
Rat1p maintains RNA polymerase II CTD phosphorylation balance

SILVIA JIMENO-GONZÁLEZ,¹ MANFRED SCHMID, FRANCISCO MALAGON,² LINE LINDEGAARD HAANING,³ and TORBEN HEICK JENSEN⁴

Center for mRNP Biogenesis and Metabolism, Department of Molecular Biology and Genetics, Aarhus University, DK-8000, Aarhus, Denmark

ABSTRACT

In *S. cerevisiae*, the 5′-3′ exonuclease Rat1p partakes in transcription termination. Although Rat1p-mediated RNA degradation has been suggested to play a role for this activity, the exact mechanisms by which Rat1p helps release RNA polymerase II (RNAPII) from the DNA template are poorly understood. Here we describe a function of Rat1p in regulating phosphorylation levels of the C-terminal domain (CTD) of the largest RNAPII subunit, Rpb1p, during transcription elongation. The *rat1-1* mutant exhibits highly elevated levels of CTD phosphorylation as well as RNAPII distribution and transcription termination defects. These phenotypes are all rescued by overexpression of the CTD phosphatase Fcp1p, suggesting a functional relationship between the absence of Rat1p activity, elevated CTD phosphorylation, and transcription defects. We also demonstrate that *rat1-1* cells display increased RNAPII transcription kinetics, a feature that may contribute to the cellular phenotypes of the mutant. Consistently, the *rat1-1* allele is synthetic lethal with the *rpb1-E1103G* mutation, causing increased RNAPII speed, and is suppressed by the *rpb2-10* mutation, causing slowed transcription. Thus, Rat1p plays more complex roles in controlling transcription than previously thought.

Keywords: RNA polymerase II; CTD phosphorylation; transcription elongation; transcription termination

INTRODUCTION

The transcription cycle of RNAPII comprises three main phases—initiation, elongation, and termination, each of which is associated with specific phosphorylation patterns of the C-terminal domain (CTD) of Rpb1p. The “CTD code” proposes that differential CTD phosphorylation regulates the recruitment of transcription and mRNA processing factors to the RNAPII machinery and is best understood for phosphorylation of serine2 (Ser2P) and serine5 (Ser5P) residues. During initiation, the CTD is hypophosphorylated, which allows binding of transcription initiation factors and assembly of the preinitiation complex (PIC). Promoter escape and early transcription elongation occur concomitantly with Ser5 phosphorylation, which facilitates pre-mRNA 5′-end capping and the stable transition from initiation to elongation (promoter release) (Rasmussen and Lis 1993; Komarnitsky et al. 2000; Schroeder et al. 2000; Kim et al. 2010). Ser5P is gradually removed during transcription elon-

gation, due to the actions of the CTD phosphatases Ssu72p and Rtr1p and is only completely removed at the polyadenylation (pA) site (Kim et al. 2010; Bataille et al. 2012). Ser2P, which is absent during transcription initiation, gradually increases along transcription units and remains high until transcription termination. Ser2P plays a central role in coupling pre-mRNA processing to transcription elongation, and substitution of CTD-Ser2 residues to alanine impairs splicing and 3′ end processing in mammalian cells (Gu et al. 2013). A major Ser2 kinase in *Saccharomyces cerevisiae* is Ctk1p, whose inactivation leads to strongly decreased Ser2P levels, a severely impaired recruitment of 3′ end processing factors and an accumulation of RNAPII at the 3′ ends of genes with weak pA sites (Ahn et al. 2004; Kim et al. 2010). Ser2P is removed during or after transcription termination by the CTD-phosphatase Fcp1p to prepare RNAPII for new initiation events (Kobor et al. 1999; Cho et al. 2001; Mandal et al. 2002). In addition, Fcp1p dampens Ser2P levels during normal transcription elongation as *fcp1* mutants display increased Ser2P levels inside genes (Archambault et al. 1997; Kobor et al. 1999; Cho et al. 2001; Ghosh et al. 2008).

¹Present address: Departamento de Biología Molecular, Centro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER), E-41092, Seville, Spain

²Present address: Laboratory of Molecular Biology, Developmental Genetics Section, National Cancer Institute, Bethesda, MD 20892, USA

³Present address: Taconic Europe, DK-8600, Silkeborg, Denmark

⁴Corresponding author

E-mail thj@mb.au.dk

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.041129.113>.

© 2014 Jimeno-González et al. This article is distributed exclusively by the RNA Society for the first 12 months after the full-issue publication date (see <http://rnajournal.cshlp.org/site/misc/terms.xhtml>). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 3.0 Unported), as described at <http://creativecommons.org/licenses/by-nc/3.0/>.

Apart from coupling transcription with RNA processing, Ser2P may also influence the speed by which RNAPII proceeds through chromatin. For example, mammalian RNAPII, harboring Ser2 to alanine substitutions, shows decreased elongation rates (Gu et al. 2013). Furthermore, in human cells the speed of RNAPII increases toward the gene 3' end, which could be related to increasing Ser2P (Danko et al. 2013). Similarly in *S. cerevisiae*, mutation of the multi-purpose transcription factor Sub1p leads to altered CTD phosphorylation levels and cotranscriptional recruitment of CTD kinases/phosphatases, which is paralleled by decreased transcription elongation rates (Calvo and Manley 2005; Garcia et al. 2010). Thus, although mechanistic details need to be elucidated, a link between CTD phosphorylation and transcription elongation appears to exist.

The mechanistic details underlying transcription termination are not well worked out. A current model postulates that Rat1p targets the 5' end of the nascent downstream RNA fragment appearing after pA site cleavage. This causes, in an unknown fashion, the dissociation of RNAPII from the DNA template (Kuehner et al. 2011). RNAPII transcription stalling at or near the pA site may help in this process. CTD phosphorylation also impacts transcription termination. Most importantly, Ser2P facilitates binding of the pre-mRNA 3' end processing-factor and transcription termination-factor Pcf1p and the Rat1p-cofactor Rtt103p (Lunde et al. 2011). The cotranscriptional recruitment of the Ser2P kinase Ctk1p ceases around the pA site, suggesting that Ser2P is lost in the downstream region (Mayer et al. 2010). However, to what extent Ser2P, or its removal, is functionally important for transcription termination in vivo is unclear. In addition to its role in transcription termination at gene 3' ends, Rat1p also aids in terminating transcription events inside the gene body, provided that they are engaged with inefficiently capped pre-mRNA (Jimeno-Gonzalez et al. 2010).

The majority of the in vivo studies of Rat1p function rely on the thermosensitive *rat1-1* mutant, the activity of which is poorly characterized. It contains a single mutation (Y657C) situated outside of the enzyme's exonuclease domain, and since the Rat1-1p protein is stable at the restrictive temperature, it may retain some exonuclease activity. Even so, growth and transcription termination phenotypes of *rat1-1* cells are not rescued by the coexpression of the catalytically inactive *rat1-D325A* point mutant (Kim et al. 2004; Luo et al. 2006; Mayer et al. 2010). This has been taken as evidence that 5'-3' exonucleolysis is essential for Rat1p function and that this activity is, at least partly, compromised in the *rat1-1* background.

Here, we find that overexpression of Fcp1p suppresses the thermosensitivity of *rat1-1*. Surprisingly, *rat1-1* cells not only exhibit a transcription termination defect but also display highly elevated CTD Ser2P levels as well as decreased RNAPII occupancy within genes. These phenotypes are all partially restored by Fcp1p overexpression. High Ser2P levels in *rat1-1* cells are not due to decreased Fcp1p levels around

transcribed chromatin but rather to an increased cotranscriptional recruitment of Ctk1p. Finally, *rat1-1* cells show increased transcription elongation rates. The data suggest that Rat1p plays a CTD-modulatory role during transcription elongation, which needs to be considered when interpreting molecular phenotypes of the *rat1-1* mutant.

RESULTS AND DISCUSSION

Increased phosphorylation of the Rpb1p CTD in *rat1-1* cells

To explore mechanisms used by Rat1p to promote transcription termination, we screened for multicopy suppressors of the *rat1-1* mutant at its nonpermissive temperature of 34°C (see Materials and Methods). In addition to the *RAT1* gene, we found that overexpression of the CTD Ser2P phosphatase, Fcp1p, was able to rescue *rat1-1* thermosensitivity at 34°C (Fig. 1A). This suggested that reduction of CTD Ser2P phosphorylation levels might help overcome growth-limiting defects of the *rat1-1* mutant. We therefore analyzed the global CTD phosphorylation status of Rpb1p in the *rat1-1* background with or without Fcp1p overexpression. Western blotting analysis of whole-cell extracts using antibodies recognizing CTD-Ser2P, -Ser5P, or -Ser7P residues demonstrated elevated Ser2P and Ser5P levels in the *rat1-1* strain,

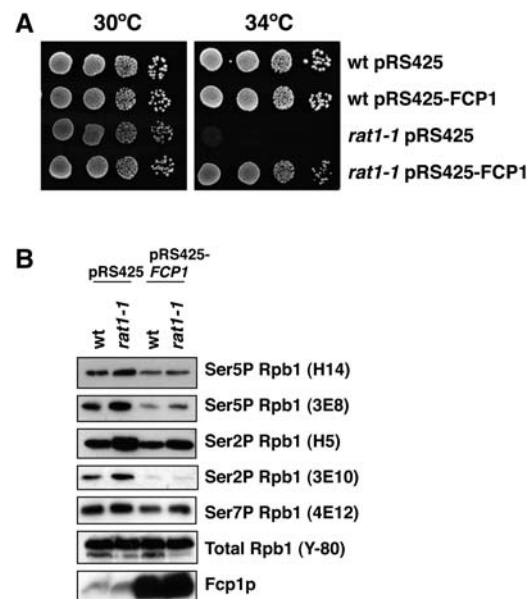


FIGURE 1. Excess Fcp1p rescues growth deficiency of *rat1-1* cells. (A) Tenfold dilution series of wt and *rat1-1* strains transformed with pRS425 or pRS425-*FCP1* plasmids as indicated and spotted onto AA-Leu plates. Strains were grown for 3 d at 30°C or 34°C. (B) Rpb1p CTD phosphorylation status in whole-cell extracts measured by Western blotting analysis. Strains from A were grown at 30°C in AA-Leu medium to an OD₆₀₀ of 0.6, followed by a subsequent incubation for 1 h at 34°C, before being harvested for protein extraction. Membranes were probed with H14, H5, 3E8, 3E10, 4E12, Rpb1p (Y-80), or Fcp1p antibodies as indicated.

whereas Ser7P levels were not affected (Fig. 1B). Both Ser2P and Ser5P levels were decreased by Fcp1p overexpression.

Fcp1p overexpression reduces elevated CTD-Ser2P levels at active chromatin in *rat1-1* cells and partly suppresses related RNAPII distribution phenotypes

We next tested whether increased CTD phosphorylation in *rat1-1* cells was also manifested during active transcription and, if so, which impact excess Fcp1p might have. For this analysis we utilized the Ser2P antibody H5, whose epitope is increased in *rat1-1* cells and dampened to roughly wt levels upon Fcp1p overexpression (Fig. 1B). Notably, H5 has highest affinity for a CTD Ser2P epitope containing neighboring Ser5P (Chapman et al. 2007). Chromatin immunoprecipitation (ChIP) experiments using H5 as well as Rpb1p (Y-80) antibodies were carried out and immunoprecipitated chromatin was interrogated by PCR amplicons distributed at different positions along the reporter gene *GAL1-YLR454W* (Fig. 2A; Mason and Struhl 2005). In line with previous reports (Komarnitsky et al. 2000; Bataille et al. 2012), we found that in wt conditions, Ser2P levels were low at the promoter, increased in the coding region, and declined immediately after RNAPII passage of the pA site (Fig. 2B, left panel). In contrast, *rat1-1* cells displayed abnormally high levels of Ser2P inside the *YLR454W* gene body, whereas the presence of this epitope disappeared downstream from the pA site. Such increased Ser2P levels were not simply due to the presence of more chromatin-associated RNAPII in *rat1-1* cells as evidenced by ChIP assays conducted with the phosphorylation-insensitive Y-80 antibody (Fig. 2C, left panel). Indeed, the Ser2P/Rpb1p signal ratio increased robustly (up to sixfold) upon Rat1p mutation (Fig. 2D, left panel). A similar phenotype is observed at the *PMA1* gene (Supplemental Fig. S1A,B), which, together with the Western blot analysis, suggests that increased CTD phosphorylation is a hallmark of transcription in *rat1-1* cells. As predicted, Ser2P levels were reduced upon Fcp1p overexpression, which restored high Ser2P ChIP signals of *rat1-1* cells to close to wt levels (Fig. 2B,D, cf. right and left panels).

Which molecular phenotypes of *rat1-1* cells may be associated with elevated CTD-Ser2P levels? Consistent with previous reports (Kim et al. 2004; West et al. 2004; Luo et al. 2006; Jimeno-Gonzalez et al. 2010), the *rat1-1* mutation caused RNAPII transcription read-through of the *YLR454* pA site (amplicons 8.5 and 9) (Fig. 2C, left panel, and E, right panel). Interestingly, RNAPII accumulated to similar levels near the pA site (8.2) in the wt and *rat1-1* strains, despite lower levels within the *YLR454W* gene body of *rat1-1* cells (2 to 8 regions) (Fig. 2C, left panel). A possible interpretation

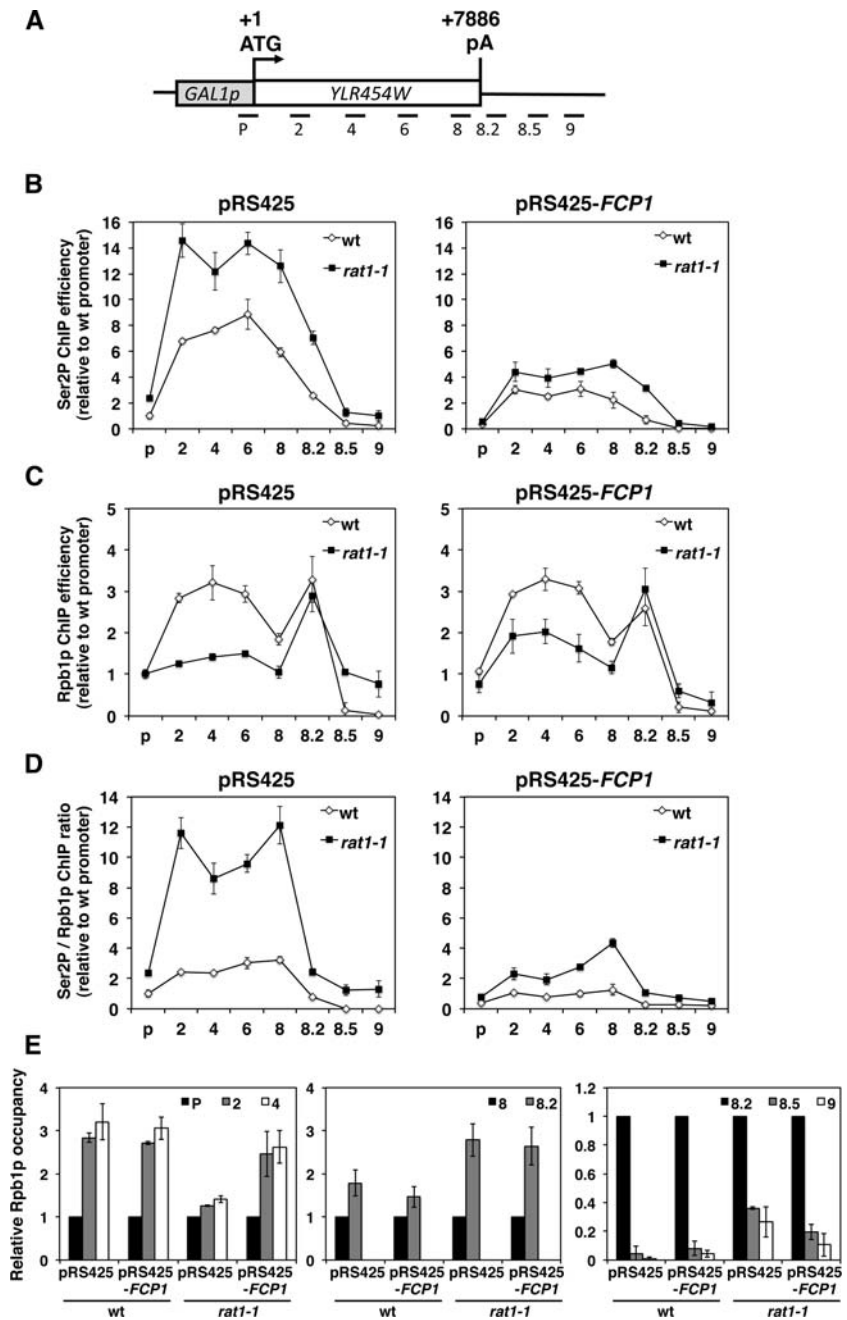


FIGURE 2. (Legend on next page)

of these data is that RNAPII stalls in the vicinity of the pA site to be targeted by Rat1p-dependent “torpedo” termination (Kim et al. 2004; Luo et al. 2006). Failure to terminate efficiently in the *rat1-1* context leads to an enhanced accumulation of stalled RNAPII versus transcribing polymerases within the gene body (Fig. 2E, middle and right panel). In addition, only in mutant cells can RNAPII escape the stalling site to “read-through” and reach downstream regions 8.5 and 9.

A strong *rat1-1*-dependent phenotype could also be observed at the *YLR454W* 5' end; while RNAPII immunoprecipitation (IP) efficiencies at the promoter were similar in the two backgrounds, only ~40%–50% of RNAPII levels were detected along the *YLR454W* gene body in *rat1-1* compared with wt cells (Fig. 2C, left panel, cf. amplicon p with 2, 4, 6, and 8; Fig. 2E, left panel). A similar phenotype was described for the *PMA1* gene in previous reports (Kim et al. 2004; Kawachi et al. 2008). This effect may be explained by the faster transcription elongation rates in the *rat1-1* mutant (see below). Importantly, Fcp1p overexpression partially suppressed both the decreased levels of RNAPII in the gene body (ratio between promoter and gene body ChIP amplicons) (Fig. 2E, left panel) and the transcription read-through (ChIP signals at amplicons 8.5 and 9) (Fig. 2E, right panel) phenotype of the *rat1-1* mutant, without markedly changing levels of RNAPII “pA site stalling” (8.2) (Fig. 2E, middle panel).

To rule out that the observed effects would be specific for the *GAL1-YLR454W* reporter, we also analyzed RNAPII distribution at various positions along the *PMA1* gene (Supplemental Fig. S1A). Gratifyingly, both decreased polymerase in the gene relative to the promoter (Supplemental Fig. S1C, cf. 5 to middle amplicons), and transcription termination (Supplemental Fig. S1C, cf. 3' UTR and T1 amplicons) phenotypes of *rat1-1* cells were observed at the *PMA1* locus. Note that RNAPII pA site-stalling and read-through effects are difficult to distinguish for the *PMA1* gene due to the presence of multiple pA sites spread over a 450-bp region

(Ozsolak et al. 2010). Even so, a clear suppression of both gene 5'- and 3'-end defects could be measured upon Fcp1p overexpression (Supplemental Fig. S1B).

We previously reported that *rat1-1* thermosensitivity is also suppressed by the *rpb1-N488D* allelic background conferring slow transcription rates (Jimeno-Gonzalez et al. 2010). The molecular mechanism underlying this suppression was not elucidated. Interestingly, introduction of the *rpb1-N488D* mutation caused decreased Ser2P levels over the *YLR454W* reporter gene in both the wt and *rat1-1* mutant contexts (Supplemental Fig. S2A, cf. left and right panels). This effect was not due to a change in RNAPII levels in the *rpb1-N488D* background (Supplemental Fig. S2B). Since both suppressors of *rat1-1* thermosensitivity, the *rpb1-N488D* allele and high-copy *FCP1*, are linked to decreased CTD phosphorylation, this may underlie the growth defect of *rat1-1* cells. Indeed, the *rpb1-N488D* allele also suppressed the decreased RNAPII levels inside the gene and transcription read-through phenotypes of *rat1-1* (Supplemental Fig. 2C, left and right panels; Jimeno-Gonzalez et al. 2010). Taken together, these data suggest that abnormally high Ser2P levels contribute to altered transcription elongation and termination in *rat1-1* cells. Reducing Ser2P levels by excess Fcp1p or the *rpb1-N488D* mutant can correct these defects. The increased levels of pA-site-stalled RNAPII in *rat1-1* cells (8.2/8) (Fig. 2E; Supplemental S2C, middle panel) are not decreased by *FCP1* overexpression or in the *rpb1-N488D* mutant. Hence, unusually high levels of Ser2P appear to specifically impact a subset of RNAPII that either fails to stall or is capable of resuming transcription after stalling.

Rat1p displaces Ctk1p

Increased CTD phosphorylation in *rat1-1* cells and its suppression by extracopy Fcp1p could be explained by defective recruitment of Fcp1p in the absence of wt Rat1p activity. To test this possibility, we conducted *YLR454W* ChIP using myc-tagged Fcp1p, which yielded specific signals at amplicons within the gene (Supplemental Fig. S3A). Unexpectedly, the relative ChIP efficiency of Fcp1-myc at the *YLR454* and *PMA1* genes was not decreased but was rather comparable to that of Rpb1p in both wt and *rat1-1* cells at all measured positions (Fig. 3A; Supplemental Fig. S3B). Thus, Fcp1p recruitment per transcription event is not affected by the *rat1-1* mutation. While it is possible that the catalytic capacity of Fcp1p may be compromised in the *rat1-1* background, we deemed it more likely that increased recruitment of a relevant kinase may explain the observed phenotype. We therefore chose to monitor levels of the main Ser2P kinase, Ctk1p, using

FIGURE 2. Transcriptional phenotypes of the *rat1-1* mutation are rescued by Fcp1p overexpression. (A) Schematic representation of the *GAL1::YLR454W* system. Promoter is shaded gray, and the transcript unit is white. The transcription start site (TSS) is depicted by an arrow; the major pA site, by a vertical line. Distances (kb) of ChIP amplicons to the reporter TSS are indicated. (B–E) ChIP experiments at the *GAL1::YLR454W* reporter gene. Cells containing the indicated plasmids were grown in AA-Ura-Leu/2% raffinose at 30°C to an OD₆₀₀ of 0.6. The *GAL1* promoter was induced with 2% galactose for 3 h at 30°C and *rat1-1* inactivated by an additional incubation for 1 h at 34°C before fixation. ChIP values are normalized to the signal at the *YLR454W* promoter region (P) in wt cells containing empty plasmid. Averages and standard deviations of all ChIP data were calculated from three independent biological experiments, each subjected to triplicate quantitative PCR analysis. Data from cells containing empty plasmids (*left panels*) and cells containing high-copy *FCP1* plasmids (*right panels*) are averages from the same experiments and only split for better visualization. (B) Rpb1 Ser2P distribution measured by the H5 antibody. (C) Total Rpb1p distribution measured by the Y-80 antibody. (D) Ratio between Ser2P and Rpb1p ChIP values in wt and *rat1-1* strains. Positions 8.5 and 9 in the wt strain are set to zero since the Rpb1 ChIP levels used for normalization are at background levels in wt cells. (E) ChIP values from C normalized separately for each strain to reveal differences in local changes of Rpb1p distribution at *GAL1::YLR454W*. (*Left*) Promoter escape shows occupancy at fragments 2 and 4 normalized to promoter p. (*Middle*) pA site stalling shows occupancy at fragments 8.2 normalized to fragment 8. (*Right*) Read-through is fragments 8.5 and 9 normalized to fragment 8.2 separately for each strain.

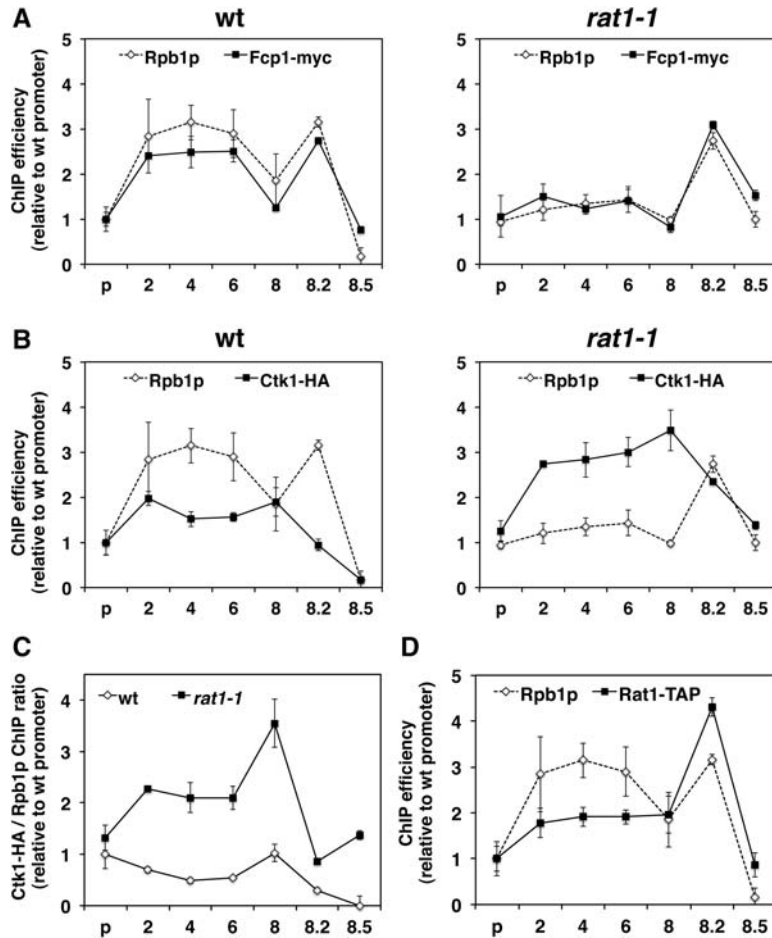


FIGURE 3. Ctk1-HA recruitment at *GAL1::YLR454W* is increased in *rat1-1* cells. Fcp1-myc (A), Ctk1-HA (B), and Rat1-TAP (D) distributions along the *GAL1::YLR454W* gene as measured by ChIP (solid lines). Parallel Rpb1p ChIP values in the indicated strains are shown as dotted lines. Cells were grown as described in the legend of Figure 2B. ChIP values are relative to the wt signal at the *YLR454W* promoter region (P). (C) Ctk1-HA ChIP normalized to Rpb1p in wt and *rat1-1* strains. Position 8.5 in the wt strain is set to zero since Rpb1 ChIP levels are at background in wt cells (see Fig. 2B). Averages and standard deviations of all ChIP data were calculated from three independent biological experiments, each subjected to triplicate quantitative PCR analysis.

HA-tagged Ctk1p. Indeed, elevated Ctk1-HA occupancy per RNAPII could be observed in *rat1-1* relative to wt cells at the *YLR454W* and *PMA1* genes (Fig. 3B,C; Supplemental Fig. S3B). Hence, high Ser2P levels at chromatin in *rat1-1* cells are likely due to increased recruitment of Ctk1p. This suggests a possible competition between cotranscriptional recruitment of Rat1p and Ctk1p. Indeed, Ctk1p becomes excluded from chromatin when RNAPII reaches the pA site where Rat1p levels peak (Fig. 3, cf. 8.2 amplicon in B, left panel, and D; Kim et al. 2004; Luo et al. 2006; Mayer et al. 2010). Notably, Ctk1p disappearance in that region is less efficient in *rat1-1* cells; at the 8.2 position, a threefold increase of Ctk1p per RNAPII can be detected compared with the wt strain (Fig. 3C). The molecular basis for the competition between Rat1p and Ctk1p is unclear, but we speculate that they may compete for a common binding site, possibly on the CTD.

RNAPII elongation rate is increased in the *rat1-1* mutant

Rat1p partakes in pre-mRNA 5' capping quality control and causes premature transcription termination of RNAPII engaged with uncapped RNA (Jiao et al. 2010; Jimeno-Gonzalez et al. 2010). Under such conditions, the *rat1-1* mutation leads to increased RNAPII levels inside gene bodies. Hence, the decreased RNAPII occupancy within gene bodies of *rat1-1* cells, observed here without any impaired capping, is not explained by this role of Rat1p. On the other hand, Gu et al. (2013) recently found that substitution of CTD-Ser2 residues with alanines slows down transcription in mammalian cells. Thus, we speculated that CTD hyperphosphorylation in *rat1-1* cells might cause an increased transcription elongation rate. In fact, this could help explain the lowered levels of gene body RNAPII in *rat1-1* cells; i.e. the same amount of RNAPII is loaded at the promoter but spends less time transcribing along the coding region. To investigate this possibility, we monitored transcription kinetics of the *GAL1-YLR454W* system, where transcription rate can be extrapolated from the time it takes RNAPII to leave the gene template after turning off transcription by the addition of glucose to the culture (Mason and Struhl 2005). Hence, such a time course of the last wave of transcription was conducted by Rpb1p ChIP analysis at different positions along the gene.

Indeed, RNAPII took a shorter time to leave the *YLR454W* gene in the *rat1-1* compared with the wt context (Fig. 4A). Therefore, RNAPII speed is higher in *rat1-1* cells.

If an altered RNAPII elongation rate in the *rat1-1* strain contributes to its growth defect, one might expect genetic interactions with other mutants affecting RNAPII speed. Indeed, combining the *rat1-1* allele with the *rpb1-E1103G* mutation, which causes a faster transcription rate in vivo and in vitro (Malagon et al. 2006; Hazelbaker et al. 2013), resulted in an enhanced growth defect (Fig. 4B). Conversely, the *rpb1-N488D* or *rpb2-10* alleles, which both cause a slower RNAPII elongation rate in vivo and in vitro (Powell and Reines 1996; Mason and Struhl 2005; Jimeno-Gonzalez et al. 2010), suppressed *rat1-1* thermosensitivity (Fig. 4C; Jimeno-Gonzalez et al. 2010). As a slowdown of RNAPII transcription downstream from the pA site is believed to

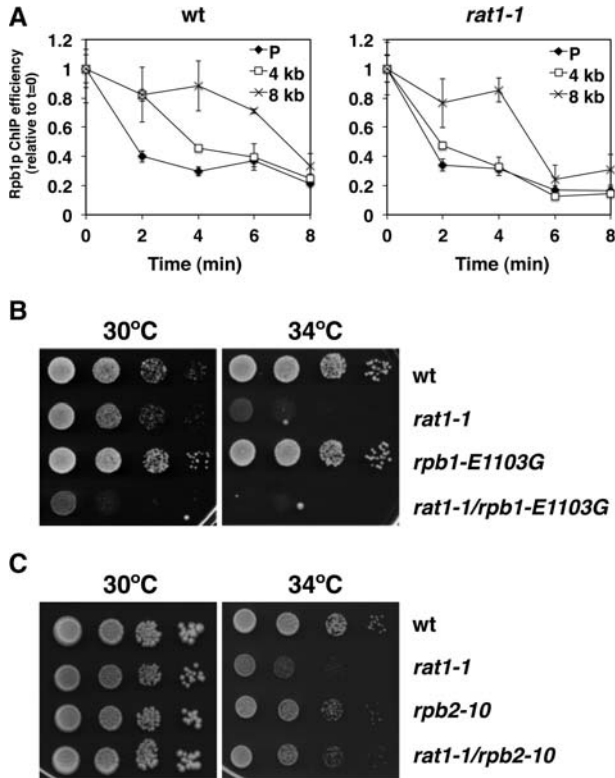


FIGURE 4. Increased RNAPII transcription rate in *rat1-1* cells. (A) Transcription elongation rate assay measuring the last wave of transcription. Cells were grown in AA-Ura/2% raffinose at 30°C to an OD₆₀₀ of 0.6. The *GAL1* promoter was then induced with 2% galactose for 3 h at 30°C. After an additional incubation for 1 h at 34°C, the promoter was turned off by the addition of 2% glucose to the cultures, and samples were cross-linked at the indicated time points. Values correspond to Rpb1p ChIP signals, obtained with the indicated amplicons, at each time point, and normalized to signals in galactose (time point 0). Averages and standard deviations of all ChIP data were calculated from three independent biological experiments, each subjected to triplicate quantitative PCR analysis. (B,C) Growth capabilities measured by 10-fold dilution series of the indicated strains spotted onto YM-1 plates. Strains were grown for 2 d at 30°C or 34°C as indicated.

be a key step during the transcription termination process, an increased elongation rate may contribute to the termination defect observed in *rat1-1* cells. Consistently, rescued growth of *rat1-1* cells by introducing *rpb1* alleles slowing RNAPII progress is paralleled by a correction of the *rat1-1* transcription termination phenotype (Jimeno-Gonzalez et al. 2010).

Rat1p is widely accepted to be involved in transcription termination. In addition, the enzyme is required for quality control of RNA 5'-end capping (Jiao et al. 2010; Jimeno-Gonzalez et al. 2010). Previous reports all suggest a causal role for exonucleolytic attack of nascent RNA 5' ends by Rat1p. Consistently, coexpression of a Rat1p catalytic dead mutant, in the *rat1-1* strain, did not rescue CTD hyperphosphorylation (Supplemental Fig. S4), and exonucleolysis thus appears to also be required for the molecular phenotypes described here. As CTD phosphorylation impacts all steps of the transcription cycle, we suggest that the RNAPII CTD hyperphosphorylation phenotype of *rat1-1* cells is taken into account when molecular phenotypes upon Rat1p inactivation are considered. How altered CTD phosphorylation status impinges on the various roles of Rat1p in transcription will be the focus of further investigation.

MATERIALS AND METHODS

Yeast strains and plasmids

Yeast manipulations and growth conditions were as previously described (Malagon et al. 2006). All strains used in this study are derived from a S288C background and listed in Table 1. The *GAL1::YLR454W* reporter was introduced by crossing with the strain BY4741 *GAL1p::YLR454W* (Mason and Struhl 2005). To isolate suppressors of *rat1-1*, we used the severe thermosensitivity phenotype conferred by the *rat1-1/rpb1-E1103G* double mutant at 34°C. The strain Y2823 was transformed with a genomic library (a kind gift of François Lacroute) cloned into the *URA3* containing

TABLE 1. Yeast strains

Name	Strain	Genotype	Source
<i>rat1-1 rpb1-E1103G</i>	Y2823	<i>MATa rat1-1 rpb1-E1103G</i>	This study
wt	Y2400	<i>MATa pGAL1-YLR454::URA3 ura3Δ</i>	Jimeno-Gonzalez et al. (2010)
<i>rat1-1</i>	Y2402	<i>MATa rat1-1 pGAL1-YLR454::URA3 ura3Δ</i>	Jimeno-Gonzalez et al. (2010)
<i>rpb1-N488D</i>	Y2401	<i>MATa rpb1-N488D pGAL1-YLR454::URA3 ura3Δ</i>	Jimeno-Gonzalez et al. (2010)
<i>rat1-1 rpb1-N488D</i>	Y2403	<i>MATa rat1-1 rpb1-N488D pGAL1-YLR454::URA3 ura3Δ</i>	Jimeno-Gonzalez et al. (2010)
<i>rpb1-E1103G</i>	Y2820	<i>MATa rpb1-E1103G pGAL1-YLR454::URA3 ura3Δ</i>	This study
<i>rat1-1 rpb1-E1103G</i>	Y2823	<i>MATa rat1-1 rpb1-E1103G pGAL1-YLR454::URA3 ura3Δ</i>	This study
<i>rpb2-10</i>	Y2571	<i>MATa rpb2-10 pGAL1-YLR454::URA3 ura3Δ</i>	This study
<i>rpb2-10 rat1-1</i>	Y2572	<i>MATa rat1-1 rpb2-10 pGAL1-YLR454::URA3 ura3Δ</i>	This study
FCP1-myc	Y2517	<i>MATa FCP1-(myc)13::KAN pGAL1-YLR454::URA3 ura3Δ</i>	This study
<i>rat1-1 FCP1-myc</i>	Y2518	<i>MATa rat1-1 FCP1-(myc)13::KAN pGAL1-YLR454::URA3 ura3Δ</i>	This study
CTK1-HA	Y2515	<i>MATa CTK1-(HA)3::TRP1 pGAL1-YLR454::URA3 ura3Δ</i>	This study
<i>rat1-1 CTK1-HA</i>	Y2516	<i>MATa rat1-1 CTK1-(HA)3::TRP1 pGAL1-YLR454::URA3 ura3Δ</i>	This study
RAT1-TAP	Y2411	<i>MATa RAT1-TAP::HIS3 pGAL1-YLR454::URA3 ura3Δ</i>	Jimeno-Gonzalez et al. (2010)

All strains are isogenic or congeneric (S288C background) and *his3Δ leu2Δ trp1Δ::hisG*.

multicopy plasmid pFL44L (Bonneaud et al. 1991). Out of approximately 60,000 transformants, 14 clones suppressed the lack of growth at 34°C after reisolation of the original clones and retransformation. Sequencing of the clones identified 13 identical insertions containing the *RAT1* gene (cXV coordinates 717670-721951; plasmid pRES21), while one clone carried a genomic fragment (cXIII 818791-822802) containing the *FCP1* gene (plasmid pRES7). Suppression was also observed in the *rat1-1* single mutant, and no effect of *RAT1* or *FCP1* overexpression was observed for the *rpb1-E1103G* single mutant. Hence, *FCP1* is a high-copy suppressor of *rat1-1*. All results shown here were obtained using the *FCP1* gene subcloned into the multicopy *LEU2* containing vector pRS425. The plasmid expressing the *rat1* exonuclease mutant pRS315 *rat1-D235A* was kindly supplied by Steve Buratowski.

ChIP analysis

ChIP reactions were performed as previously described (Jimeno-Gonzalez et al. 2006) by cross-linking with 1% formaldehyde for 20 min at room temperature. IPs were carried out using the following reagents: Rpb1p, Sepharose-protein A beads (GE Healthcare) and the rabbit polyclonal antibody Rpb1-y80 (Santa Cruz Biotechnology); Rpb3, Dynabeads M-280 sheep anti-Mouse IgG (Invitrogen) and the mouse monoclonal antibody anti-POLR2C (Abcam); Rat1-TAP, Sepharose-IgG beads (GE Healthcare); Rpb1p CTD-Ser2P, Dynabeads M-280 sheep anti-Mouse IgM (Invitrogen) and the mouse monoclonal antibody H5 (Covance); Fcp1-myc, Dynabeads M-280 sheep anti-Mouse IgG (Invitrogen) and the mouse monoclonal antibody c-myc (Santa Cruz); and Ctk1-HA, Dynabeads M-280 sheep anti-Mouse IgG (Invitrogen) and the mouse monoclonal antibody anti-HA (Santa Cruz). Purified DNA was analyzed as previously described (Jimeno-Gonzalez et al. 2010). Oligonucleotide sequences used for qPCR analysis of the *GAL-YLR454W* reporter gene are described by Jimeno-Gonzalez et al. (2010) and for *PMA1* are as follows: 5', TCAGCTCATCAGCCAACCTCAAG and CGTCGACACCGTGATTAGATTG; middle, TTGCCAGCTGTCGTTACCAC and TCGACA CCAGCCAAGGATTC; 3' UTR, TCTCTGGATGGTACTTTTTTCTTTCTTG and TGCGTGTGTGAATTGTGCTC; and T1, GC GCCATACAGACA and CTTGTAGAATGGCCT.

Antibodies for Western blotting

The following antibodies were used for Western blotting analysis: mouse monoclonal antibody H14 (Covance); rat monoclonal antibodies 3E8, 4E12, and 3E10 (a gift from Dirk Eick); and rabbit polyclonal anti-Fcp1p (a gift from Steve Buratowski). The other antibodies are as described for ChIP.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

We thank Steve Buratowski and Dirk Eick for antibodies and plasmids. This work was supported by the Danish National Research Foundation (grant DNRF58) and the Novo Nordisk Foundation

(T.H.J.). M.S. is the recipient of a grant from the Lundbeck foundation.

Received July 4, 2013; accepted December 23, 2013.

REFERENCES

- Ahn SH, Kim M, Buratowski S. 2004. Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. *Mol Cell* **13**: 67–76.
- Archambault J, Chambers RS, Kobor MS, Ho Y, Cartier M, Bolotin D, Andrews B, Kane CM, Greenblatt J. 1997. An essential component of a C-terminal domain phosphatase that interacts with transcription factor IIF in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci* **94**: 14300–14305.
- Bataille AR, Jeronimo C, Jacques PE, Laramée L, Fortin ME, Forest A, Bergeron M, Hanes SD, Robert F. 2012. A universal RNA polymerase II CTD cycle is orchestrated by complex interplays between kinase, phosphatase, and isomerase enzymes along genes. *Mol Cell* **45**: 158–170.
- Bonneaud N, Ozier-Kalogeropoulos O, Li GY, Labouesse M, Minvielle-Sebastia L, Lacroute F. 1991. A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae/E. coli* shuttle vectors. *Yeast* **7**: 609–615.
- Calvo O, Manley JL. 2005. The transcriptional coactivator PC4/Sub1 has multiple functions in RNA polymerase II transcription. *EMBO J* **24**: 1009–1020.
- Chapman RD, Heidemann M, Albert TK, Mailhammer R, Flatley A, Meisterernst M, Kremmer E, Eick D. 2007. Transcribing RNA polymerase II is phosphorylated at CTD residue serine-7. *Science* **318**: 1780–1782.
- Cho EJ, Kobor MS, Kim M, Greenblatt J, Buratowski S. 2001. Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-terminal domain. *Genes Dev* **15**: 3319–3329.
- Danko CG, Hah N, Luo X, Martins AL, Core L, Lis JT, Siepel A, Kraus WL. 2013. Signaling pathways differentially affect RNA polymerase II initiation, pausing, and elongation rate in cells. *Mol Cell* **50**: 212–222.
- García A, Rosonina E, Manley JL, Calvo O. 2010. Sub1 globally regulates RNA polymerase II C-terminal domain phosphorylation. *Mol Cell Biol* **30**: 5180–5193.
- Ghosh A, Shuman S, Lima CD. 2008. The structure of Fcp1, an essential RNA polymerase II CTD phosphatase. *Mol Cell* **32**: 478–490.
- Gu B, Eick D, Bensaude O. 2013. CTD serine-2 plays a critical role in splicing and termination factor recruitment to RNA polymerase II *in vivo*. *Nucleic Acids Res* **41**: 1591–1603.
- Hazelbaker DZ, Marquardt S, Wlotzka W, Buratowski S. 2013. Kinetic competition between RNA polymerase II and Sen1-dependent transcription termination. *Mol Cell* **49**: 55–66.
- Jiao X, Xiang S, Oh C, Martin CE, Tong L, Kiledjian M. 2010. Identification of a quality-control mechanism for mRNA 5'-end capping. *Nature* **467**: 608–611.
- Jimeno-Gonzalez S, Gomez-Herreros F, Alepuz PM, Chavez S. 2006. A gene-specific requirement for FACT during transcription is related to the chromatin organization of the transcribed region. *Mol Cell Biol* **26**: 8710–8721.
- Jimeno-Gonzalez S, Haaning LL, Malagon F, Jensen TH. 2010. The yeast 5'-3' exonuclease Rat1p functions during transcription elongation by RNA polymerase II. *Mol Cell* **37**: 580–587.
- Kawauchi J, Mischo H, Braglia P, Rondon A, Proudfoot NJ. 2008. Budding yeast RNA polymerases I and II employ parallel mechanisms of transcriptional termination. *Genes Dev* **22**: 1082–1092.
- Kim M, Krogan NJ, Vasiljeva L, Rando OJ, Nedea E, Greenblatt JF, Buratowski S. 2004. The yeast Rat1 exonuclease promotes transcription termination by RNA polymerase II. *Nature* **432**: 517–522.
- Kim H, Erickson B, Luo W, Seward D, Graber JH, Pollock DD, Megee PC, Bentley DL. 2010. Gene-specific RNA polymerase II

- phosphorylation and the CTD code. *Nat Struct Mol Biol* **17**: 1279–1286.
- Kobor MS, Archambault J, Lester W, Holstege FC, Gileadi O, Jansma DB, Jennings EG, Kouyoumdjian F, Davidson AR, Young RA, et al. 1999. An unusual eukaryotic protein phosphatase required for transcription by RNA polymerase II and CTD dephosphorylation in *S. cerevisiae*. *Mol Cell* **4**: 55–62.
- Komarnitsky P, Cho EJ, Buratowski S. 2000. Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. *Genes Dev* **14**: 2452–2460.
- Kuehner JN, Pearson EL, Moore C. 2011. Unravelling the means to an end: RNA polymerase II transcription termination. *Nat Rev Mol Cell Biol* **12**: 283–294.
- Lunde BM, Horner M, Meinhart A. 2011. Structural insights into *cis* element recognition of non-polyadenylated RNAs by the Nab3-RRM. *Nucleic Acids Res* **39**: 337–346.
- Luo W, Johnson AW, Bentley DL. 2006. The role of Rat1 in coupling mRNA 3'-end processing to transcription termination: Implications for a unified allosteric-torpedo model. *Genes Dev* **20**: 954–965.
- Malagon F, Kireeva ML, Shafer BK, Lubkowska L, Kashlev M, Strathern JN. 2006. Mutations in the *Saccharomyces cerevisiae* *RPB1* gene conferring hypersensitivity to 6-azauracil. *Genetics* **172**: 2201–2209.
- Mandal SS, Cho H, Kim S, Cabane K, Reinberg D. 2002. FCP1, a phosphatase specific for the heptapeptide repeat of the largest subunit of RNA polymerase II, stimulates transcription elongation. *Mol Cell Biol* **22**: 7543–7552.
- Mason PB, Struhl K. 2005. Distinction and relationship between elongation rate and processivity of RNA polymerase II in vivo. *Mol Cell* **17**: 831–840.
- Mayer A, Lidschreiber M, Siebert M, Leike K, Soding J, Cramer P. 2010. Uniform transitions of the general RNA polymerase II transcription complex. *Nat Struct Mol Biol* **17**: 1272–1278.
- Ozsolak F, Kapranov P, Foissac S, Kim SW, Fishilevich E, Monaghan AP, John B, Milos PM. 2010. Comprehensive polyadenylation site maps in yeast and human reveal pervasive alternative polyadenylation. *Cell* **143**: 1018–1029.
- Powell W, Reines D. 1996. Mutations in the second largest subunit of RNA polymerase II cause 6-azauracil sensitivity in yeast and increased transcriptional arrest *in vitro*. *J Biol Chem* **271**: 6866–6873.
- Rasmussen EB, Lis JT. 1993. *In vivo* transcriptional pausing and cap formation on three *Drosophila* heat shock genes. *Proc Natl Acad Sci* **90**: 7923–7927.
- Schroeder SC, Schwer B, Shuman S, Bentley D. 2000. Dynamic association of capping enzymes with transcribing RNA polymerase II. *Genes Dev* **14**: 2435–2440.
- West S, Gromak N, Proudfoot NJ. 2004. Human 5'→3' exonuclease Xrn2 promotes transcription termination at co-transcriptional cleavage sites. *Nature* **432**: 522–525.