Dismantling Promoter-driven RNA Polymerase II Transcription Complexes in Vitro by the Termination Factor Rat1*

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Background: Rat1 is an exoribonuclease that functions in Pol II transcription termination.

Results: Rat1 releases stalled Pol II in vitro, and Rtt103 restores termination activity to an exonuclease-deficient Rat1 mutant.

Conclusion: Exonucleolytic activity is not the Rat1 function that is ultimately responsible for dislodging Pol II.

Significance: Understanding molecular details governing termination is key to understanding how it contributes to correct gene expression.

Proper RNA polymerase II (Pol II) transcription termination is essential to generate stable transcripts, to prevent interference at downstream loci, and to recycle Pol II back to the promoter (1–3). As such, termination is an intricately controlled process that is tightly regulated by a variety of different cis- and trans-acting factors (4, 5). Although many eukaryotic termination factors have been identified to date, the details of the precise molecular mechanisms governing termination remain to be elucidated. We devised an in vitro transcription system to study specific Pol II termination. We show for the first time that the exonucleolytic Rat1-Rai1 complex can elicit the release of stalled Pol II in vitro and can do so in the absence of other factors. We also find that Rtt103, which interacts with the Pol II C-terminal domain (CTD) and with Rat1, can rescue termination activity of an exonuclease-deficient Rat1 mutant. In light of our findings, we posit a model whereby functional nucleolytic activity is not the feature of Rat1 that ultimately promotes termination. Degradation of the nascent transcript allows Rat1 to pursue Pol II in a guided fashion and arrive at the site of RNA exit from Pol II. Upon this arrival, however, it is perhaps the specific and direct contact between Rat1 and Pol II that transmits the signal to terminate transcription.

The yeast RAT1 gene was initially identified by a temperature-sensitive allele, rat1-1, that resulted in accumulation of poly(A) RNA transcripts within the nucleus (6). This nuclear RNA retention phenotype has been observed in mutations in cleavage and polyadenylation factors (7, 8), thus linking Rat1 activity with mRNA 3′-end processing. Other studies revealed that Rat1 possesses a 5′-3′-exoribonuclease activity (9, 10) and participates in RNA polymerase II (Pol II)2 transcription termination downstream of poly(A) sites, as described by the so-called torpedo model of termination (11, 12). According to the torpedo model, cleavage of the nascent RNA at the poly(A) site by the cleavage and polyadenylation machinery generates a 5′-unprotected monophosphate that provides a point of entry for Rat1. In a manner reminiscent of the interaction of the helicase Rho with bacterial RNA polymerase (13), Rat1 travels toward Pol II by degrading the unprotected RNA still bound to the polymerase. Upon reaching the polymerase, Rat1 somehow serves as a molecular torpedo to dislodge Pol II, thereby effectively terminating transcription.

Other models of transcription termination have been posited to describe the role of Rat1 in this process. For example, the allosteric model assigns more emphasis to the modulation of Pol II activity via release and binding of elongation and termination factors, respectively, to the differentially phosphorylated Pol II CTD and does not provide a central role for Rat1 in the molecular events, leading to transcription termination (14). A hybrid model, however, combines elements of both the torpedo and allosteric models and provides Rat1 with an adjunct role in recruiting additional protein factors, such as the cleavage/polyadenylation protein Pcf11, in addition to its role in cotranscriptional degradation of RNA downstream of the cleavage site (12).

Rat1 is evolutionarily conserved, and its higher eukaryotic ortholog, Xrn2, is also involved in termination of Pol II transcription (15, 16). With the aid of its stabilizing partner Rai1, Rat1 can also gain access to 5′-triphosphate transcripts via the pyrophosphorolysis activity of Rai1 (17) or to capped transcripts via the decapping endonuclease activity of Rai1 (18). By this means, Rat1 is thought to participate in mRNA quality control by promoting early termination of Pol II that has synthesized uncapped transcripts (19). The Rat1-Rai1 complex can form a trimeric complex with Rtt103, which aids in the recruitment of Rat1 to the transcription complex through its CTD-interacting domain (CID) (11, 12, 20).

In addition to promoting Pol II termination, Rat1 is also required for termination of ribosomal precursor RNA transcribed by Pol I (21). To date, it remains unclear as to how exactly Rat1 dislodges RNA polymerases. To illuminate the

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2 The abbreviations used are: Pol II, RNA polymerase II; CTD, C-terminal domain; CID, C-terminal interacting domain; PIC, pre-initiation complex; SEC, stalled elongation complex; NTP, nucleoside triphosphate; exo-Rat1, exonuclease-deficient Rat1; Δ-CID Rtt103, Rtt103 lacking the CID.
molecular details of this yet elusive process, we generated stalled elongation complexes (SECs) from transcribing pre-initiation complexes (PICs) assembled on a natural Pol II promoter in yeast whole cell extract. We challenged the SECs with purified recombinant yeast Rat1 in complex with its activating partner Rai1, with or without its other partner, Rtt103. In addition we employed variants of Rat1 and Rtt103 to probe the specific contributions of these proteins to promote Pol II release. Whereas previous efforts to dislodge Pol II from a scaffolded transcription complex in vitro resulted in very little Rat1-mediated release (22), we show here for the first time that Rat1 can release stalled Pol II in vitro. Our results also support a model that additional aspects of Rat1, beyond its nucleolytic capabilities, are ultimately responsible for promoting transcription termination.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The strain yBC25 expressing the yRpb2-TAP plasmid (23) was a kind gift of Benoit Coulombe (Institut de Recherches Cliniques de Montréal). Yeast strains were grown in rich medium containing glucose and adenine (YPAD) at 30 °C. Rat1, Rai1, and Rtt103 ORF were amplified from yeast FY23 genomic DNA. The Rat1 ORF was cloned between the Nhel and Xhol sites of pET24b, resulting in a His$_6$ tag at the ORF C terminus. Site-directed mutagenesis of this recombinant expression plasmid was used to generate the exo-Rat1 variant (D235A). The Rai1 and Rtt103 ORFs were cloned between the Ndel and Xhol sites of pET24b. A stop codon was inserted immediately following the Rai1 ORF, so as to maintain an untagged version of Rai1. For the Δ-CID Rtt103 variant, the first 130 amino acids were deleted from the Rtt103 ORF. The Gal4-VP16 His$_6$ expression plasmid was a kind gift of Stephen Buratowski (Harvard Medical School).

Protein Purification—Recombinant Rat1(His$_6$), Rai1, Rtt103(His$_6$), and their variants were overexpressed in the Escherichia coli Rosetta expression strain via autoinduction overnight at 18 °C, as described previously by Studier (24). Rat1(His$_6$) and Rai1 extracts were mixed and were co-purified first with nickel affinity chromatography and then with heparin affinity chromatography. Rtt103(His$_6$) and its variant were purified first with nickel affinity chromatography and then with DEAE-ion exchange chromatography. Recombinant Gal4-VP16(His$_6$) was purified via nickel affinity chromatography.

Whole Cell Extract Preparation—Yeast strains were grown to an A$_{600}$ of 2–3, harvested, and resuspended in one volume of AGK buffer (20 mM HEPES-KOH, pH 7.9; 200 mM potassium chloride; 1.5 mM magnesium chloride; 20% glycerol; 0.5 mM DTT) supplemented with protease inhibitors (EDTA-free mixture, Roche Applied Science). Cells were frozen in droplets in liquid nitrogen and lysed with cryogrinding. The slurry was subsequently cleared with ultracentrifugation first for 30 min at 31,000 rpm and then for 1 h at 65,000 rpm in a TLA 100.3 rotor. Proteins in the cleared lysate were precipitated with 0.35 mg/ml finely ground ammonium sulfate with stirring on ice for 30 min. The ammonium sulfate pellet was collected with ultracentrifugation for 20 min at 31,000 rpm in a TLA 100.3 rotor and was carefully resuspended in 100 μl of dialysis buffer (20 mM HEPES-KOH, pH 7.9; 75 mM potassium acetate; 1.5 mM magnesium acetate; 20% glycerol; 1 mM DTT plus protease inhibitors) per ml of sample volume prior to ammonium sulfate precipitation. The resuspension was dialyzed against three exchanges of 500 ml dialysis buffer for 1 h each. The resuspension was cleared briefly in a tabletop microcentrifuge to remove any insoluble material, flash-frozen in liquid nitrogen, and stored in aliquots at −80 °C.

DNA Templates for Transcription Complex Formation—The DNA templates utilized in this study are based upon a circular template, pN1742, developed in the Kornberg laboratory (25) and kindly provided by Dr. Hampsey (University of Medicine and Dentistry of New Jersey). The ~1-kb linear template was PCR-amplified with a pair of primers, one of which is 5’-biotinylated, to include the following elements (listed in the 5’ to 3’ direction): three tandem Gal4 upstream activation sequences located 290 bp downstream of the biotin moiety; a CYC1 TATA element; and a 65-bp G-less cassette followed by a 460-bp downstream G-rich segment (Fig. 1A). To generate template 2 (Fig. 3A), a portion of the natively G-less CYC1 poly(A) sequence (40 bp) was inserted between the unique NcoI and SacI restriction sites located in the G-rich segment of the original circular pN1742 template. A unique PacI site was included in the 5’ region of this inserted segment. Site-directed mutagenesis was then used to alter the NcoI site to maintain the G-less segment up to the SacI site. Template 2 was amplified with the same primers used to produce template 1.

In Vitro Transcription Reactions—All steps were carried out at ambient temperature unless otherwise indicated. The buffers used are as follows: binding buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA; 1 mM NaCl; 0.003% Nonidet P-40); transcription buffer (50 mM HEPES-KOH, pH 7.5; 100 mM potassium glutamate; 10 mM magnesium acetate; 5 mM EGTA; 10% glycerol; 2.5 mM DTT); blocking buffer (transcription buffer, 5 mg/ml polyvinyl pyrrolidone, 0.1 mg/ml BSA); transcription wash buffer (transcription buffer, 0.005% Nonidet P-40); quench buffer (10 mM Tris-Cl, pH 7.9; 0.3 mM NaCl; 5 mM EDTA; 0.5% SDS).

For each 25-μl transcription reaction, 0.25 pmol of biotinylated promoter DNA template were coupled to 39 μg of streptavidin-coated magnetic Dyna beads for 30 min in binding buffer with gentle agitation. The beads were then blocked for 15 min with gentle agitation in blocking buffer. The blocked beads were washed three times in transcription wash buffer and resuspended to 5 mg/ml in transcription buffer.

The template was activated by incubation with 140 ng of recombinant Gal4-VP16 for 10 min. Simultaneously, 80 μg of whole cell extract were incubated with HaeIII-digested bacterial genomic DNA (26) for 10 min in transcription buffer. The activated template was combined with the whole cell extract, and the mixture was allowed to incubate for 40 min with gentle agitation to form PICs. Upon magnetic precipitation of the beads, the PICs were washed three times with transcription wash buffer and were resuspended in 25 μl of transcription buffer containing a G-less NTP pool (0.4 mM ATP, 0.4 mM CTP, 4 μM UTP, and 10 μM [α-32P]UTP (800 Ci/mmol)). The purified PICs + NTP mixture was incubated for 10 min at ambient temperature to form SECs. The SECs were washed as described above and were resuspended in 200 μl of quench buffer. In a subset of “chase” experiments, the SECs were incubated with a

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1 mM solution of all four NTPs for 10 min prior to quenching. As a negative control, α-amanitin (10 μg/ml) was added to PICs prior to incubation with the 1 mM NTP pool (including 10 μCi [α-32P]UTP). Proteinase K (100 μg) was added, and the reaction was incubated for 30 min at 30 °C. The radiolabeled RNA was precipitated with 20 μg of glycogen + 0.6 ml of 100% ethanol, and the ethanol-washed RNA pellet was resuspended in 80% formamide in standard 1× TBE buffer. The RNA products were elcrophoresed on an 8% polyacrylamide/7 M urea gel. RNA gels were dried, exposed to a phosphorimager intensifying screen, and analyzed with ImageQuant software.

Pol II Release Assay—For the release assays, purified SECs generated from 50-μl transcription reactions (double the amount described above) were resuspended in exonuclease buffer (20 mM Tris-Cl, pH 7.9; 50 mM sodium chloride; 10 mM magnesium chloride; 2.5 mM DTT). In a subset of experiments, the SECs were resuspended in transcription buffer instead of release buffer. The SECs were then incubated with 75 nM ribonuclease (Rat1-Rai1, exo-Rat1-Rai1, or RNase A) or with CTD-binding protein Rtt103 or Δ-CID Rtt103 variant. The reactions proceeded for 15 min at ambient temperature with gentle agitation. The “bound” (precipitated) and “released” (supernatant) complexes were fractionated by magnetic precipitation and analyzed by 8% SDS-PAGE. Protein gels were transferred to PVDF membranes, immunoblotted for Pol II CTD with the pan-CTD antibody 4H8 (Santa Cruz Biotechnology), and analyzed with Gene Systems software.

Transcription Complex Characterization—To probe the phosphorylation status of the Pol II CTD in the complexes, both PICs and SECs were immunoblotted with anti-hypophosphorylated CTD (8WG16, Covance), anti-Ser5-P CTD (H14, Covance), anti-Ser2-P CTD (3E10), and anti-Ser7-P CTD (4E12). The 3E10 and 4E12 antibodies were kind gifts of Dirk Eick via the Specific Phosphotyrosine Database. Blotting with an antibody specific for Ser5 phosphorylation reveals that only the higher molecular weight form is phosphorylated at Ser5 in the SECs (Fig. 1 right panel). This result indicates that our complexes undergo active phosphorylation of the Rpb1 CTD when the PICs are incubated with NTPs and allowed to extend to a stalled elongation complex. When we blotted with Ser5- and Ser7-specific phosphoantibodies, we did not detect any appreciable levels of phosphorylation of these CTD residues in our SECs (data not shown). Taken together, these results demonstrate that our in vitro transcription system behaves in a manner consistent with the known biochemistry of the CTD in the transcription cycle, namely that hypophosphorylated Pol II initiates transcription and that Ser5 is phosphorylated shortly after Pol II promoter escape (28, 29).

Nascent Complex-bound Transcripts Are Sensitive to Rat1-Rai1 in a Salt-dependent Manner—To elucidate the mechanism by which Rat1 elicits transcription termination, we overexpressed and co-purified recombinant Rat1 with its activating partner Rai1 (Fig. 2A). We first tested the ability of the Rat1-Rai1 complex to digest and/or promote release of RNA transcripts bound to SECs assembled on template 1. In these assays, the Rat1-Rai1 complex is present in a 1000-fold molar excess of SECs. Although we have not explicitly determined whether any fraction of the purified Rat1-Rai1 complex is inactive, we assume that we are working at saturating levels of Rat1-Rai1 in our system, given the extent of observed transcript degradation (see text below; Fig. 2B). After 15 min of incubation, we separated the bead-bound fractions from the superna-
tant fractions via magnetic precipitation (Fig. 2B). Transcripts located in the bead-bound fraction are associated with the DNA template and hence are still held in the transcription complex. Transcripts located in the supernatant fraction are considered to be released from the transcription complex.

We first incubated SECs with the Rat1-Rai1 complex in transcription buffer, which is a glutamate-based buffer (Fig. 2B, left panel). However, only ~50% of the template-bound RNA was degraded. To improve the efficiency of Rat1-mediated degradation, we took into account several factors. First, early in vitro work with purified Rat1 indicated that Rat1 exonucleolytic activity was sensitive to salt conditions (9). Additionally, studies of transcript cleavage via endogenous or factor-mediated E. coli RNA polymerase hydrolysis activity suggested that the identity and concentration of the counter anion of a buffer influenced the efficiency of hydrolysis (30). For example, chloride counter anions greatly enhanced RNA cleavage over glutamate counter anions. Additionally, the hydrolysis reaction was more favorable under lower salt concentrations. Based on the findings of these studies, we changed to a low ionic strength, chloride-
based buffer (referred to as exonuclease buffer) and found that Rat1 degraded the SEC-bound transcript to near completion (Fig. 2B, right panel). Furthermore, no RNA was released in reactions that lacked Rat1/H18528Rai1, indicating that the SECs are stable under the digestion conditions. Because the counter cation is potassium in the transcription buffer and sodium in the exonuclease buffer, we repeated the transcript degradation experiment in a potassium-based exonuclease buffer and achieved identical results to those obtained in the original sodium-based exonuclease buffer (data not shown).

Catalytically Active Rat1 Is Able to Dislodge Stalled Pol II Transcription Complexes in Vitro—Due to the fact that Rat1 efficiently degrades Pol II-associated RNA in our system, we cannot use the release of transcript as an indicator of terminated transcription complexes. Instead, we assayed for the release of Pol II itself by probing with an antibody that recognizes all forms of CTD phosphorylation or hypophosphorylation. We first tested the ability of Rat1/H18528Rai1 to release Pol II in the glutamate and chloride buffers (Fig. 2C). Negligible amounts of release are observed in the mock reactions lacking Rat1-Rai1. C, Western blot analysis of buffer effects on Rat1-mediated Pol II release. Pol II complexes present on template 1 after transcription in the absence of GTP were challenged with 75 nM Rat1/H18528Rai1 complex in the indicated buffers. The bound (B) and released (R) fractions were resolved on an 8% SDS-polyacrylamide gel, transferred to a PVDF membrane, and blotted with the anti-pan-CTD antibody 4H8. D, Western blot of Pol II pre-initiation (left four lanes) and post-transcription (right four lanes) complexes challenged with Rat1-Rai1. Pol II is examined as described in C. The percent of released Pol II after Rat1-Rai1 treatment is indicated.
presence of glutamate, these results suggest that fully functional Rat1 nucleolytic activity is essential to its ability to dissolve a stalled transcription complex.

In light of the evidence that susceptibility to RNA degradation correlated with release of stalled Pol II (Fig. 2, B and C), we next asked whether RNA-free Pol II could be released from DNA in the presence of the Rat-Rai1 complex. We thus tested the susceptibility of pre-initiation complexes to Rat1-mediated termination activity and compared this result with the release of Pol II from post-transcription complexes. When pre-initiation complexes were challenged with the Rat1-Rai1 complex, we detected very little release of Pol II (Fig. 2D). The extent of pre-initiated Pol II release in the presence of Rat1 (~5%) is typical of what we observe in a mock post-transcription release reaction in the absence of Rat1. When pre-initiated Pol II molecules were allowed to transcribe in the absence of GTP and form post-transcription complexes, the Pol II complexes became 5-fold more sensitive to Rat1-mediated activity (Fig. 2D). We conclude that only Pol II complexes that have engaged in active transcription are competent for release.

Mapping the Distribution of Pol II Complexes along the DNA Template—The results in Fig. 2 suggested that although Rat1 efficiently degrades complex-bound transcripts, a high percentage of the observable Pol II population appeared to remain unsusceptible to release in the presence of Rat1. Given that pre-initiated Pol II molecules were insensitive to Rat1 (Fig. 2D), we next asked what percentage of the observable Pol II remains promoter-bound upon incubation with NTPs. To determine the distribution of Pol II along the DNA template, we formed stalled transcription complexes on template 2 (Fig. 3A). This template is similar to template 1 (Fig. 1A), except that the G-rich cassette was extended in the 3’ direction by 40 bp and a portion of the G-rich segment was removed during cloning to accommodate the G-less extension. At the junction of this extension and the original G-less cassette is a unique PacI site, which when cut, results in 420-bp and 555-bp products (Fig. 3B).

Upon forming transcription complexes on template 2, we digested the Pol II-bound template with the restriction endonuclease PacI, separated the bead-bound fractions from the supernatant fractions via magnetic precipitation, and probed for Pol II with the pan-CTD antibody (Fig. 3C). Pol II complexes that were engaged in productive elongation and have traversed the PacI site appeared in the supernatant fraction, whereas those that remained promoter-bound were found in the bead-bound fraction (Fig. 3C). The results of Fig. 3C indicated that ~40% of the total Pol II escaped the promoter and reached the stall site at the end of the G-less cassette. To be sure that transcription complexes assembled on template 2 behaved in the same manner when assembled on template 1, we challenged template 2 complexes with Rat1-Rai1 complex and found the same extent of Rat1-mediated release (Fig. 3D).

We conclude that only about half of the observable Pol II population in our elongation/stalling assays have engaged in active transcription and are thus competent for release in the presence of Rat1. This result has consequences for the analysis of the extent of Pol II release. For example, in Fig. 2D, the apparent extent of Pol II release is 25%. However, when factoring in the fraction of Pol II that is competent for release, the actual Rat1-mediated release of Pol II is closer to 50%.

Rtt103 Restores Termination-inducing Capabilities to an Exonucleolytically Deficient Rat1 Mutant in a CTD-dependent Manner—To explore further the nature of the termination capabilities of Rat1, we next asked whether a variant of Rat1 containing a point mutation at an evolutionarily conserved catalytic residue (D235A) can also release stalled Pol II. Denoted as exo- Rat1, this mutant is reported to be defective in nuclease activity in vitro and insufficient for mediating transcript termination in vivo (11, 22). In our release assay, exo-Rat1, in complex with Rai1 (Fig. 4A, left panel), is incapable of dismantling stalled transcription complexes. A representative Pol II immunoblot is shown in Fig. 4B, and quantification of repeats of these experiments is shown in Fig. 4F. This result indicates that Rat1 catalytic activity is indeed important in mediating termination. We also examined the elongation complex-associated transcripts during incubation with the two Rat1 species (Fig. 4E). Whereas degradation of the transcript occurs in the presence of wt Rat1-Rai1, the transcript is largely intact in the presence of exo-Rat1-Rai1.

We then explored the possibility that complete transcript decay is sufficient for Pol II release in our system. To test this idea, we challenged stalled complexes with RNase A and observed no release of Pol II relative to the mock reaction (Fig. 4, B and F). This result is in agreement with earlier studies demonstrating that ribonucleolytic activity alone is insufficient to dislodge stalled transcription complexes in vitro or in vivo (12, 31, 32). Together, these results indicate that specific, fully functional Rat1 activity is necessary for Pol II release and hence transcription termination. However, there may be other functional aspects of Rat1 in addition to its nucleolytic capabilities that contribute to efficient termination because the nonspecific degradation of the transcript does not promote Pol II release.

The experiments presented above allowed us to explore the specific contribution of the Rat1-Rai1 complex to transcription termination in our defined system. The Rat1-Rai1 complex can form a trimeric complex with the Rtt103 protein (11). However, Rtt103 does not enhance the catalytic activity of Rat1 in vitro (22), and Rtt103 is dispensable for termination in vivo (11). Therefore, a role for Rtt103 in Rat1 function has not been defined. Because Rtt103 interacts directly with the CTD of Pol II through its CID (11, 12, 20), it could aid in the recruitment of Rat1 to the transcription complex.

To test the effect of Rtt103 in our in vitro release assay, we first overexpressed and purified Rtt103 (Fig. 4A, middle panel). We then challenged stalled complexes on template 1 (Fig. 4C) with recombinant Rtt103 alone, or in complex with Rat1-Rai1 or with exo-Rat1-Rai1. Transcription complexes show only the background level of release in the presence of Rtt103 alone (Fig. 4, C and Fig. F). Additionally, the trimeric Rat1-Rai1-Rtt103 complex does not enhance Pol II release when compared with that seen with the dimeric Rat1-Rai1 complex (Fig. 4, C and F). Surprisingly, when Rtt103 is added to the exonucleolytically deficient Rat1 complex, the level of Pol II release from template 1 is comparable with that of wild-type Rat1-Rai1-mediated release (Fig. 4, C and F). In other words, Rtt103 restores termi-
nation activity to the exo-Rat1 mutant, which by itself is incapable of dissolving stalled transcription complexes (Fig. 4).

We next sought to elucidate the mechanism by which Rtt103 restores the termination activity of exo-Rat1. Because the Rtt103 CID is known to interact with the Pol II CTD, we removed the CID from Rtt103 (Δ-CID Rtt103, Fig. 4A, right panel) and tested the ability of this Rtt103 variant to restore exo-Rat1-mediated termination. When the CID is deleted, Rtt103-mediated recovery of exo-Rat1 activity is greatly reduced (Fig. 4, D and E). This result suggests that the Pol II CTD interaction provided by Rtt103 facilitates transcription termination by the exo-Rat1 mutant. To explore the possibility that Rtt103 enhances exo-Rat1 catalytic activity, we examined the transcripts for enhanced degradation when Rtt103 is added to the exo-Rat1-Rai1 complex. However, Rtt103 does not stimulate transcript degradation by exo-Rat1 (Fig. 4E, compare lanes 3 and 6). Therefore, the restored termination capability of exo-Rat1 is not due to recovered exonucleolytic activity of this mutant. As expected, Rtt103 itself does not exhibit any nuclease activity (Fig. 4E, lane 4).

**DISCUSSION**

In this work, we have described an *in vitro* transcription system that can be employed to explore the poorly understood molecular mechanisms governing Pol II transcription termination. Specifically, we have interrogated the details surrounding
Rat1-mediated release of promoter-driven Pol II transcription complexes stalled at the end of a G-less cassette. We have found that Rat1, in complex with Rai1, can degrade nascent transcripts associated with these promoter-initiated complexes and promote release of Pol II, thereby dissolving stalled transcription complexes. This termination effect is dependent on fully functional Rat1 exonucleolytic activity, as an exonuclease-deficient Rat1 mutant (exo-Rat1) is incapable of promoting Pol II release. Additionally, transcript degradation alone is insufficient to facilitate termination, as the Pol II complex remains intact upon challenge with RNase A. Our findings differ from those of a previous study, which found that Pol II scaffolded complexes challenged with Rat1-Rai1 or RNase A, with or without different forms of Rtt103. Exonuclease-deficient transcription complexes are incubated with the indicated recombinant proteins. RNA products, indicated by arrows, are separated on a 7 M urea/8% polyacrylamide gel. Exonuclease-deficient transcription complexes are incubated with the indicated recombinant proteins. RNA products, indicated by arrows, are separated on a 7 M urea/8% polyacrylamide gel. F. Quantification of chemiluminescent band intensities from immunoblots such as those displayed in A, C, and D. The fraction of Pol II released from template 1 is plotted for the indicated conditions and represents an average of two to five experiments. All Western blots (B, C, and D) are performed with the 4H8 antibody.

FIGURE 4. Catalytically active Rat1 is able to dislodge stalled Pol II transcription complexes in vitro. A, purified recombinant exo-Rat1-Rai1 complex (left panel) is shown on an 8% SDS-polyacrylamide gel stained with silver. Purified recombinant wt Rtt103 (middle panel) and Δ-CID Rtt103 (right panel) are shown on 12% SDS-polyacrylamide gels stained with silver. B, Western blot of Pol II elongation complexes stalled on template 1 and challenged with Rat1-Rai1, exo-Rat-Rai1, or RNase A. C, Western blot of Pol II elongation complexes challenged with Rat1-Rai1 or exo-Rat-Rai1, with or without Rtt103. D, Western blot of Pol II elongation complexes challenged with exo-Rat-Rai1, with or without different forms of Rtt103. E, nascent transcripts bound to Pol II elongation complexes are incubated with the indicated recombinant proteins. RNA products, indicated by arrows, are separated on a 7 M urea/8% polyacrylamide gel. F, quantification of chemiluminescent band intensities from immunoblots such as those displayed in A, C, and D. The fraction of Pol II released from template 1 is plotted for the indicated conditions and represents an average of two to five experiments. All Western blots (B, C, and D) are performed with the 4H8 antibody.

Rat1-mediated release of promoter-driven Pol II transcription complexes stalled at the end of a G-less cassette. We have found that Rat1, in complex with Rai1, can degrade nascent transcripts associated with these promoter-initiated complexes and promote release of Pol II, thereby dissolving stalled transcription complexes. This termination effect is dependent on fully functional Rat1 exonucleolytic activity, as an exonuclease-deficient Rat1 mutant (exo-Rat1) is incapable of promoting Pol II release. Additionally, transcript degradation alone is insufficient to facilitate termination, as the Pol II complex remains intact upon challenge with RNase A. Our findings differ from those of a previous study, which found that Pol II scaffolded complexes challenged with Rat1 were resistant to release from the DNA template (22). Scaffolded complexes are synthesized by first annealing an RNA oligonucleotide to a single-stranded DNA template, by next binding Pol II to these hybridized oligonucleotides, and by then binding the single-stranded non-templated DNA to the Pol II-nucleic acid complex. Perhaps the fundamental properties of scaffolded complexes and of promoter-driven complexes are wholly different, especially when the scaffolded complexes undergo no, or at most a single, NTP incorporation event. This hypothesis may explain why our promoter-derived Pol II complexes (and only those populations that have actually engaged in active elongation) are sensitive to the effects of Rat1.

In comparing the two complexes, we must also consider that promoter-driven complexes may contain multiple transcription-associated proteins not present on scaffolded complexes formed from purified Pol II and that these proteins could assist Rat1-mediated Pol II release. Recent studies have shown that two proteins linked to termination are enriched in pre-initiation complexes assembled in vitro (33, 34). One is Sub1, which is thought to repress the termination activity of cleavage factor IA (35), and thus unlikely to promote release in our assay. The other is the mediator complex subunit, Srb5/Med18, which stimulates termination on a subset of yeast genes (36). Given the physical and functional linkages that have been established between the promoter and terminator regions of mRNA encod-
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ing genes (37), there may be other initiation factors with similar dual, but as yet unrecognized, functions. A strength of our in vitro assay is that it will allow rapid and direct analysis of stalled complexes assembled from extract defective for proteins such as Srb5/Med18.

In this study, we also examined the role of Rtt103, a protein that copurifies with the Rat1-Rai1 complex and also binds the Pol II CTD (11). Comparison of Pol II initiation complexes and Pol II elongation complexes demonstrates that the CTD is actively phosphorylated upon promoter escape in our in vitro system. It has been suggested that, by binding to hyperphosphorylated CTD, Rtt103 can recruit Rat1 to the transcription complex, thereby facilitating Rat1-mediated termination (11). However, we detect no enhancement of wild-type Rat1-mediated Pol II release in the presence of Rtt103. This result is consistent with the observations that Rtt103 is not essential for Pol II termination (11) and that there is probably redundancy in the contacts that help recruit Rat1 to the elongation complex (12, 20). As is clear from our in vitro assay, by following the trail of an unprotected nascent transcript to the site of RNA exit on Pol II, Rat1 can reach and dislodge Pol II without the assistance of CTD-bound Rtt103. Under typical in vivo conditions, Rat1 may perhaps choose this canonical route of transcript degradation to locate Pol II and to effect transcription termination.

What then is the function of Rtt103? The answer to this question may in fact shed light on how Rat1 disrupts transcription upon reaching Pol II. Our experiments indicate that the presence of Rtt103 can restore Rat1-mediated termination capability to an exonuclease-deficient Rat1 mutant, exo-Rat1, in a manner dependent on the Rtt103 CTD interaction domain. However, this restoration is not at the level of enhanced Rat1 catalytic activity. Because exo-Rat1 cannot degrade the nascent transcript, it therefore has no guided path to pursue a transcribing Pol II molecule. Rtt103 appears to allow exo-Rat1 to bypass this unavailable route by directing exo-Rat1 to the CTD, where Rtt103 is presumably anchored. Previous work has suggested that CTD-binding alone, even when the CTD-binding molecule is the 8WG16 CTD-specific antibody, is sufficient to promote full termination of in vitro tailed Pol II transcription complexes (38). However, our work indicates that CTD binding alone is insufficient to effect termination of promoter-initiated Pol II but may instead function primarily to recruit active termination factors. Because the CTD is located in the vicinity of the RNA exit channel (39), CTD-bound Rtt103 may facilitate the access of exo-Rat1 to the natural nucleolytic end point of wild-type Rat1, the site of transcript extrusion from Pol II. This model strongly suggests that features of Rat1, other than its nucleolytic activity, are ultimately responsible for triggering Pol II release. Degradation of the nascent transcript therefore is simply a means to an end. Work in the field of bacterial transcription has provided a precedent for factors eliciting termination by directly contacting RNA polymerase in the vicinity of the RNA exit channel (40, 41). In a similar fashion, Rat1 may also make specific contacts with Pol II that promote efficient dissolution of the transcription complex. The mechanistic insights gained in this work bolster the value of employing defined in vitro systems to elucidate the molecular details of transcription termination.

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