RESEARCH COMMUNICATION

A physiological role for gene loops in yeast

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DNA loops that juxtapose the promoter and terminator regions of RNA polymerase II-transcribed genes have been identified in yeast and mammalian cells. Loop formation is transcription-dependent and requires components of the pre-mRNA 3'-end processing machinery. Here we report that looping at the yeast *GAL10* gene persists following a cycle of transcriptional activation and repression. Moreover, *GAL10* and a *GAL1p*-*SEN1* reporter undergo rapid reactivation kinetics following a cycle of activation and repression—a phenomenon defined as “transcriptional memory”—and this effect correlates with the persistence of looping. We propose that gene loops facilitate transcriptional memory in yeast.

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Topological analyses of *Saccharomyces cerevisiae* chromatin have identified gene loops that juxtapose promoter and terminator regions of genes transcribed by RNA polymerase II (RNAP II) (O'Sullivan et al. 2004; Ansari and Hampsey 2005; Singh and Hampsey 2007; El Kaderi et al. 2009). Gene loops are dynamic structures whose formation is dependent on RNAP II transcription and also requires the general transcription factor TFIIB and components of the pre-mRNA 3'-end processing complex. Looping appears to be a general phenomenon of RNAP II transcription, not restricted to any particular class of genes.

Gene loops are not unique to yeast. The HIV provirus forms a loop between the 5' long terminal repeat (LTR) and 3' LTR poly(A) signal, also in a transcription-dependent manner (Perkins et al. 2008). Dynamic promoter-terminator loops have also been described for the breast cancer *BRCA1* gene (Tan-Wong et al. 2008), and at the gene encoding the immunohistological marker CD68 in mammalian cells (O'Reilly and Greaves 2007). In the case of *BRCA1*, different loop structures are formed in response to estrogen stimulation, and in normal versus breast cancer cell lines. These results suggest that looping might affect gene regulation. Nonetheless, no physiological role has been demonstrated for gene loops in either yeast or mammalian cells.

Genes of the yeast *GAL* regulon are repressed in glucose medium, but are strongly induced in the presence of galactose as the sole carbon source. Interestingly, the kinetics of *GAL* gene activation are dramatically different depending on prior cell exposure to galactose: Whereas galactose induction is slow, requiring up to 2 h for full activation, reinduction following a cycle of activation and repression occurs in minutes (Brickner et al. 2007; Kundu et al. 2007; Zacharioudakis et al. 2007). This effect has been referred to as “transcriptional memory.” *GAL* gene memory has been shown to be cytoplasmically inherited, conferred by the Gal1 protein (Zacharioudakis et al. 2007), and also requires the histone variant H2A.Z and the SWI/SNF chromatin remodeling complex (Brickner et al. 2007; Kundu et al. 2007). Translocation of genes to the nuclear periphery has been implicated in memory (for review, see Brickner 2009). However, the mechanism by which the transcriptional apparatus “remembers” prior transcriptional activity, resulting in rapid reactivation, remains unresolved.

Here we report that gene looping is associated with transcriptional memory. We demonstrate that gene loops persist at the *GAL10* and *GAL1p*-*SEN1* genes following a cycle of activation and repression, and that rapid reactivation kinetics are dependent on the persistence of looping. In a related study, Proudfoot and colleagues (Tan-Wong et al. 2009) report that rapid reactivation of the galactose-responsive *HXX1* and *GAL1p*-*FMP27* genes is also dependent on looping, and that looping requires the perinuclear myosin like protein 1 (Mlp1) protein. These results define a physiological role for gene loops in yeast, and suggest that looping might be required for the transcriptional burst associated with specific physiological or developmental stimuli.

Results and Discussion

Gene looping persists following a cycle of GAL10 activation and repression

Gene looping is induced by transcriptional activation (Ansari and Hampsey 2005). To further investigate the relationship between transcription and looping, we characterized the *GAL10* gene (Fig. 1A), exploiting the ability to readily activate and repress *GAL10* transcription in response to carbon source. Transcript levels were assayed by RT-PCR, and gene looping was monitored by a modified version of the chromosome conformation capture (3C) assay as described previously (Ansari and Hampsey 2005; Singh and Hampsey 2007; Singh et al. 2009). 3C detects and quantifies the frequency of interaction between any two genomic loci by converting physical chromatin interactions into specific ligation products (Dekker et al. 2002). To determine the stability of the *GAL10* loop, we subjected a wild-type yeast strain to a cycle of galactose activation and glucose repression, according to the scheme summarized in Figure 1B. As expected, *GAL10* transcript levels were elevated following 2.5 h of exposure to galactose, but returned to repressed levels following a 0.5-h glucose chase (Fig. 1C). The dynamic range of *GAL10* expression relative to *ACT1* is comparable with the dynamic range of *GAL1* expression as quantified by real-time PCR (Bryant and Ptashne 2003). Chromatin immunoprecipitation (ChIP)

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Figure 1. Gene looping persists following a cycle of *GAL10* activation and repression. (A) Schematic depiction of the *GAL* locus. The positions of the divergent P1 and T1 primer pairs used in 3C analysis and the positions of the PCR products generated in RT-PCR and ChIP analyses are indicated. (B) Time course of *GAL10* activation and repression. Cells were grown in 2% glucose (Glc) to mid-log phase, shifted to 2% galactose (Gal) for 2.5 h, then shifted back to 2% glucose. The numbers in parentheses above the line correspond to time points below the line and to lane numbers in C–E. (C) *GAL10* and *ACT1* (control) mRNA levels were assayed by RT-PCR. (Lane 1) *GAL10* transcript levels were quantified by normalizing to the preinduction level, set at 1. (D) RNAP II occupancy of the *GAL10* promoter was determined by ChIP using an antibody (8WG16) to the unphosphorylated form of RNAP II. (Lane 1) The data were quantified by dividing the immunoprecipitation:input ratio for *GAL10* to the immunoprecipitation:input ratio for an intergenic region on chromosome V (Komarnitsky et al. 2000; Singh and Hampsey 2007; data not shown) and were normalized to the preinduction sample. (E) Gene looping between the *GAL10* promoter and terminator regions was determined by 3C using the P1 and T1 primer pairs as described previously (Singh and Hampsey 2007). Control PCR represents the intergenic region of chromosome V, generated using convergent primers, confirming that equal amounts of template DNA were present in all reactions. (Lane 1) The data were quantified by dividing the P1–T1 PCR signals by the control PCR signals for each sample and were normalized to the ratio for the preinduction sample. Results are presented as fold increase below each lane in C–E.

indicated that RNAP II association and dissociation from the *GAL10* promoter coincided with induction and repression of *GAL10* transcript levels (Fig. 1D). Loop formation also coincided with galactose induction. Surprisingly, however, the *GAL10* loop was maintained following glucose repression, diminishing only after cells had been exposed to glucose for >4 h (Fig. 1E). These results demonstrate that looping at *GAL10* persists following a cycle of activation and repression. Furthermore, the persistence of looping is not dependent on retention of RNAP II at the promoter.

Rapid reactivation kinetics of *GAL10* is associated with looping

What is the significance of the persistence of the *GAL10* loop following glucose repression? Conceivably, gene

loops that juxtapose the promoter and terminator regions could facilitate subsequent rounds of transcription. Indeed, this possibility would be consistent with the recent demonstration of “transcriptional memory” at *GAL* genes following a cycle of galactose activation and glucose repression (Brickner et al. 2007; Kundu et al. 2007; Zacharioudakis et al. 2007).

To determine whether gene looping is related to galactose memory, we first determined the kinetics of *GAL10* activation in a looping-defective mutant. For this analysis, we used the *sua7-1* mutant, which encodes a glutamic acid-to-lysine replacement at position 62 (E62K) of the general transcription factor TFIIB (Pinto et al. 1994; Bushnell et al. 2004). The TFIIB E62K defect impairs looping at *GAL10* and other genes, but does not affect mRNA levels (Singh and Hampsey 2007). Using an isogenic wild-type and *sua7-1* strain pair, we monitored the kinetics of *GAL10* activation following the glucose → galactose shift according to the scheme outlined in Figure 2A. We observed relatively slow kinetics of *GAL10* induction, requiring >1 h for peak transcript levels in

Figure 2. The rapid reactivation kinetics of *GAL10* is associated with gene looping. (A) Schematic depiction of the time course of *GAL10* activation, corresponding to the data presented in B and C. (B,C) Transcript levels (RT-PCR) and *GAL10* looping (3C) were determined as described in Figure 1. The *sua7-1* mutant encodes the TFIIB E62K replacement and is isogenic to the wild-type (WT) strain. (D) Schematic depiction of the time course of *GAL10* activation, repression, and reactivation, corresponding to the data presented in E and F. (E,F) Analogous to B and C, extended to include a cycle of glucose repression and subsequent reactivation. (G,H) Quantification of the RT-PCR and 3C data for *GAL10* activation and reactivation. In A and D, the numbers in parentheses above the line correspond to time points below the line and to lane numbers in B, C, E, and F. The data in G and H summarize the data shown in B, C, E, and F.

the wild-type strain (Fig. 2B, lanes 1–7). Essentially identical results were obtained with the *sua7-1* mutant (Fig. 2B, lanes 8–14). However, 3C analysis revealed that looping occurred coincident with galactose induction in the wild-type strain (Fig. 2C, lane 1 vs. lanes 2–7), but was defective in the *sua7-1* mutant (Fig. 2C, lanes 8–14). Results are summarized in Figure 2, G and H, and demonstrate that the initial kinetics of *GAL10* activation is relatively slow and unaffected by looping.

Next, we determined the kinetics of *GAL10* reactivation following a cycle of activation and repression (Fig. 2D). In marked contrast to the kinetics of activation (Fig. 2B), the kinetics of *GAL10* reactivation in the wild-type strain was very rapid, with maximum transcript levels observed 2 min after the galactose shift (Fig. 2E, lanes 1–7). The kinetics of reactivation in the *sua7-1* mutant, however, was much slower, requiring >1 h for maximum reactivation (Fig. 2E, lanes 8–14), essentially identical to the kinetics of the initial activation (Fig. 2B). 3C analysis confirmed that looping persists throughout the time course of reactivation in the wild-type strain (Fig. 2F, lanes 2–7), whereas looping was not observed under the same conditions in the *sua7-1* mutant (Fig. 2F, lanes 8–14). These results are summarized in Figure 2, G and H, and demonstrate that cells previously exposed to galactose exhibit very rapid *GAL10* reactivation kinetics and do so in a manner that correlates with the persistence of gene looping.

GAL1p-SEN1 exhibits looping-dependent rapid reactivation

To assess whether looping-dependent transcriptional memory is idiosyncratic to *GAL10* or might be a more general effect, we assayed transcription and looping at the *GAL1p-SEN1* locus (Fig. 3A). Previous results from our laboratory demonstrated induction of looping at *GAL1p-SEN1* when cells were shifted from glucose to galactose medium (Ansari and Hampsey 2005). We now monitored the kinetics of *GAL1p-SEN1* activation in an isogenic wild-type and *sua7-1* strain pair following the glucose → galactose shift according to the scheme outlined in Figure 3A. We observed the same relatively slow kinetics of *GAL1p-SEN1* induction as at *GAL10*, requiring >1 h for full activation in the wild-type strain and *sua7-1* strains (Fig. 3C, lanes 1–8 and 9–16, respectively). Again, loop formation occurred coincident with galactose induction in the wild-type strain (Fig. 3D, lanes 1–8), but was defective in the *sua7-1* mutant (Fig. 3D, lanes 9–16). Thus, the initial kinetics of *GAL1p-SEN1* activation is relatively slow and unaffected by looping, comparable with the *GAL10* results.

To determine whether looping affects *GAL1p-SEN1* reactivation, we determined the kinetics of reactivation (Fig. 3E). Once again, we observed very rapid reactivation kinetics following a cycle of galactose activation and glucose repression, with maximum transcript levels observed 2 min after the galactose shift (Fig. 3F, lanes 1–8). This effect was abolished in the *sua7-1* mutant, where reactivation occurred with the same kinetics as activation (Fig. 3F, lanes 9–16). 3C analysis confirmed that looping persists throughout the time course of reactivation in the wild-type strain (Fig. 3G, lanes 1–8), but not in the *sua7-1* mutant (Fig. 3G, lanes 8–14). Results are summarized in Figure 3, H and I. Thus, cells previously exposed to galactose exhibit very rapid reactivation ki-

Figure 3. *GAL1p-SEN1* undergoes rapid reactivation and correlates with the persistence of looping. (A) Schematic depiction of the *GAL1p-SEN1* gene, depicting HindIII sites (H) and positions of the primer pairs (P1 and T1) used in 3C analysis, and the region amplified by RT-PCR analysis of mRNA levels. (B) Time course of *GAL1p-SEN1* activation, corresponding to the data presented in C and D. (C,D) *SEN1* transcript levels (RT-PCR) and *GAL1p-SEN1* looping (3C) were determined as described in Figure 1. (E) Schematic depiction of the time course of *GAL10* activation, repression, and reactivation, corresponding to the data presented in F and G. (F,G) Analogous to C and D, extended to include a cycle of glucose repression and subsequent reactivation. (H,I) Quantification of the RT-PCR and 3C data for *GAL1p-SEN1* reactivation. In B and E, the numbers in parentheses above the line correspond to time points below the line and to lane numbers in C, D, F, and G.

netics for *GAL1p-SEN1*, and this effect is lost in a looping-defective mutant, comparable with effects observed at *GAL10*. Furthermore, these results demonstrate that looping-associated transcriptional memory is not unique to the structure of *GAL10* or dependent on the *GAL10* terminator.

The Set1 histone methyltransferase does not affect *GAL10* gene looping or reactivation kinetics

The Set1 histone H3 Lys 4 (H3K4) methylase is targeted to transcriptionally active genes. Interestingly, H3K4 methylation persists at the *GAL10* gene through a cycle of activation and repression, leading to the proposal that H3K4 methylation provides “memory” of recent transcriptional activity (Ng et al. 2003). Whether this transcriptional mark affects subsequent *GAL10* reactivation was not reported. To determine whether transcriptional memory associated with gene looping is related to H3K4

methylation, we asked whether the kinetics of *GAL10* reactivation and the persistence of looping are affected by deletion of *SET1*. We repeated the cycle of activation → repression → reactivation (Fig. 4A). The kinetics of *GAL10* reactivation and the persistence of looping were identical in the *set1Δ* and isogenic wild-type strain (Fig. 4B,C, cf. lanes 1 and 6,7, and lanes 1 and 8,9). These results are distinctly different from those associated with the *sua7-1* mutant, which exhibits slow *GAL10* reactivation kinetics and the absence of looping (Fig. 4B,C, cf. lanes 4,5 and 2,3). We conclude that Set1-catalyzed H3K4 methylation does not affect the rapid reactivation kinetics of *GAL10* and does not contribute to looping.

Snf2 acts downstream from gene looping to affect GAL10 transcriptional memory

To determine whether gene looping is related to SWI/SNF-dependent transcriptional memory (Kundu et al. 2007), we assayed *GAL10* transcript levels in isogenic wild-type and *snf2Δ* strains, as described above for *set1Δ*. In this case, the *GAL10* reactivation kinetics was distinctly slower, comparable with the effect of the *sua7-1* mutation (Fig. 4B, cf. lanes 4,5 and 14,15). To determine whether *GAL10* looping is dependent on Snf2, we repeated the cycle of activation and repression (Fig. 4D) and assayed looping by 3C. In contrast to the effect of *sua7-1*, which blocked loop formation at *GAL10* (Figs. 2C), looping occurred and persisted in the *snf2Δ* mutant (Fig. 4E, cf. lanes 3,4). Thus, Snf2 is required for the rapid reactivation

kinetics of *GAL10*, but is not required for looping. These results suggest that gene looping is fundamental to transcriptional memory at the *GAL* locus and that loop formation occurs upstream of SWI/SNF in the pathway of rapid reactivation.

Gal4 persists at the GAL10 promoter following a cycle of activation and repression

To further investigate the relationship between gene looping and transcription, we asked whether the looping-defective *sua7-1* mutation affects RNAP II occupancy of the *GAL10* promoter during the cycle of activation → repression → reactivation (Fig. 5A). ChIP of RNAP II using an antibody directed against the Rpb3 subunit revealed that RNAP II was recruited to the promoter coincident with galactose activation and diminished following glucose repression in wild-type and *sua7-1* cells (Fig. 5B, lanes 1–3). RNAP II rapidly reassociated with the promoter upon reactivation in the wild-type strain, whereas the kinetics of RNAP II reassociation was distinctly slower in the *sua7-1* mutant (Fig. 5B, lanes 4,5). These results mirror the reactivation kinetics of *GAL10* and *GAL1p-SEN1* in the wild-type and *sua7-1* strains (Fig. 2G, 3H). We conclude that (1) RNAP II recruitment to the *GAL10* promoter during the initial round of activation is unaffected by gene looping, (2) RNAP II is not required for the persistence of looping following glucose repression, and (3) looping facilitates the rapid association of RNAP II with the *GAL10* promoter upon reactivation.

We next asked whether the Gal4 activator remains associated with the *GAL_{UAS}* elements, located between the divergently transcribed *GAL1* and *GAL10* genes, through a cycle of activation and repression, and whether its association is affected by looping. As expected, Gal4 is recruited to the *GAL_{UAS}* region in response to galactose, but in contrast to RNAP II, remains associated following glucose repression (Fig. 5C). The *sua7-1* mutation does not affect Gal4 recruitment, but adversely affects its retention following glucose repression. Thus, Gal4 persists at *GAL_{UAS}*, and this effect is associated with the persistence of gene looping.

The results presented here define a physiological role for gene loops in yeast. Earlier work demonstrated that looping is transcription-dependent and requires components of the initiation and termination machineries. We now report that looping correlates with transcriptional memory, defined as rapid reactivation following a cycle of activation and repression. This conclusion offers an explanation for why the looping-defective *sua7-1* mutant exhibits no apparent defect in transcription: Gene looping would not necessarily confer a transcriptional advantage for expression of most yeast genes, 60% of which are expressed at less than one mRNA copy per cell (Holstege et al. 1998). Instead, gene loops might be important for the transcriptional burst—in some cases by as much as two to three orders of magnitude—of a subset of genes under specific physiological or developmental conditions.

How might the persistence of gene loops enable rapid reactivation? One possibility is that looping could facilitate the handoff of RNAP II from the terminator back to the promoter of the same gene (Ansari and Hampsey 2005; Singh and Hampsey 2007). However, the absence of RNAP II at the *GAL10* promoter following the cycle of activation and repression, despite promoter–terminator

Figure 4. Effects of Set1 and Snf2 on *GAL10* looping and memory. (A) Schematic depiction of the time course of *GAL10* activation and repression, corresponding to the data presented in B and C. (B,C) Transcript levels (RT-PCR) and *GAL10* looping (3C), as described in Figure 1, were assayed using isogenic *SUA7* (WT), *sua7-1*; *SET1* (WT), *set1Δ*; and *SNF2* (WT), *snf2Δ* strain pairs. (D) Schematic depiction of the time course of *GAL10* activation and repression for the data presented in E. (E) *GAL10* looping (3C) using the same wild-type and *snf2Δ* strain pair as in B. All data were quantified as described in Figure 2 and results are presented as fold change below each lane in B, C, and E.

Figure 5. Gal4 persists at the *GAL10* promoter and is associated with looping. (A) Schematic depiction of the time course of *GAL10* activation and reactivation, corresponding to the data presented in B and C. The numbers in parentheses above the line correspond to time points below the line and to the lane numbers in B and C. (B) ChIP analysis of RNAP II occupancy of the *GAL10* regulatory region in isogenic wild-type and *sua7-1* strains using an antibody to the Rpb3 subunit of RNAP II. (C) ChIP analysis of Gal4 occupancy of the *GAL10* regulatory region. All data were quantified as described in the legend for Figure 1D.

juxtaposition, suggests that RNAP II might not be recycled from terminator to promoter. If RNAP II recruitment is the rate-limiting step in *GAL10* activation, RNAP II must be recruited by a different and more efficient pathway than de novo assembly of the initiation complex. There is a precedent for this scenario in vitro: A “scaffold” structure, which appears to include most components of the transcription initiation complex, but neither RNAP II nor TFIIB, persists at the promoter through the transcription cycle and facilitates higher rates of activator-mediated reinitiation (Yudkovsky et al. 2000). The structure of the gene loop might include a subset of transcription factors, as well as termination factors, perhaps stabilized by promoter–terminator juxtaposition that promotes RNAP II recruitment and rapid reactivation.

The persistence of the *GAL10* gene loop for several hours, presumably through cell division, is consistent with earlier reports of galactose-induced transcriptional memory (Brickner et al. 2007; Kundu et al. 2007; Zacharioudakis et al. 2007). A heterokaryon assay established that galactose memory is cytoplasmically inherited and that the Gal1 galactokinase is the heritable determinant (Zacharioudakis et al. 2007; for review, see Ptashne 2008). Our results imply that gene looping lies downstream from Gal1 in the pathway that establishes transcriptional memory. It will be interesting to determine whether cytoplasmic inheritance of transcriptional memory no longer occurs in looping-defective mutants.

Transcriptional memory is likely to involve gene translocation to the nuclear periphery (for review, see Brickner 2009). Several yeast genes have been shown to translocate to the nuclear periphery upon activation, to remain tethered there following repression, and to undergo rapid

reactivation as a consequence (Brickner and Walter 2004; Casolari et al. 2004, 2005; Abruzzi et al. 2006; Dieppl et al. 2006; Taddei et al. 2006; Brickner et al. 2007; Chekanova et al. 2008; Vodala et al. 2008). We propose that gene loops create a structure that enables the transcription and pre-mRNA processing machineries to be anchored to the nuclear pore, and that this architecture facilitates subsequent rounds of RNAP II recruitment. This view is consistent with data from the Proudfoot laboratory (Tan-Wong et al. 2009) showing that gene loops are associated with the Mlp1 located on the nucleoplasmic face of the nuclear pore complex, and that Mlp1 is required for looping and rapid reactivation of the *HXK1* gene. Gene loops might also be coupled to nuclear export of mRNA: Deletion of the Hpr1 component of the TREX complex, which couples transcription with mRNA export, blocks looping, and *hpr1Δ* is suppressed by *sua7* mutations (*soh4* alleles of *SUA7*) (Fan et al. 1996) encoding altered forms of TFIIB (BN Singh, unpubl.). Although the results presented here demonstrate that gene loops underlie transcriptional memory, gene loops might also prove to be fundamental to coupled RNAP II transcription and pre-mRNA processing, including nuclear export of the transcript.

Materials and methods

Yeast strains

The yeast strains used in this study are listed in Supplemental Table S1. Strain pairs T16 (WT) and YDW546 (*sua7-1*); YMH965 (WT *SNF2-TAP*) and YMH966 (*sua7-1 SNF2-TAP*); YMH1034 (*GAL1p-SEN1 SUA7**), YMH1035 (*GAL1p-SEN1 sua7-1*); MBY1198 (WT) and MBY1217 (*set1Δ*); and BY4734 (WT) and (*snf2Δ*) are isogenic. The *GAL1p-SEN1* strains were derived from T16 and YDW546 by integrating the *GAL1* promoter upstream of the *SEN1* gene at its normal chromosomal locus as described previously (Ansari and Hampsey 2005). The *Snf2* TAP-tagged strains YMH965 and YMH966 were derived from T16 and YDW546, respectively, by transformation with DNA that was PCR-amplified from pBS1539 (*URA3* marker) (Puig et al. 2001).

3C

DNA loops were analyzed by a modified version of 3C (Dekker et al. 2002; Dekker 2006), as described elsewhere (Singh et al. 2009). Juxtaposition of the *GAL10* and *GAL1p-SEN1* promoter–terminator regions were detected as P1–T1 PCR products. PCR primer sequences are listed in Supplemental Table S2. Control reactions were performed to establish that P1–T1 PCR products are dependent on formaldehyde cross-linking, HindIII restriction digestion, and subsequent ligation (data not shown). Control PCR reactions were also carried out using a convergent primer pair corresponding to a chromosome V intergenic region (Supplemental Table S2). PCR products were fractionated on a 1.5% agarose gel and visualized by ethidium bromide staining using an AlphaImager 2000.

ChIP

Cross-linking and isolation of chromatin were performed as described for 3C analysis. Immunoprecipitation reactions were performed as described previously (Singh and Hampsey 2007) using antibodies directed against TFIIB (Pinto et al. 1994), RNAP II (8WG16 or α -Rpb3), or Gal4 (Santa Cruz Biotechnologies). PCR reactions were performed and analyzed as described for 3C analysis using the *GAL10* or *SEN1* primer pairs indicated in Supplemental Table S2.

RT-PCR analysis

RT-PCR analysis was performed as described previously (Singh and Hampsey 2007). Cell pellets obtained from 50-mL cultures, grown in

parallel with the cultures used for 3C analysis, were dissolved in 400 μ L of RLT buffer. RT-PCR was done using 1 μ g of total RNA and gene-specific forward and reverse primer pairs (Supplemental Table S2) according to the One-Step RT-PCR system. PCR products were analyzed as described above for 3C.

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