, RNA decay does not play a direct role in the glucose response, but rather functions as a surveillance mechanism that ensures prompt removal of the no longer needed untranslated RNA. It remains unclear whether or not selective RNA degradation may directly contribute to the signalling cascades of the glucose response.

In order to evaluate the contribution of RNA degradation to glucose sensing the impact of known yeast ribonucleases including Rnt1p (10), the nuclear exoribonuclease Rrp6p (44) and the cytoplasmic exoribonuclease Xrn1p (45) on the expression of glucose-dependent genes was examined. The results indicate that the repression of glucose sensing genes is regulated by targeted RNA degradation, and is largely unaffected by the generic machinery of RNA decay. The Rgt1p glucose sensing regulatory loop that includes both the activator Mth1p and the transcription repressor Mig2p was selectively regulated by Rnt1p. Surprisingly, Rnt1p altered not only the RNA stability, but also influenced the transcription of the Rgt1p-associated genes. Indeed, Rnt1p inhibited gene expression in a promoter-dependent manner, and influenced the promoter activity independent of the RNA sequence. Together, the results suggest a model in which the promoters of Rgt1p-associated genes recruit Rnt1p in order to control glucose-dependent steady-state expression and to potentiate a rapid response to any changes in the glucose concentration.

MATERIALS AND METHODS

Strains and plasmids

Yeast strains were grown and manipulated using standard procedures (46,47). All strains used in this study are listed in Supplementary Table S1. Unless specified otherwise, all strains were grown at 26°C in YEP media supplemented with either 2% dextrose or 4% galactose, as specified in each experiment. LacZ-transformed strains were grown in YC(-)ura media. The inactivation of *rat1-1* allele was accomplished by growing the cells at 26°C, then shifting them to the restrictive temperature (37°C) for 4 h before harvesting as previously described (48). P_{ACT1}-*MIG2*, P_{ACT1}-*MTH1* and P_{ACT1}-*RGT1* strains were created by replacing the respective promoter sequences of *MIG2*, *MTH1* and *RGT1* with the *ACT1* promoter using standard gene replacement procedures (49). First, a 500 nt PCR fragment corresponding to the *ACT1* promoter was amplified using yeast genomic DNA as a template. The PCR product was then inserted downstream of the KanMX gene using the SacI and SpeI restrictions sites in the pCM224 vector (49). The resulting KanMX-*ACT1* promoter cassette was further amplified by PCR using probes containing sequence homology with the region located upstream of the target gene (i.e. 400–500 nt upstream of the translation start codon of the *MIG2*, *MTH1* or *RGT1*). Finally, the resulting PCR fragments were transformed into both wild-type (*RNT1*) and *rnt1Δ* strains, and the transformants selected for growth on G418-containing media. Adequate integration of the exogenous promoter was verified by PCR reaction followed by restriction enzyme profile analysis. The p*MIG2*pr-LacZ, p*MTH1*pr-LacZ and p*RGT1*pr-LacZ plasmids were generated as described before (37) by inserting the PCR-amplified promoter regions of *MIG2* (500 bp), *MTH1* (495 bp) and *RGT1* (711 bp) between the BamHI and EcoRI restriction sites of yEP357R vector (50). The resulting plasmids were then transformed into W303 and *rnt1Δ* strains. All oligonucleotides used in this study are listed in Supplementary Table S2.

In vitro RNA cleavage

Cleavage of total RNA extracted from both wild-type and *rnt1Δ* cells was conducted essentially as described previously (51). Briefly, 30 µg total RNA was incubated with 4 pmol purified Rnt1p (48,52) for 20 min at 30°C in 100 µl of reaction buffer [30 mM Tris–HCl (pH 7.5),

Figure 1. Continued
mRNAs where used to detect both the full transcripts and 5′-end cleavage products. Act1 mRNA was used as loading control, and the position of the 25S and 18S ribosomal RNAs are indicated on the left as size markers. P1 and P2 indicate the positions of the cleavage products. The positions of the long (L) and short (S) forms of each gene are indicated on the right. The asterisk represents a non-specific band observed in strands carrying a knockout in *RGT1* (data not shown). Primer extension using probes located downstream of the predicted cleavage sites are shown on the right, and the positions of the two cleavage sites (C1 and C2) are indicated on the right. The predicted Rnt1p cleavage signal, the detected cleavage sites and the position of the different probes used are illustrated schematically on top of the gels.

5 mM spermidine, 0.1 mM DTT, 0.1 mM EDTA (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl]. The reactions were stopped by phenol:chloroform extraction, and the RNA collected by salted ethanol precipitation for analysis.

RNA analysis

Northern blots were performed as described previously (48) using 15 µg of total RNA and a 1% denaturing agarose gel. The RNA was visualized by autoradiography using randomly labelled probes corresponding to specific genes (a labelled oligonucleotide probe was used in the case of *LacZ*). The RNA was quantified using a Storm 825 scanner (GE Healthcare) and the ImageQuant software (Molecular Dynamics). The primer extension reactions used to map the cleavage sites of Rnt1p *in vitro* were performed using 5 µg of cleaved total RNA and 1 ng of ^{32}P end-labelled oligonucleotide as described (53). The primers used to generate the probes are listed in Supplementary Table S2.

Chromatin immunoprecipitation

Chromatin extraction and immunoprecipitation were performed as described previously (48). Monoclonal anti-Rpb1 8WG16 (Covance, Berkeley, CA, USA) was used to pull down the RNA polymerase II complex. Quantitative PCR analysis was performed according to the method previously described (54). The radioactivity of each PCR fragment was quantified using a Storm 825 scanner (GE Healthcare). All signals were normalized using an internal control derived from an unexpressed region of chrV and RNAPII occupancy was calculated by comparing the signals from the immunoprecipitated samples relative to that of the input samples for each primer pair.

RESULTS

Rnt1p selectively degrades the mRNAs associated with the Snf3p–Rgt2p glucose sensing pathway

It has previously been shown that Rnt1p degrades the mRNA encoding the glucose-dependent transcription factor Mig2p (12). This suggests that post-transcriptional gene regulation may play an important role in the glucose response pathway. In order to evaluate this hypothesis were identified all of the genes associated with glucose signalling (29), glucose response and transport (55) and their expression patterns determined in the absence of different yeast ribonucleases. Previously generated genome-wide expression profiles (12) of both wild-type cells and cells carrying deletions of the three main non-essential ribonucleases in yeast, namely *RNT1*, *RRP6* and *XRN1*, were used to identify potential targets for RNA degradation. As shown in Figure 1B, the deletion of *RNT1* gene increased the expression of ~20% of the genes associated with glucose response, while deletion of either the nuclear 3′–5′ exoribonuclease *RRP6* or the 5′–3′ cytoplasmic exoribonuclease *XRN1* increased the expression of only one or two of these genes. This suggests that Rnt1p is preferentially implicated in regulating the expression of the glucose-associated genes, and that the expression of these genes is not highly dependent on exonucleolytic RNA degradation. In general, the genes up-regulated by *RNT1* deletion where comparably distributed across the different classes of glucose-dependent genes. In order to identify direct targets of Rnt1p all genes associated with the glucose response were examined for the presence of NGNN stem loop structures (11,48), which constitute Rnt1p cleavage signals. As shown in Figure 1B, mRNAs of all glucose-associated genes, with the exception of six genes, exhibited local structures (loop) that may be recognized by Rnt1p. However, *in vitro* cleavage assay using recombinant Rnt1p indicated that only three RNAs are direct substrates of Rnt1p. This result was not unexpected as the majority of local stem loops do not fold in this context and thus cannot support cleavage by the recombinant enzyme (48,56). Two of the RNA substrates that were cleaved by Rnt1p encode Mth1p (26) and Rgt1p (27), transcription factors associated with the Snf3p–Rgt2p glucose induction pathway (Figure 1B). The third encodes Mig2p (57), which has previously been shown to be regulated by both Rgt1p (39) and Rnt1p (12). These data indicate that Rnt1p does not generically influence the RNA stability of the glucose-dependent genes, but instead selectively targets a tightly linked glucose sensing regulatory loop.

In order to confirm the impact of Rnt1p on the expression and cleavage of the two newly identified substrates, the impact of Rnt1p on both Mth1 and Rgt1 mRNA *in vivo* and *in vitro* was examined using northern blot analysis. As shown in Figure 1C, Rgt1 expression was detected in wild-type (*RNT1*) cells in two forms corresponding to long (Rgt1-L) and short RNA (Rgt1-S) transcripts. Based on previous tiling array expression profiles (58), Rgt1-L is likely a 3′-extended polycistronic transcript arising from transcription termination after the downstream gene (*AIM26*). As expected, both forms increased in *rnt1Δ* cells. Reverse transcription using a primer complementary to the sequence downstream of the predicted loop confirmed the position of the cleavage site and ensured the specificity of the cleavage reaction. In the case of *MTH1* (Figure 1D), which is not expressed in cells grown on standard media containing glucose, the mRNA was only detected in *rnt1Δ* cells, clearly indicating that Rnt1p is required for the glucose-dependent shut down of *MTH1*. Similar to Rgt1, two transcripts (Mth1-S and Mth1-L) were detected, and further investigation confirmed that the longer form is a polycistronic transcript consisting of *MTH1* and the downstream *PMP3* gene (Figure 5 and data not shown). Once again, northern blot and primer extension analysis of RNA incubated in the presence of recombinant Rnt1p confirmed cleavage at the predicted site. Clearly Rnt1p directly regulates the expression of both *RGT1* and *MTH1 genes,* at least in part, by endonucleolytic cleavage of their messenger RNAs.

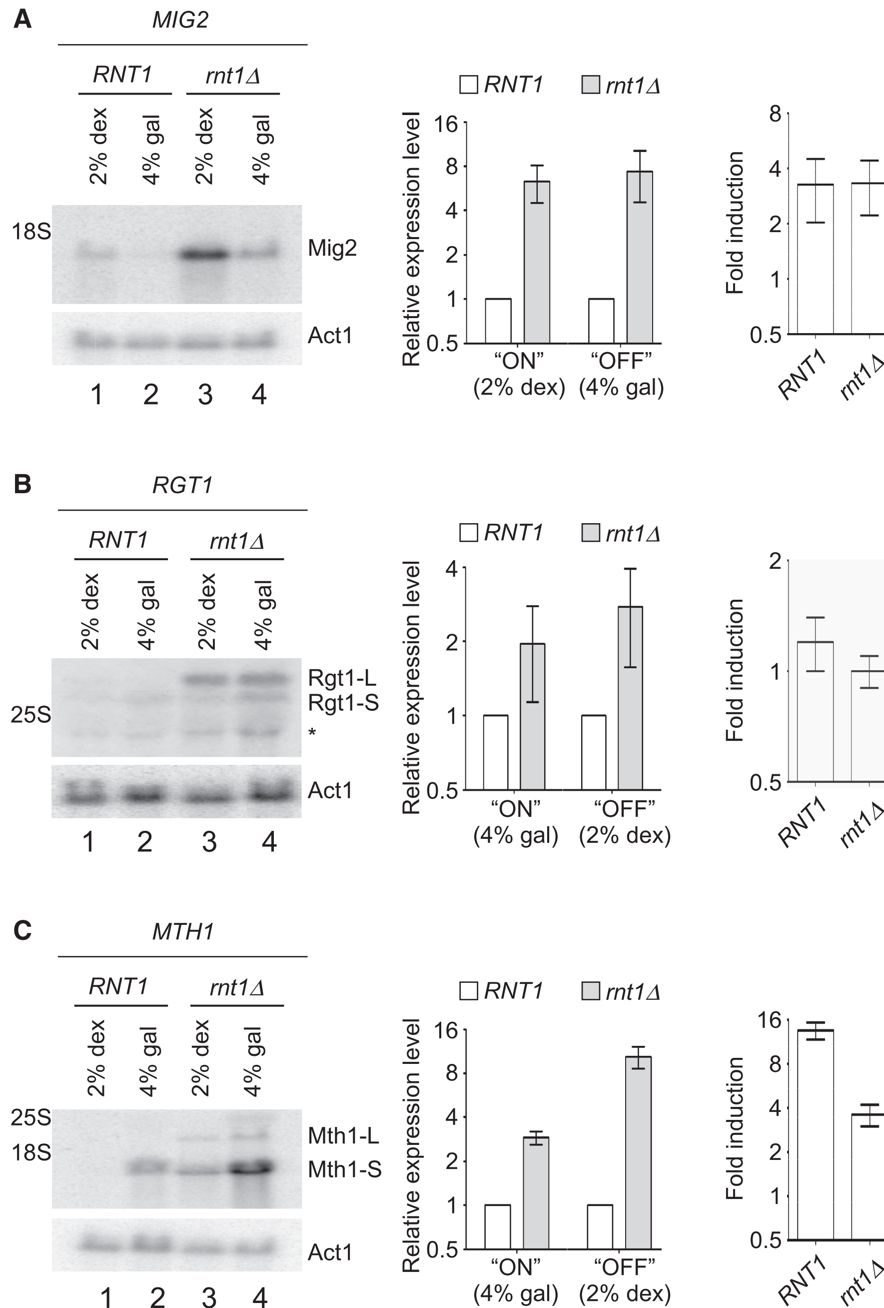

Figure 2. Rnt1p optimizes the expression of the Rgt1-associated transcription factors. Total RNA was extracted from either *RNT1* or *rnt1Δ* cells grown in the presence of either glucose or galactose and the expression levels of the Mig2 (**A**), Rgt1 (**B**) or Mth1 (**C**) mRNAs were detected by northern blot (left panels). Act1 mRNA was used as a loading control. The bands were quantified, and the relative RNA amount of three biological replicas was calculated. Bar graphs (middle panels) illustrate the impact of *RNT1* deletion on the expression of each gene in the presence of different sugars relative to that of wild-type cells. Bar graphs on the right illustrate the sugar-dependent fold induction (i.e. ratio of mRNA amount detected in 'ON' condition over 'OFF' condition) of each mRNA in both *RNT1* and *rnt1Δ* cells.

Rnt1p promotes glucose-dependent repression of Rgt1p-associated factors

In order to determine the impact of Rnt1p on glucose-dependent gene expression, *RNT1* and *rnt1Δ* cells where grown in media containing either glucose or galactose and the expression levels of Mig2, Rgt1 and Mth1 mRNAs were analysed by northern blot. As reported earlier (12), the expression of *MIG2* in *RNT1* cells was detected when the cells were grown in the presence of glucose (ON condition), and the expression was inhibited when the cells were grown in the presence of galactose (OFF condition) (Figure 2A). The deletion of *RNT1* (*rnt1Δ*) increased the expression of *MIG2* in both the ON and OFF conditions to a similar extent (Figure 2A, right panel). The glucose-mediated induction was found to be about three fold in both *RNT1* and *rnt1Δ* cells (Figure 2A, left panel),

suggesting that Rnt1p is equally required in both conditions. In the case of *RGT1* (Figure 2B), expression was detected in the presence of both glucose (OFF condition) and galactose (ON condition), as expected, as *RGT1* is known to be regulated at the protein level (59). The deletion of *RNT1* increased the expression of the long form of Rgt1 (Rgt1-L) in both growth conditions without affecting the expression of the short form (Rgt1-S). Thus, Rnt1p regulates both the quantity and pattern of *RGT1* expression in a glucose independent manner. In contrast, *MTH1* expression was detected only in the presence of galactose (ON condition), and the effect of *RNT1* deletion was found to be more pronounced when the gene was OFF (Figure 2C). This indicates that unlike *RGT1*, Rnt1p plays an important role in regulating the glucose-dependent repression of *MTH1*. Together, these results indicate that Rnt1p plays different roles in regulating gene expression rates that vary from constitutive (e.g. *MIG2*) to condition enhanced inhibition (e.g. *MTH1*) of gene expression.

Rnt1p mediates the promoter-dependent repression of gene expression

Glucose-dependent genes are primarily regulated at the transcriptional level by promoter-specific transcription factors (60,61). For this reason the impact of the promoter sequence on the *RNT1*-dependent expression of Mig2, Rgt1 and Mth1 mRNAs was tested. Each gene's promoter was replaced by that of the house keeping gene *ACT1* (62), and the expression was monitored using total RNA extracted from both *RNT1* and *rnt1Δ* cells grown in different sugar conditions. As expected, the expression of Mig2 mRNA driven from ACT1 promoter abolished most of the glucose-dependent response (compare Figures 3A and 2A), demonstrating that the endogenous promoter is essential for conditional repression. The same trend was also observed with P_{ACT1}-*MTH1* where promoter replacement also abolished the glucose-dependent repression (compare Figures 3C and 2C). In the case of *RGT1,* whose expression is not regulated by glucose, the promoter replacement increased the relative expression level in both sugar conditions (compare Figures 3B and 2B). The deletion of *RNT1* increased the expression levels of all three genes, even when they were expressed from exogenous promoters, regardless of the sugar conditions (Figure 3, right panels). Consistently, mutations that alter Rnt1p cleavage signal increased the Mth1 mRNA half-life (Supplementary Figure S1B) (12). This confirms that at least part of the Rnt1p inhibition of gene expression is dependent on the sequence harboring Rnt1p cleavage site in good agreement with Rnt1p targeted RNA degradation. Indeed, the deletion of *RNT1* increases the half-life of both the Mig2 (12) and the Mth1 mRNAs (Supplementary Figure S1A). Surprisingly, the increase in the expression levels upon *RNT1* deletion was more pronounced in genes expressed from their endogenous promoters, suggesting that the promoter enhances Rnt1p-dependent repression. Interestingly, in the case of *MTH1*, the impact of the promoter on the *RNT1*-mediated repression was only observed under the OFF condition, suggesting that Rnt1p inhibits the accumulation of Mth1 mRNA in a glucose-dependent manner *in vivo*. We propose that Rgt1p-associated factors are regulated via a coordinated mechanism of gene repression that combines both transcriptional and post-transcriptional levels of gene regulation.

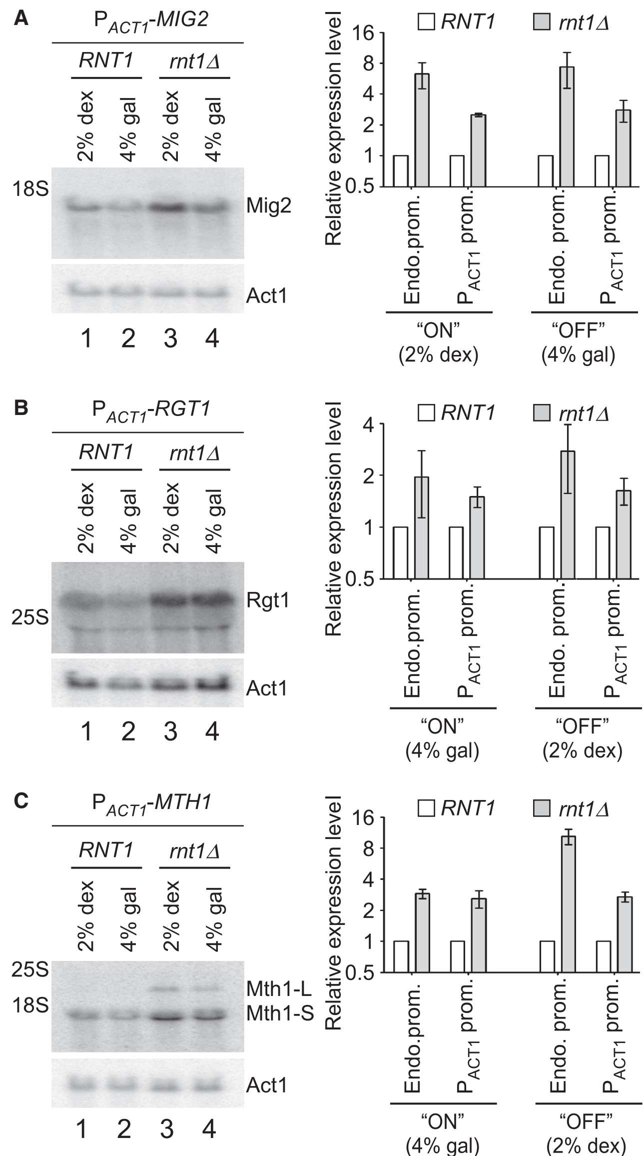

Figure 3. Promoters partially mediate the Rnt1p-dependent repression of gene expression. The expression of *MIG2* (**A**), *RGT1* (**B**) or *MTH1* (**C**) driven by either the endogenous or the *ACT1* promoters where assayed by northern blots in both *RNT1* and *rnt1Δ* strains grown in media containing either glucose or galactose (left panels). The positions of the 25S and 18S ribosomal RNAs are indicated on the left, and the loading control is shown at the bottom. The averages mRNA levels of the different genes in each sugar condition were quantified and plotted in order to illustrate the effect of RNT1 deletion relative to wild-type cells (right panels). The data from Figure 2 (endogenous promoters) was repeated so as to facilitate comparison. The data shown are the average of three independent experiments. ON and OFF indicate the conditions that are either inducing or repressing the expression of each gene, respectively.

RNT1 mediates cleavage signal independent transcription inhibition

In order to directly examine the contributions of the *MIG2*, *RGT1* and *MTH1* promoters to both glucose and Rnt1p-dependent repression, the endogenous coding sequence starting from the translation start codon (AUG) was replaced with that of a reporter gene (*LacZ*) and the promoters' activities were monitored under different conditions. Replacement (p*MIG2*pr-*LacZ*) of the coding sequence reduced the *MIG2* response to glucose to 1.4-fold instead of 3-fold (Figures 4A and 2A) when Rnt1p was present, suggesting that RNA degradation plays an important role the regulation of this gene. Therefore, the presence of the *MIG2* promoter is necessary (Figure 3A, left panel, lanes 1 and 2), but not sufficient (Figure 4A, left panel, lanes 1 and 2) for optimal glucose response. Surprisingly, the deletion of *RNT1* increased the expression of the *MIG2* promoter (Figure 4A, left panel, lanes 3 and 4) in the absence of the RNA cleavage site detected *in vitro*. When driven from *MIG2* promoter (p*MIG2*pr-*LacZ*), expression of LacZ mRNA in *rnt1Δ* was more pronounced in the ON condition than that in the OFF condition, suggesting that Rnt1p induces the basal promoter's activity and was not simply alleviate repression (Figure 4, right panel). This increase is not due to a global increase either in the promoter activities or gene expression since the majority of genes are under transcribed in *rnt1Δ* (48) and the expression of the *LacZ* reporter gene did not increase when driven by unrelated promoter like *ACT1* (data not shown) (63). It should also be noted that Rnt1p effect is unlikely to be caused by transcription independent activity of the 5′-UTR of the transcripts since the enzyme did not cleave this region (Figure 1). However, we cannot exclude the possibility that the 5′-UTR play a role in mediating Rnt1p impact on transcription. In the case of p*RGT1*pr-*LacZ*, expression on LacZ mRNA was moderately increased in *rnt1Δ* cells grown in OFF condition, and was strongly increased in ON conditions when compared with *RNT1* cells grown under these conditions (Figure 4B). This result is unexpected since the wild-type allele of *RGT1* does not seem to respond to glucose at either the transcriptional or post-transcriptional levels [Figure 2 and (64)]. One explanation for this apparent contradiction is that Rgt1 mRNA degradation conceals the effect on the promoter activity observed in absence of Rnt1p. Indeed, Rgt1 expression from a heterologous promoter responded equally to *RNT1* deletion under both sugar conditions (Figure 3B). Unlike *MIG2* and *RGT1,* Rnt1p does not inhibit the promoter activity of *MTH1* in the ON condition, but rather specifically reduces the promoter repression under the OFF conditions (Figure 4). This result is consistent with a role for Rnt1p in regulating the glucose-dependent expression of Mth1 mRNA observed in Figure 2. The conclusion drawn is that yeast dsRNA-specific ribonuclease may influence gene expression in two non-exclusive manners: one is promoter dependent and cleavage site independent, while the other requires the original open reading frame sequence.

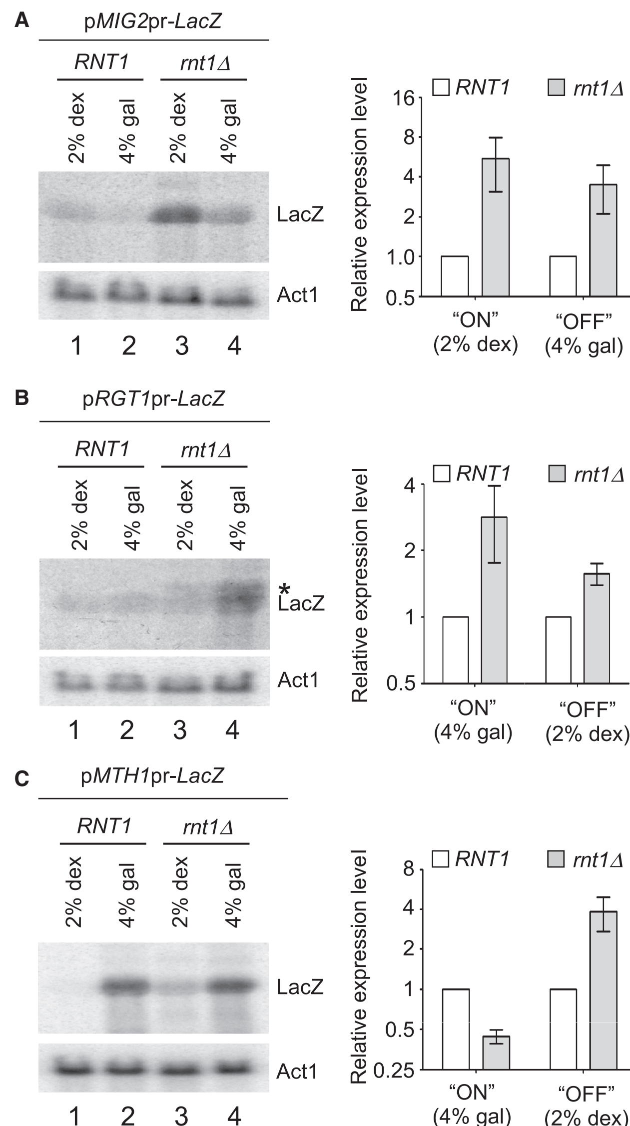

Figure 4. Rnt1p mediates cleavage site independent repression of gene expression. RNA was extracted from both *RNT1* and *rnt1Δ* strains expressing LacZ RNA from the *MIG2* (**A**), *RGT1* (**B**) or *MTH1* (**C**) promoters. The cells were grown in different media containing either glucose or galactose in order to assay the sugar effect. The relative amounts of RNA were detected by northern blot analysis using probes complimentary to LacZ (left panel), and are represented in a bar graph (right panel) as described in Figure 2. The data shown represent an average of at least three independent experiments.

Rnt1p inhibits RNAPII association with *MTH1* DNA in a glucose-dependent manner

Since *MTH1* is the only gene regulated by Rnt1p at the promoter level in a glucose-dependent manner, whether or not this regulation is directly related to an increase in transcriptional activity, and whether or not glucose regulates the Rnt1p contribution to transcription repression was investigated. Accordingly, the occupancy of the RNA polymerase II complex (RNAP II) along *MTH1* locus in both *RNT1* and *rnt1Δ* cells grown in different conditions was monitored and directly compared to the corresponding transcripts accumulation. RNAP II

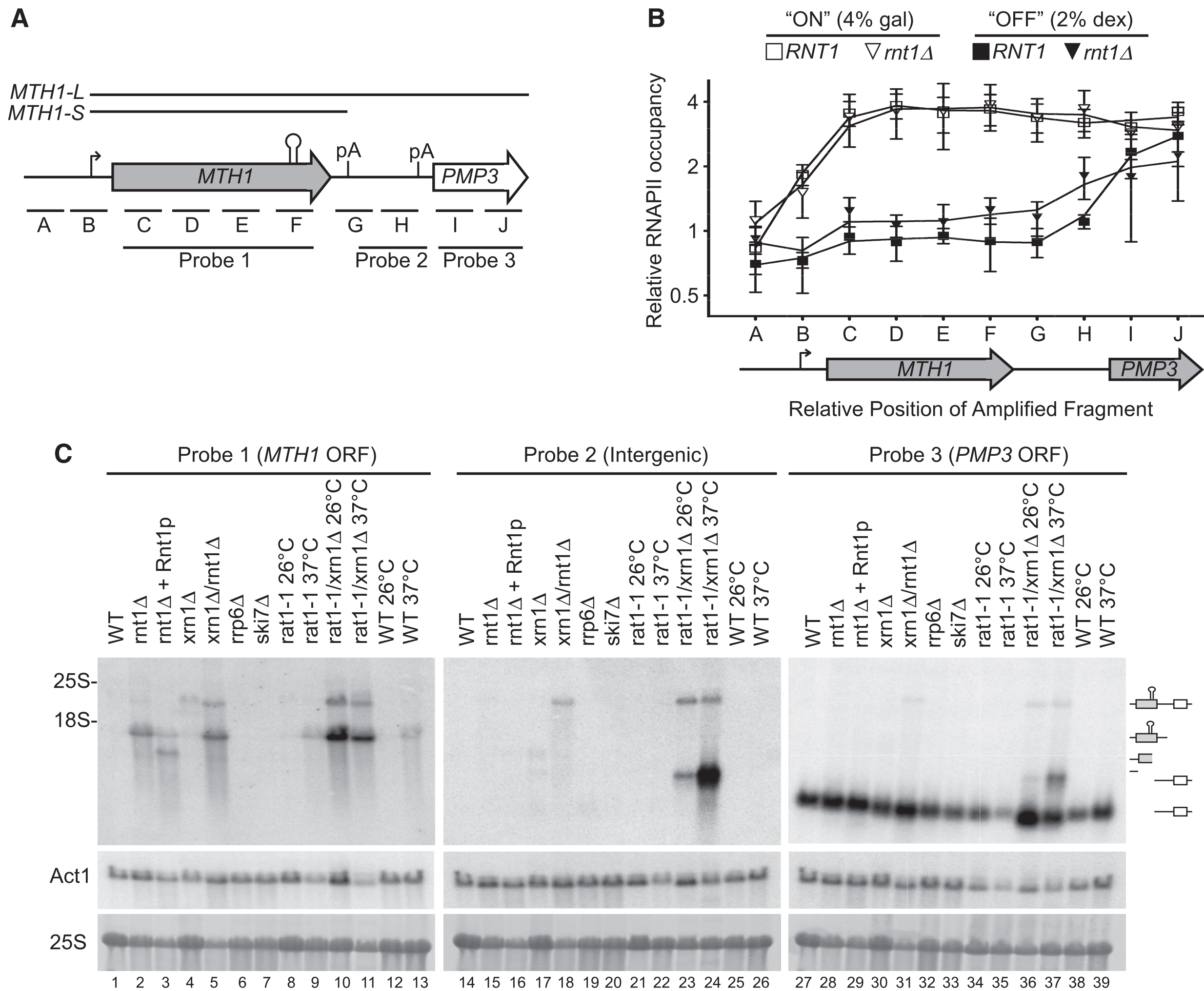

Figure 5. Rnt1p enhances the glucose-dependent transcriptional repression of *MTH1*. (**A**) Schematic representation of the *MTH1* gene locus. The two forms of Mth1 mRNAs detected by northern blot (**C**) are illustrated on top. The position of the Rnt1p cleavage signal (tetraloop) and the predicted polyadenylation signals (pA), are shown. The positions of the probes used for the northern blots shown in (**C**), and the regions amplified after the ChIP shown in (**B**) are indicated at the bottom. (**B**) ChIP was performed using antibodies against the RNAP II protein subunit Rpb1p in either *RNT1* or *rnt1Δ* cells grown in media containing either 2% dextrose (2% dex) or 4% galactose (4% gal). The precipitated DNA was amplified by quantitative radiolabelled PCR using the primers indicated in (**A**), and the average values of three independent biological replicates were used to calculate the enrichment relative to the input samples. A primer pair amplifying a known untranscribed region of chromosome V (chrV) was used to normalize the signals. (**C**) Northern blot analysis of total RNA extracted from strains either lacking or carrying mutations in different ribonucleases. The positions of the probes are indicated in (**A**). Schematics of the different RNA transcripts observed are indicated on the right. Both Act1 mRNA and 25S ribosomal RNA were used as loading controls. The extended transcripts observed in the *rat1-1*/*xrnt1Δ* RNA represent the transcriptional read-through expected upon the inactivation of *rat1-1* (84).

association with the transcription unit was examined by chromatin immunoprecipitation (ChIP) using antibodies directed against the Rpb1p subunit (65). The precipitated DNA fragments where amplified using probes covering the complete *MTH1* gene, the adjacent intergenic area and the downstream gene *PMP3* so as to clearly delineate the transcription unit (Figure 5A). As shown in Figure 5B, under ON condition, RNAPII co-immunoprecipitated, in both *RNT1* and *rnt1Δ* strains, DNA fragments corresponding to the promoter region, the coding sequence and the intergenic region downstream of *MTH1* (fragments B–H), but not the untranscribed region upstream of the promoter (fragment A). The fragments corresponding to the genes located downstream were equally associated with RNAPII suggesting that, under ON conditions, the expression of *MTH1* does not terminate efficiently and reaches levels similar to that of the downstream genes. The transcription read-through by RNAPII on *MTH1* was confirmed by the accumulation of a long RNA transcript corresponding to a 3′-end extension (Figures 1 and 5C; data not shown). Under OFF conditions, few DNA fragments corresponding to the *MTH1* sequence where immunoprecipitated with RNAPII in *RNT1* cells. In contrast, those corresponding to *PMP3* precipitated under OFF conditions to levels similar to that observed for the ON condition, as would be expected from a glucose independent gene (Figure 5B). The differences between RNAPII association in the

presence and the absence of Rnt1p under the OFF condition are statistically significant with a combined *P*-value of 0.0025. This confirms that glucose does indeed repress *MTH1* transcription. Interestingly, the deletion of *RNT1* specifically increased the RNAPII association with *MTH1* under the OFF condition, but not with *PMP3,* clearly showing that Rnt1p selectively inhibits transcription in a sugar-dependent manner.

The RNAPII association pattern with *MTH1* indicates that transcription termination and sugar-dependent repression of this gene are partly dependent on RNA degradation. Consequently, the impact of the main yeast ribonucleases implicated in both the nuclear and the cytoplasmic degradation of RNA on the expression of *MTH1* locus was investigated. The different deletion strains where grown under OFF condition in order to determine the factors that contribute to *MTH1* repression. As shown in Figure 5C, very little RNA was detected in wild-type cells (WT, lane 1), while the two forms of Mth1 (L and S) where detected in *rnt1Δ* cells and were cleaved by the recombinant enzyme (Lanes 2 and 3). As expected, Mth1-L in *rnt1Δ* cells was also weakly detected with probes downstream of *MTH1* and within *PMP3*. Interestingly, the deletion of the 5′–3′ cytoplasmic exoribonuclease *XRN1* resulted in the accumulation of Mth1-L mRNA (Lane 4). This clearly indicates that, even under OFF conditions, a certain level of Mth1 mRNA is constitutively produced and degraded in the cytoplasm. The double deletion of *RNT1* and *XRN1* increases the amount of Mth1-L (Lanes 5, 18, 31), once again confirming that Mth1-L is regulated by Rnt1p in the nucleus and Xrn1p in the cytoplasm. The deletion of the nuclear or the cytoplasmic 3′–5′ ribonucleases *RRP6* and *SKI7* did not have much effect on expression, suggesting that 3′-end degradation does not play an important role in repressing the expression of *MTH1*. In contrast, cells carrying a temperature sensitive allele of the nuclear 5′–3′ exoribonuclease *RAT1* (*rat1-1*) displayed a modest increase in the amount Mth1-S (Lanes 8 and 9). However, a significant increase in both forms of Mth1 and in RNA transcripts corresponding to 5′-end extended Pmp3 were detected at both the permissive and the restrictive temperatures in strains carrying both a deletion in *XRN1* and the *rat1-1* allele (Lanes 10, 11, 23, 24, 36 and 37). The results clearly demonstrate that post-transcriptional regulation may play a much more important role in gene expression than previously anticipated. We propose that transcriptional and post-transcriptional regulation works as a tightly integrated unit in order to achieve a rapid and complete repression of gene expression.

DISCUSSION

This study demonstrates that targeted RNA degradation plays an important role in enhancing conditional transcription repression of glucose-dependent genes. The dsRNA-specific ribonuclease III Rnt1p selectively repressed the expression of factors associated with the Snf3p–Rgt2p sensing pathway *in vivo*, and directly cleaved the associated mRNAs *in vitro* (Figure 1). In contrast, the deletion of ribonucleases like *XRN1* or *RRP6*, which are required for general RNA turnover, did not significantly alter the repression of the glucose-associated genes, underscoring the preference for the Rnt1p contribution within the glucose regulatory network. The Rnt1p-mediated repression of gene expression was partially dependent on the promoter sequence, suggesting that Rnt1p is recruited to its substrate during transcription (Figure 3). Strikingly, the promoter activity was independently suppressed by Rnt1p expression independent of the RNA sequence (Figure 4). Indeed, the association of RNAPII with the *MTH1* DNA increased in the absence of *RNT1*, confirming that Rnt1p does not only decrease gene expression by sentencing RNA for degradation, but may also repress transcription. The glucose-dependent expression pattern of the Rnt1p substrates indicates that the enzyme contributes to glucose response in a gene-specific manner that varies from the fail–safe repression of transcription (*MIG2* and *RGT1*) to direct glucose-dependent repression (*MTH1*) (Figures 2 and 6). Taken, together the results presented here reveal a new mode of gene regulation in which RNA degradation factors may simultaneously degrade nascent RNA transcripts and inhibit *de novo* transcription.

The regulation of the glucose response was mostly thought to be carried out by a well knit transcriptional network, with a few exceptions in which either protein or RNA degradation were considered to be factors in the signalling pathway (66,67). Several examples of differential RNA degradation were noted in the gluconeogenic pathway, including the Fbp1 and Pck1 mRNAs that are specifically degraded at low levels of glucose (42). The mRNAs of other genes that are not directly connected to glucose metabolism, like the iron protein subunit gene *SDH1,* were also shown to degrade in response to glucose. However, in this case, the degradation was accelerated only in the presence of high glucose levels (41). In all cases, the signal that trigger the accelerated degradation was not identified, nor was the ribonuclease identified, with the exception of the cytoplasmic 5′–3′ exoribonuclease Xrn1p that was linked to the degradation of the Sdh1 mRNA (41). Similarly, the glucose-sensing pathway was considered to be solely regulated by transcriptional activity. For example, a recent model suggested that the glucose transporter genes are differentially regulated by a transcriptional pulse of the transcription repressors Rgt1p and Mig2p in response to the amount of glucose present in the cells (67). In this mathematical model, one that considers RNA degradation as being constant, the efficiency with which Rgt1p and Mig2p repress the expression of each *HXT* gene determines which target genes have a pulse of transcription in response to glucose (67). In contrast, this study demonstrates that *RGT1* and its activator *MTH1*, as well as *MIG2*, gene expression is determined in large part by selective RNA degradation. This clearly changes the current view of how glucose sensing is achieved. As described in the model illustrated in Figure 6, RNA degradation may contribute to glucose sensing either by providing a means for fast repression, by constant surveillance, or by the conditional repression of the relevant genes.

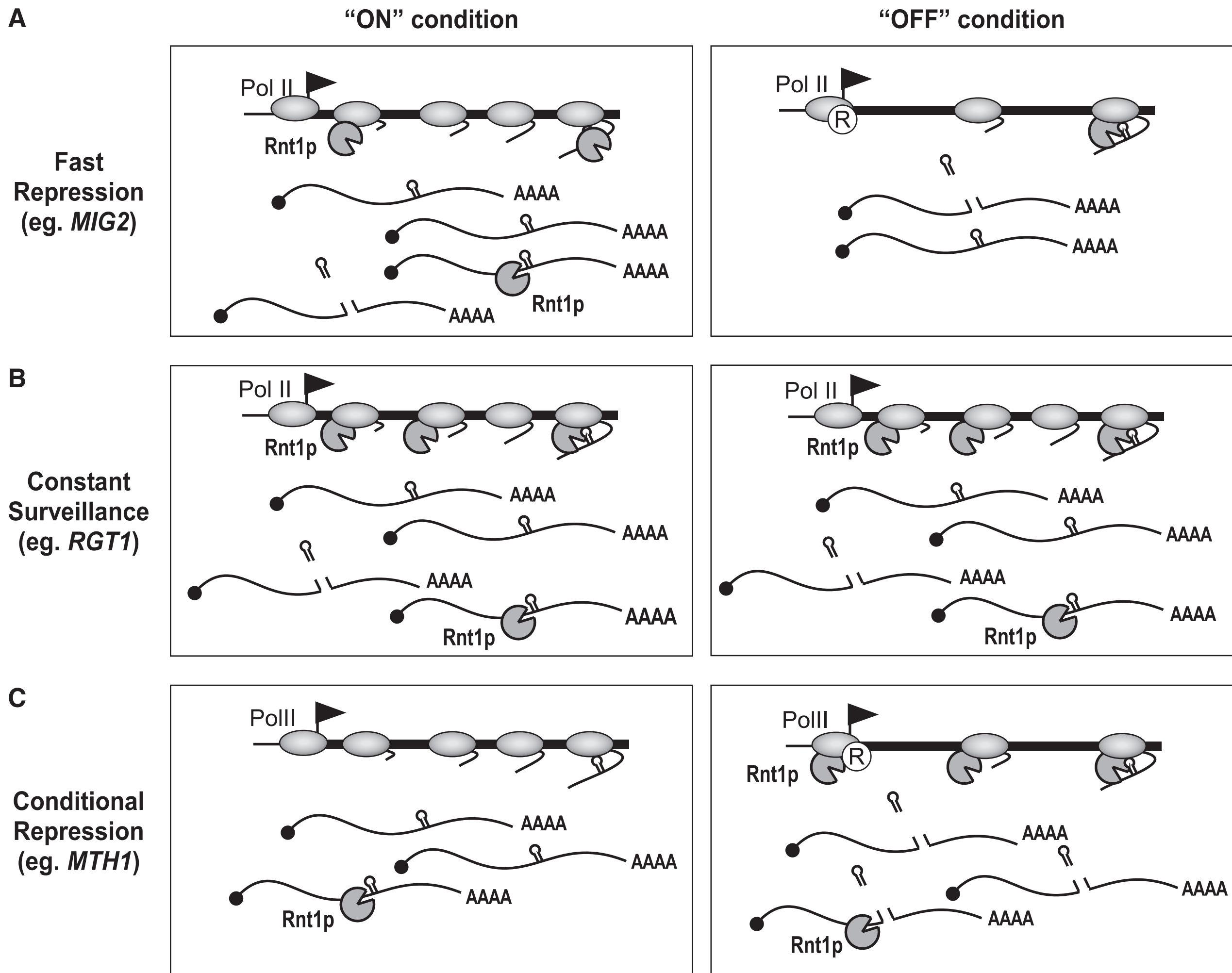

Figure 6. Proposed models of Rnt1p-dependent gene regulation. The Rnt1p contribution to the RNA degradation and transcriptional repression of *MIG2*, *RGT1* and *MTH1* genes are illustrated in both ON (genes are expressed) and OFF (expression inhibited) conditions. (**A**) Rnt1p may mediate a rapid and robust repression by down-regulating transcription and RNA stability in the ON condition, and by enhancing the effect of glucose-dependent transcription repressors (R) by degrading all nascent transcripts present in the nucleus in the OFF condition. (**B**) Constitutive down-regulation and surveillance of gene expression may be achieved by constant transcriptional repression and RNA degradation that prevents the production of aberrant RNA transcripts and maintains a constant supply of proteins for genes whose activities are regulated at the proteins level. (**C**) Rnt1p may differentially inhibit transcription and induce RNA degradation in a condition-specific manner. In this case, Rnt1p inhibits the transcription only under the OFF condition, and its impact on RNA degradation is more robust when the genes are turned OFF.

In the fast repression mode (Figure 6A), as in the case of *MIG2* gene, Rnt1p decreases the steady-state mRNA level of Mig2 mRNA by cleaving a percentage of the newly synthesized RNA co-transcriptionally and thereby reducing the transcription rate. In this case, the activity of Rnt1p appears to be constitutive, and independent of glucose, as the deletion of *RNT1* increased the expression of Mig2 mRNA to the same extent in both ON and OFF conditions (Figure 2). This mode of constant promoter coupled RNA degradation allows all transcripts to be rapidly degraded once transcription is halted by repressors like Rgt1p (39,67). In addition, Rnt1p was shown to be required for the fast degradation of the Mig2 mRNA immediately after a transition from a glucose to a glycerol containing media (12). Thus, RNA degradation ensures the fast and sustained repression of conditionally regulated genes. This mode of repression is particularly required for glucose sensors due to the constant flux of glucose cells normally experience in their natural habitat. Indeed, the short bursts of *MIG2* transcription hypothesized by the incoherent feed forward regulatory loop model (67) are difficult to envision if all nascent transcripts (i.e. transcripts that are still produced before the transcriptional repression is activated) have to be degraded post-transcriptionally in the cytoplasm as suggested for glucose sensitive genes like *SDH1* (41). Moreover, a recent study in mammalian cells demonstrated that RNA degradation is required to sharpen the transcription peak, further supporting the hypothesis that the coordination of transcriptional repression and RNA degradation is essential for producing optimal non-overlapping transcriptional pulses (68).

Rnt1p also contributed to glucose sensing by the constant surveillance of RNA transcripts that are not

conditionally repressed by glucose (Figure 6B). In this mode, represented by *RGT1*, Rnt1p constantly cleaves any excess RNA co-transcriptionally thereby preventing it from being translated. In this way, a constant amount of Rgt1p is produced allowing for a sensitive activation through the protein–protein interaction with Mth1p. The need for this method of transcriptional repression is not to increase the rate of the transcriptional repression cycle, but rather to balance the production of Rgt1p with that of its activator Mth1p. Mth1 RNA is also cleaved by Rnt1p, and the cleavage in this case appears to provide a means for the glucose-dependent conditional repression of the *MTH1* gene (Figure 6C). Unlike for the Mig2 and Rgt1 mRNAs, Rnt1p cleaves only a small fraction of the Mth1 mRNA under ON conditions without interfering with transcription. However, once the cells are moved to the OFF conditions, Rnt1p appears to specifically repress the transcription of MTH1 and cleaves its RNA in a glucose-dependent manner. This is supported by the fact that the deletion of Rnt1p had a greater effect on the repression of Mth1 mRNA in OFF condition than in the ON condition (Figures 2E and 4E). Interestingly, it was demonstrated that protein degradation by itself is not sufficient to explain the reduction in Mth1p observed after glucose addition. Moreover, even when protein decay and transcriptional repression are combined, the predicted rate of Mth1p depletion remains relatively slow (69). We propose that the conditional regulation of the Mth1 mRNA level by Rnt1p prevents any residual mRNA from escaping the nucleus, thus allowing for a faster repression of Mth1p expression. Overall, through these three different modes of gene repression, Rnt1p provides the glucose sensing network the means to fine tune transcription as mandated by the glucose availability and fluctuation.

Traditionally, eukaryotic RNA degradation was considered as an independent post-transcriptional step that takes place once transcription is complete (70). This view was fuelled by the image of RNA degradation being mostly cytoplasmic, while transcription occurs in the nucleus (71–73). However, it has become increasingly clear in recent years that certain RNA actively degrades in the nucleus (74–76), and that this degradation is not restricted to erroneous or misfolded RNA as previously thought (51,77). The degradation of RNA in the nucleus makes the distinction between transcriptional and post-transcriptional events much more difficult. It is now established that RNAPII interacts via its C-terminal domain (CTD) with the RNA modification and processing factors that are required for the maturation of mRNA (78,79). This commits the nascent transcript very early to maturation and cytoplasmic export (80), which makes the RNA degradation of mRNA difficult to achieve unless the RNA is either deliberately retained in the nucleus, or the involved ribonucleases are recruited to the transcription unit. In the cases of *MTH1*, *RGT1* and *MIG2* genes, we propose the latter scenario where Rnt1p is actively recruited to the transcription site. It was previously shown that Rnt1p associates with the chromatin of actively transcribed genes in order to promote their polyadenylation independent transcription termination (48). In parallel, the promoters of the *MTH1*, *RGT1* and *MIG2* genes seem to play an important role in enhancing the Rnt1p-dependent repression, and, as such, suggest that Rnt1p is linked to the transcriptional activity. In addition, ChIP-on-CHIP assays suggest that Rnt1p is recruited to the DNA of many genes (48), including *MIG2* (data not shown). This recruitment to the transcription site also permits Rnt1p to directly influence transcription as was noted in the cases of *MTH1* (Figure 5) and other genes (48). The sequence elements required for Rnt1p-dependent transcription repression appears to be embedded in the core promoter since deletion analysis failed to separate Rnt1p repression from basic transcription (data not shown). Other ribonucleases like Rrp6p, Xrn1p and Rat1p were shown to affect transcription by silencing bidirectional promoters and triggering transcription termination (81–84). However, in all cases, these activities were associated with the degradational activity of these enzymes. Conversely, in the case of Rnt1p, transcription is altered even in the absence of its RNA cleavage site (Figure 4). It is unlikely that the effect of Rnt1p on transcription is generic or indirect due to a general perturbation of transcription since *RNT1* deletion only increases the transcription of a minority of genes, and most of these are related to Rnt1p substrates (12). In fact, very few genes display differential increase in transcription when *RNT1* is deleted (48). It is possible, however, that Rnt1p conditionally associates with the RNAP II complex and thus triggers conformational changes in the transcriptional machinery leading to changes in transcription pattern. Alternatively, Rnt1p may function as genuine transcription repressor independent of RNA cleavage. There is no direct evidence for this possibility, but this may explain why the enzyme does not directly cleave, *in vitro*, a large number of genes that are up-regulated upon the deletion of *RNT1* (Figure 1 and data not shown). In all cases, the data reported here cement Rnt1p as an integral part of the transcription repression machinery that blurs the borders between the transcriptional and the post-transcriptional regulation of gene expression.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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