**Torpedo Nuclease Rat1 Is Insufficient to Terminate RNA Polymerase II in Vitro**

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Termination of RNA polymerase (pol) II transcription in vivo requires the 5′-RNA exonuclease Rat1. It was proposed that Rat1 degrades RNA from the 5′-end that is created by transcript cleavage, catches up with elongating pol II, and acts like a *Torpedo* that removes pol II from DNA. Here we test the *Torpedo* model in an *in vitro* system based on bead-coupled pol II elongation complexes (ECs). Recombinant Rat1 complexes with Rai1, and with Rai1 and Rtt103, degrade RNA extending from the EC until they reach the polymerase surface but fail to terminate pol II. Instead, the EC retains an ~18-nucleotide RNA that remains with its 3′-end at the active site and can be elongated. Thus, pol II termination apparently requires a factor or several factors in addition to Rat1, Rai1, and Rtt103, post-translational modifications of these factors, or unusual reaction conditions.

Transcription termination involves the dissociation of template DNA from RNA polymerase (pol) that is engaged in an elongation complex (EC). Termination is important for proper gene expression, because defects lead to interference of nonterminated ECs with the initiation events of downstream genes (1, 2). In contrast to pol I and pol III, pol II does not terminate transcription at a specific sequence at the end of a gene (reviewed in Refs. 3, 4). Instead, termination sites seem to be randomly located up to 1 kb downstream of the poly(A) site (Ref. 5 and references therein), where 3′-processing of the transcript is conducted by factors that interact with the C-terminal domain of pol II (6).

Two different models for pol II termination were suggested. The “anti-terminator model” postulates that binding of 3′-processing factors induces a change in the EC that recruits a termination factor or displaces an anti-termination factor (7). The “*Torpedo* model” was suggested in 1988 (5, 8) and postulates that the 5′-end of the RNA that is generated after poly(A) site-dependent cleavage is used as a substrate by a nuclease, which catches up with elongating pol II and dissociates the polymerase from DNA.

There is evidence for the *Torpedo* model and for Rat1 being the *Torpedo* nuclease. pol II shows termination defects in yeast cells that are deficient in Rat1 or lack Rai1 (9), a factor that binds Rat1 in vivo and stabilizes the Rat1 exonuclease activity in vitro (10). Similar termination defects occur in mammalian cells after knockdown of the Rat1 homolog Xnr2 (11, 12). Rat1-Rai1 localizes to 3′-regions of genes together with Rtt103, a protein that contains a C-terminal domain-interacting domain and is likely involved in Rat1 recruitment to ECs (9). The gene encoding Rat1 is essential and was identified in genetic screens for mutants in nuclear export (13) and transcription initiation by RNA polymerase III (14).

Rat1 is a nuclear exoribonuclease that processively degrades RNA from the 5′-end and is similar in sequence and activity to the cytoplasmic nuclease Xrn1 (15–17). Xrn1 was the first 5′–3′-exoribonuclease characterized in yeast (18–21). Xrn1 can rescue the temperature-sensitive growth phenotype of a mutation in Rat1 (rat1-I-1) when directed to the nucleus (22) but does not complement the termination defects (23). pol II transcription of small nuclear RNAs, small nucleolar RNAs, and cryptic unstable transcripts is terminated by a different mechanism (24), but termination of pol I transcription is also dependent on Rat1–Rai1 activity (25).

Some evidence argues against the *Torpedo* model. Pol(A) site-induced cleavage of the transcript does not seem to be prerequisite for termination (26–30). RNA immunoprecipitation suggested that Rat1 and Xrn2 are responsible for co-transcriptional degradation of RNA 3′ of the pol(A) site but not for termination (23). ECs that are treated with RNase remain stable and capable of elongation (31), showing that degradation of the RNA emanating from the polymerase is insufficient to terminate transcription. Conventionally, it is unclear how a nuclease, even if it is processive, could exert a force that would be sufficient to disrupt the highly stable EC. The *Torpedo* model could thus far not be tested directly, because of a lack of a pure and defined biochemical *in vitro* system.

To test the *Torpedo* termination model, we established a defined *in vitro* pol II elongation system and prepared recombinant, highly purified Rat1 and its complexes Rat1-Rai1 and Rat1-Rai1-Rtt103. We show that the latter two Rat1 complexes degrade RNA in ECs from the 5′-end until they reach the pol II surface but do not induce termination. Instead, the EC remains intact with the RNA 3′-end at the active site. Thus, the recombinant Rat1 complexes contain nuclease activity but do not trigger release of DNA and RNA from pol II *in vitro*. These data suggest that an additional factor or additional factors, post-translational modification of Rat1, Rai1, and/or Rtt103, or reac-

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The atomic coordinates and structure factors (code 3H3V) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://wwpdb.org/).

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2 The abbreviations used are: pol, polymerase; EC, elongation complex; nt, nucleotide; DTT, dithiothreitol.
tion conditions very different from those in our in vitro assays, are required for efficient pol II termination in vivo.

**EXPERIMENTAL PROCEDURES**

Production of Recombinant Exonuclease Complexes—Coding sequences for Rat1, Rai1, and Rtt103 were amplified from *Saccharomyces cerevisiae* genomic DNA by PCR and cloned into pET expression vectors (pET21b with Amp' and a C-terminal hexahistidine tag for Rat1 and Rtt103 and pET24b with Kana' untagged for Rai1, Novagen). Site-directed mutagenesis was carried out with the overlap extension method. Plasmids were used for transformation of *Escherichia coli* BL21-CodonPlus (DE3)RIL cells (Stratagene). Expression was carried out using autoinducing medium (32). After cell lysis by sonification, cell extracts were cleared by centrifugation (two times for 20 min at 24,000 × g), and supernatants were applied to nickel-nitrilotriacetic acid column equilibrated with 50 mM Tris/HCl, pH 8.0, at 4 °C, 100 mM NaCl, 1 mM MgCl2, and 5 mM DTT. Bound protein was eluted with a salt gradient containing recombinant Rat1 or Rat1-Rai1 were pooled and used for transformation of *Escherichia coli* BL21-CodonPlus (DE3)RIL cells (Stratagene). Impurities were removed by centrifugation (two times for 20 min at 24,000 × g), and supernatants were applied to nickel-nitrilotriacetic acid-agarose (Qiagen) equilibrated with 50 mM Tris/HCl, pH 8.0, at 4 °C, 100 mM NaCl, 5 mM DTT. Bound protein was eluted with a salt gradient from 100–1000 mM NaCl. Pure proteins were applied to a Superose 6 10/300 GL size-exclusion chromatography column (GE Healthcare) equilibrated with RNase-free 50 mM Tris/HCl, pH 8.0, at 4 °C, 200 mM NaCl, 2 mM MgCl2, 2 mM DTT. In case of the Rat1-Rai1-Rtt103 complex, pure Rat1-Rai1 was incubated with a 2-fold molar excess of partially purified Rtt103 (elutions from the nickel-nitrilotriacetic acid column) for 30 min at 20 °C and purified by size-exclusion chromatography. Pure recombinant proteins were flash-frozen in liquid nitrogen in 25 mM Tris/HCl, pH 8.0, at 4 °C, 100 mM NaCl, 1 mM MgCl2, 1 mM DTT, 10% (v/v) glycerol, and stored at −80 °C.

**RNase Activity Assay**—For RNase assays, a 27-nt RNA (5'-UCCCAAUAAUAGCUAAGACCAGGC-3') was labeled at the 3'-end using T4 RNA ligase (Fermentas) and [32P]2'-deoxyctydine-3',5'-diphosphate at a concentration of 3 µCi/ pmol RNA in ligase buffer provided by the producer. We did not use RNA oligonucleotides with a 5'-monophosphate modification because this gave rise to multimers of RNA molecules due to the activity of T4 RNA ligase. Excess radioactive nucleotides were removed with MicroSpin™ G-25 columns (GE Healthcare), and 3'-end-labeled RNA was used as a substrate for 5'-end digestion. 3 pmol of labeled RNA were mixed with an equimolar amount of pure Rat1-Rai1 or Rat1-Rai1-Rtt103 in reaction buffer (20 mM Tris/HCl, pH 8.0, at 30 °C, 150 mM NaCl, 2 mM MgCl2, 1 mM DTT), in a total volume of 30 µL and incubated at 30 °C. After 30, 60, and 180 min, 9 µL of each sample was removed, and the reaction was stopped by mixing with an equal volume of RNA loading buffer (20% (v/v) 10× TBE, 8 M urea, 0.03% (w/v) bromphenol blue, 0.03% (w/v) xylene cyanol FF) and incubating at 95 °C for 5 min. The samples were analyzed by denaturing PAGE containing 8 M urea. Remaining RNA was detected by autoradiography, and the relative amounts were quantified with ImageQuant. Radioactive nucleotides were added to a storage phosphor screen (GE Healthcare) for several hours, and scanning of storage screens was carried out with a STORM 860 imaging system (GE Healthcare). All experiments were carried out in triplicate.

Termination Assay on Beads—pol II core complex from *S. cerevisiae* was purified as described (33) except that the anion exchange step was omitted. Transcription-competent elongation complexes, containing a fully complementary transcription bubble, were assembled as described (34). Equimolar amounts of template DNA and RNA were mixed in RNase-free TE buffer. The oligonucleotides were annealed by heating to 95 °C for 2 min and slowly cooling to room temperature. pol II was incubated with a 2-fold molar excess of the annealed hybrid for 15 min at 20 °C while shaking. A 4-fold molar excess of non-template DNA, containing biotin at the 5'-end, was added, and the mixture was incubated for 20 min at 25 °C while shaking. Control elongation complexes, lacking the biotinylated non-template strand, were assembled identically, omitting the last step. The complete 12-subunit pol II was purified as described (35). Recombinant Rpb4/7 was purified as described (35) and added to the sample at a 5-fold molar excess to avoid dissociation. 1–3 pmol of the assembled elongation complexes were bound to magnetic, streptavidin-coated beads (Dynabeads® MyOne™ streptavidin T1, Dynal Biotech, distributed by Invitrogen). Beads were prepared by washing twice with beads breaking buffer (50 mM Tris/HCl, pH 8.0, at 25 °C, 150 mM NaCl, 0.1% (w/v) Triton X-100, 5% (w/v) glycerol 0.5 mM DTT) followed by incubation in 500 µL of beads blocking buffer (50 mM Tris/HCl, pH 8.0, at 25 °C; 150 mM NaCl, 2 mM EDTA, pH 8.0, 0.1% (w/v) Triton X-100, 5% (w/v) glycerol, 0.5% (w/v) bovine serum albumin, 200 µg/ml insulin, 0.1 mg/ml heparin, 0.5 mM DTT) overnight at 4 °C. After washing again twice with breaking buffer, beads were resuspended in the original volume of breaking buffer. 1–3 pmol of elongation complexes were added per reaction (~10 µL of blocked and washed beads) followed by an incubation for 30 min at 25 °C and gently shaking. Unbound complexes were removed by washing with beads breaking buffer, washing buffer (20 mM Tris/HCl, pH 8.0, at 30 °C, 500 mM NaCl, 2 mM MgCl2, 1 mM DTT), and reaction buffer. Beads were resuspended in 19 µL of reaction buffer. RNA in the elongation complex was labeled at the 3'-end with the use of pol II activity, by adding 1 µL of [α-32P]UTP (10 mCi/ml), followed by an incubation at 28 °C for 20 min, and slowly shaking. Unincorporated nucleotides were washed away by applying 50 µL of reaction buffer two times. Beads were resuspended in reaction buffer, and nuclease complexes were added at a 2-fold molar excess over the elongation complex concentration used for binding to the beads, followed by incubation for 1 h at 30 °C. As a single strand-specific control nuclease, *RnaseF* (100 units, catalog number M0243S, New England Biolabs) was used. After the reaction, nuclease was washed away with washing buffer and reaction buffer. Beads were resuspended in 20 µL of reaction buffer. To test the ability of ECs to elongate the transcript after nuclelease digestion, beads were resuspended in 19 µL, and 1 µL of NTP mix was added at a concentration of 1 mM and incubated at 28 °C for 30 min. The reactions were stopped by adding 20 µL of 2× loading buffer and incubating for 5 min at 95 °C. Samples were analyzed by 6 M urea-PAGE, and radioactively gels were exposed to a storage phosphor screen (GE Healthcare) for several hours, and scanning of storage screens was carried out with a STORM 860 imaging system (GE Healthcare). All experiments were carried out in triplicate.
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**TABLE 1**

X-ray diffraction data and refinement statistics for the complete Pol II EC containing a poly(A)site sequence within the DNA-RNA hybrid

| Data collection | Source | Value |
|-----------------|--------|-------|
| Space group     | C222(1)|       |
| Unit cell axes lengths | 222.5, 391.6, 284.1 Å |       |
| Wavelength      | 0.918905 Å |       |
| Resolution      | 50 to 4.0 Å |       |
| \(R_{\text{sym}}\) | 10.6% (51.6%) |       |
| \(I/\sigma\)    | 9.6 (2.7) |       |
| Completeness    | 99.9% (100%) |       |
| Redundancy      | 4.3 (4.3) |       |

**Refinement**

| Resolution | 50-4.0 Å |       |
| Unique reflections (anomalous pairs unmerged) | 202,368 (33,630) |       |
| No. of atoms | Protein: 31,102, Ions: 9, Nucleic acids: 671, B-factors (Å²): Protein: 137.1, Ions: 137.8, Nucleic acids: 196.8 |       |
| Root mean square deviations | Bond lengths: 0.008 Å, Bond angles: 1.5° |       |

Rat1 activity requires Rai1 but not Rtt103—The substrate specificity of Rat1 and the homologous cytoplasmic Xrn1 have been studied extensively (16–19, 53). Both enzymes prefer RNA substrates with a 5′-hydroxyl group with radioactive phosphate (see “Experimental Procedures”). The Rat1-Rai1 complex was very stable, and could be purified over several columns. To purify a recombinant trimeric Rat1-Rai1-Rtt103 complex, we incubated the pure Rat1-Rai1 complex with partially purified recombinant full-length Rtt103 and subjected the mixture to size-exclusion chromatography. We obtained a symmetric peak for the trimeric complex at a shorter retention than the peak for the dimeric Rat1-Rai1 complex (Fig. 1B). The identities of the three proteins were confirmed by mass spectrometry (data not shown). This work provided Rat1 and the complexes Rat1-Rai1 and Rat1-Rai1-Rtt103 in a pure, bacterially expressed, and recombinant form. The results also confirmed the protein-protein interactions that were previously inferred from co-purifications of endogenous proteins (9, 16) and co-expression of Rat1 and Rai1 in yeast (10, 38).

**RESULTS**

Preparation of Recombinant Rat1 Complexes—To investigate the function of the Rat1 nuclease, we established a protocol to obtain the protein in recombinant form after overexpression in *E. coli* (Fig. 1, A and B, and see under “Experimental Procedures”). After optimization of the procedure, about 0.5 mg of the 116-kDa, 1006-residue Rat1 protein could be obtained from 1 liter of bacterial cell culture. We further established a protocol to obtain a stoichiometric pure complex of Rat1 with its cellular partner, Rai1 (Fig. 1, and see under “Experimental Procedures”). We co-expressed Rat1 and Rai1 from individual plasmids with different antibiotic resistance in *E. coli* cells with the use of autoinducing medium (32).

Affinity purification of Rat1, which carried a C-terminal hexahistidine tag, led to co-purification of Rai1 (Fig. 1, and see under “Experimental Procedures”). The Rat1-Rai1 complex was very stable, and could be purified over several columns. To purify a recombinant trimeric Rat1-Rai1-Rtt103 complex, we incubated the pure Rat1-Rai1 complex with partially purified recombinant full-length Rtt103 and subjected the mixture to size-exclusion chromatography. We obtained a symmetric peak for the trimeric complex at a shorter retention than the peak for the dimeric Rat1-Rai1 complex (Fig. 1B). The identities of the three proteins were confirmed by mass spectrometry (data not shown). This work provided Rat1 and the complexes Rat1-Rai1 and Rat1-Rai1-Rtt103 in a pure, bacterially expressed, and recombinant form. The results also confirmed the protein-protein interactions that were previously inferred from co-purifications of endogenous proteins (9, 16) and co-expression of Rat1 and Rai1 in yeast (10, 38).
showed weak, badly reproducible activity. In contrast, the recombinant Rat1-Rai1 complex showed reproducible and stable nuclease activity. This is consistent with the published stabilizing effect of Rai1 on Rat1 activity (10, 52). The complex could be stored at −80 °C for several months without significant decrease of activity.

We next compared the robust nuclease activity of the recombinant Rat1-Rai1 complex to that of the trimeric Rat1-Rai1-Rtt103 complex. The dimeric and trimeric complexes had indistinguishable activities (Fig. 1C). This suggested that Rtt103 is not a regulator of Rat1 activity, consistent with the results that Rtt103 is involved in the recruitment of Rat1-Rai1 to the transcription machinery via its C-terminal domain-interacting domain (9). To test if the observed nuclease activity stemmed from Rat1, and not from a possible impurity, we prepared a point mutant of recombinant Rat1 that was previously shown to compromise Rat1 nucleolytic activity (D235A, see Ref. 9). As expected, purified samples of the mutant showed defective nuclease activity (data not shown).

**An Improved in Vitro Elongation Assay**—To establish a defined biochemical system for testing the *Torpedo* model *in vitro*, we assembled ECs containing pol II, DNA with a fully complementary transcription bubble, and RNA with a 5′-monophosphate as described previously (34) (Fig. 2A, step a, and B, lanes 1 and 7). After washing away unincorporated [α-32P]UTP and addition of all four NTPs (Fig. 2A, step b), these ECs were able to elongate the labeled RNA (Fig. 2B, lanes 2 and 8). RNA transcripts of various lengths were observed, up to the expected 68-nt run-off prod-

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**FIGURE 1. Preparation of recombinant, active Rat1 complexes.** A, expression and purification scheme for Rat1, Rat1-Rai1, and Rat1-Rai1-Rtt103. At the bottom, the resulting proteins were visualized by Coomassie-stained SDS-PAGE. Ni-NTA, nickel-nitrilotriacetic acid. B, size-exclusion chromatography profiles of the nuclease complexes from A. Shown is a plot of the absorption at 280 nm against the retention time. Profiles are from three separate experiments using the same conditions. C, relative ribonucleolytic activity of Rat1-Rai1 and Rat1-Rai1-Rtt103 (compare with “Experimental Procedures”). The intensity of signals corresponding to the 27-mer RNA were quantified with the ImageQuant software resulting in a mean value from three replicates of each experiment. The intensity of the input RNA was set to 100%, the decrease because of degradation by Rat1 was calculated relative to that value and plotted against the duration of the reaction.
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A

step a

biontin

5' -GGCTACCCAGCTAGTCTAAGGCCCGAGGTAGTAACTGAGGTCCAGCTGACACTGAGG- 3'

3' -GGCTGCTGATGTGGTCCTGCTGACTCTAGCTCATTA

CTGTGACTG

AGTTGATGCACTGACCTCAGTCACTGACCTGACCC- 5'

5' AUACUCCUAUAUAUACUAUAAAGACCAGGC

Pol II

step a'

+α(32P)UTP ☆

+ wash

+ Rat1

step b

+ wash +NTPs

+ extended RNA

step b'

+ wash +NTPs

RNA extension?

B

12 su EC

run-off 68 nt

run-off after 55 nt

Rat1/Rai1

31 nt

Rat1/Rai1: – 18 nt

core EC

run-off 68 nt

run-off after 55 nt

Rat1/Rai1

31 nt

Rat1/Rai1: – 18 nt

C

% 12 su EC Rat1/Rai1 RNase I

% core EC Rat1/Rai1 RNase I

intact ECs after nuclease digestion

elongating ECs after nuclease digestion
Rat1-Rai1 instead of Rat1-Rai1-Rtt103 degraded RNA that is accessible on the EC surface, but do not effectively dissociate pol II, and leave the ECs largely intact and transcriptionally competent, such that the RNA 3’-end remains at the active site and can be re-extended. Because transcription termination is defined as a discontinuation of the ability to extend RNA, these results show that Rat1-Rai1 is insufficient to efficiently terminate pol II in a defined in vitro system.

Poly(A) Site Sequence Does Not Trigger Termination—We next asked whether pol II could terminate in our *in vitro* system if the poly(A) site sequence was transcribed. We prepared a nucleic acid scaffold containing a poly(A) site 10 nt downstream of the incorporated [α-32P]UTP (Fig. 3A, poly(A) transcription scaffold 1), assembled an EC, and added ATP, CTP, and UTP but withheld GTP from the reaction mixture. This led to transcription of the poly(A) site plus 16 additional base pairs, and EC stalling at a defined position (CCC in the template strand; Fig. 3A). RNAs with a length of 41–46 nt were not observed (Fig. 3B), which means that the poly(A) sequence was transcribed like a control random sequence in our assay.

We tested the model that passage of a poly(A) site would alter EC stability and render pol II prone to Rat1-induced termination. We assembled ECs containing the poly(A) site sequence within the DNA-RNA hybrid at positions −1 to −6 (Fig. 3A, poly(A) transcription scaffold 2). RNA was labeled by incubation with CTP and [α-32P]UTP, which led to incorporation of three cytosines and a radioactive uridine (Fig. 3C, lane 1). Incorporation of three additional cytosines ensured that the resulting RNA length was as in the other experiments (Fig. 2 and Fig. 3, B and D) and mimicked partial polymerase passage of the poly(A) site sequence. Addition of NTPs produced the run-off RNA of 53 nt (Fig. 3C, lane 2). As before, RNA was trimmed down by Rat1-Rai1 to around 18 nt and could be re-extended after NTP addition (Fig. 3C, lanes 3 and 4).

Very similar results were obtained when RNase I was used instead of Rat1-Rai1 (Fig. 3C, lanes 5 and 6). RNAs longer than the run-off transcript were also detected, but they were excluded from Fig. 3C because they were present in all the samples of the experiment and likely result from transcript slippage as a side reaction, probably induced by the CCC sequence in the downstream template (Fig. 3A). Taken together, Rat1-Rai1 and

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**FIGURE 2.** Rat1-Rai1 does not terminate pol II *in vitro*. **A**, schematic representation of the bead-based elongation/RNA degradation assay. Top, nucleic acid scaffold that was assembled with pol II into a bead-coupled EC and used as a substrate for Rat1 is shown. Below, different steps of the protocol are shown (compare “Results” with “Experimental Procedures”). Steps a and b represent the control reaction without added nucleic acid, and steps a’ and b’ represent the reaction containing the nuclelease. B, autoradiographs of RNA from bead-based termination assays using 12-subunit pol II and 10-subunit core pol II on the left and the right side, respectively. Samples that were treated as depicted in A were separated by a 6% PAGE and exposed to a storage phosphor screen. The steps represented in A are given above the gel and lane numbers below the gel. C, quantification of bands on autoradiographs in B (see “Experimental Procedures”). Light gray bars represent relative amounts of ECs that remain attached to beads after nuclelease digestion (asterisks in lanes 1, 3, 5, 7, 9, and 11). Dark gray bars represent ECs that are still able to elongate RNA after nuclelease digestion (black squares in lanes 2, 4, 6, 8, 10, and 12). The values shown for the 12-subunit pol II ECs (left) are the means from three individual experiments, and those shown for the 10-subunit core pol II ECs are the means from two individual experiments. The apparent slight increase in the signal following RNase I treatment can be explained by impurities of the synthesized RNA sample that also assemble into ECs (dotted line). To examine which fraction of ECs that remain attached to the beads after nuclelease digestion can start into elongation, we related signals from the ECs after nuclease digestion (asterisks in lanes 1, 3, 5, 7, 9, and 11) to the intensities of the same regions of the gel in lanes of samples where NTPs were added (squares in lanes 2, 4, 6, 8, 10, and 12).
FIGURE 3. Neither a poly(A) site nor a pause sequence trigger termination. A, nucleic acid scaffolds that were assembled with pol II for the indicated experiments. B, autoradiograph of RNA extension with a bead-coupled EC, including poly(A)-transcription scaffold 1. The region where signals of RNAs would be expected if elongation had stopped at the poly(A) site sequence is indicated by dashed lines. C, autoradiograph of bead-based assay with poly(A)-transcription scaffold 2. a, b, a’, and b’ refer to steps in the protocol described in Fig. 2A. D, autoradiograph of bead-based assay with EC containing the pause site transcription scaffold. a, b, a’, and b’ refer to steps in the protocol described in Fig. 2A. For a detailed description of these experiments, refer to text.
a transcribed poly(A) site sequence alone are insufficient to trigger efficient termination of pol II in these assays.

**Hybrid with Poly(A) Site Sequence Does Not Change EC Structure**—The above results suggested that an EC that contains the poly(A) site within the DNA-RNA hybrid does not differ significantly in structure from an EC that contains a random sequence within the hybrid. To examine this, we solved the x-ray structure of *S. cerevisiae* pol II EC containing a hybrid that harbors the poly(A) site sequence in the active center cleft of the enzyme (Fig. 4). The structure could be determined at 4.0 Å resolution with the use of established protocols (44). The unbiased difference electron density in the hybrid site did not

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**FIGURE 4. Canonical binding of a poly(A) site-containing hybrid within the pol II EC.** A, used nucleic acid scaffold is shown. Filled circles indicate nucleic acids that have interpretable electron density, and empty circles indicate nucleic acids that were not ordered. The 5-bromouracil is shown in yellow. The template strand is shown in blue, the non-template strand in cyan, and the RNA in red throughout this figure. B, overview of the pol II EC structure containing the poly(A) site sequence, pol II is shown as a ribbon model in gray. Rpb2 residues 1–828 are omitted for clarity. The pol II bridge helix (residues 811–843 of Rpb1) is shown in green. Nucleic acids are shown in a stick representation and colored as in A. C, $2F_o - F_c$ electron density map contoured at 1σ for the nucleic acids in the poly(A) site-containing DNA-RNA hybrid. D, superposition of the poly(A) site nucleic acids (red) and nucleic acids of random sequence (green, Protein Data Bank accession code 1Y1W (36)). E, $2F_o - F_c$ electron density at 1σ of the upstream nucleic acids from C. An anomalous difference Fourier map at 3.5σ, showing the position of the bromine atom, is indicated in raspberry. The structure is rotated 90° clockwise respective to C.
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reveal any significant rearrangement of the nucleic acids within the pol II cleft (Fig. 4, B and C). However, we observed that the hybrid was back-stepped compared with the designed scaffold, and that the adenine at the RNA 3′-end was not paired with the DNA template at position +1 but rather was disordered (Fig. 4C). Such fraying of the 3′-terminal RNA nucleotide is typical for a paused state of the EC (45) and has been observed in other pol II structures (55). Superposition of our structure with the previous complete pol II EC structure showed a similar position and conformation of the DNA-RNA hybrid and downstream DNA (Fig. 4D). These results are consistent with the idea that the poly(A) site sequence is prone to pausing but do not provide evidence for models that postulate that the EC adopts an alternative structure upon poly(A) site passage.

A Paused EC Is Not Terminated by Rat1-Rai1—Because there is evidence that the EC goes through a paused state before termination (5, 46, 47), we also tested whether a paused EC can be terminated by Rat1 in our assay. We assembled ECs with a scaffold containing a 3′-uridine in the active site (Fig. 3A, pause site transcription scaffold). Introduction of an additional radioactively labeled uridine to the RNA results in a pause sequence with a UU RNA 3′-end at the pol II active center (Fig. 3A). In ECs that contain this pause sequence, the RNA 3′-nucleotide adopts a frayed position in the pore below the active site (45). We incubated the obtained EC with the recombinant Rat1 complexes and checked if they can still elongate the RNA after it had been partially degraded (Fig. 3D). The experiments showed that neither complete pol II (Fig. 3D, lanes 3 and 4) nor core pol II (lanes 9 and 10) was terminated. In both cases the incubation with RNase I gave similar results (Fig. 3D, lanes 5, 6, 11, and 12). Quantification of the RNA signals as above led to the same conclusions as for the data shown in Fig. 2C (data not shown). Thus, a paused EC conformation is not sufficient to allow Rat1 to terminate pol II in our system.

DISCUSSION

Here we report the preparation of bacterially expressed recombinant Rat1 nuclease in free form and as a functional complex with Rai1, and with Rai1 and Rtt103, and the characterization of the activity of these preparations in vitro. We find that recombinant Rai1 is required to obtain reproducible exonuclease activity of recombinant Rat1, consistent with previous data (10, 52), and that Rtt103 does not influence Rat1 activity. We further show that Rat1-Rai1 degrades RNA within a complete, reconstituted, and bead-coupled EC from the 5′-end until it reaches the polymerase surface, but not any further. After Rat1-Rai1 action, the EC remains active and can elongate the RNA. Because 10–20% of ECs are lost from beads after Rat1 treatment, we cannot exclude that a small fraction of ECs was terminated, but if so, the termination is very inefficient.

Thus recombinant Rat1 is not able to efficiently terminate pol II in our pure in vitro system despite the long incubation time of 1 h. Instead, RNA within the DNA-RNA hybrid and the RNA exit tunnel of pol II are apparently protected from Rat1, activity, and the growing RNA 3′-end remains at the polymerase active site. This failure to terminate persisted in the presence and absence of the pol II subcomplex Rpb4/7, in the presence of Rtt103, after transcription of a poly(A) site sequence, and when a paused EC was used.

Our results thus suggest that Rat1-dependent pol II termination requires an additional factor or several additional factors, unknown altered conditions, or post-translational modification of Rat1, Rai1, and/or Rtt103. One possibility is that the DNA helicase Sen1 is required in addition to Rat1 to efficiently terminate pol II (48–50). Unfortunately we could not test this proposal because Sen1 is not available in recombinant form. If true, however, it remains unclear why Rat1 is required at all, because a helicase activity alone (the Rho factor) is able to terminate transcription in the bacterial system (51).

Another possibility is that a cryptic RNase H-like activity of Rat1 may be involved in pol II termination. In this model, a cryptic RNase H activity is activated when Rat1 reaches the polymerase surface and degrades RNA within the DNA-RNA hybrid, destabilizing the EC and leading to termination. We refer to this model as the “triggered Torpedo model” because it implies that the Rat1 Torpedo involves an RNase H activity that must be activated (triggered). A cryptic RNase H activity of Rat1 is consistent with the structural homology to RNase H (52) and a recent study of the role of Rat1 in degrading telomeric repeat-containing RNA (54). In the latter study, overexpression of RNase H rescued telomere elongation defects in cells that lacked functional Rat1. This could in principle be explained if Rat1 had RNase H activity in vivo in certain contexts.

In the future, our in vitro system should allow identification of a missing factor that is required for termination, and testing of the triggered Torpedo model. Additional protein factors or nuclear fractions may be added to the assay, and reaction conditions may be changed at certain steps of the reaction. In our experiments thus far, however, we could not identify such an activity or altered conditions that cause efficient termination. Once termination is achieved in such a defined in vitro system, the availability of recombinant, functional Rat1 complexes opens the way to mutational analysis of these proteins and will ultimately lead to a mechanistic dissection of the mechanism of eukaryotic mRNA transcription termination.

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