The Yeast Mitochondrial RNA Polymerase and Transcription Factor Complex Catalyzes Efficient Priming of DNA Synthesis on Single-stranded DNA*

Received for publication, May 25, 2016, and in revised form, June 14, 2016; Published, JBC Papers in Press, June 16, 2016, DOI 10.1074/jbc.M116.740282

Aparna Ramachandran†, Diyva Nandakumar†‡, Aishwarya P. Deshpande§, Thomas P. Lucas*, Ramanagouda R-Bhojappa‖, Guo-Qing Tang‡, Kevin Raney‖, Y. Whitney Yin†, and Smita S. Patel‡†

From the †Department of Biochemistry and Molecular Biology, Rutgers University, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, ‡Graduate School of Biomedical Sciences, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, §Department of Pharmacology and Toxicology, Sealy Center for Structural Biology, University of Texas Medical Branch, Galveston, Texas 77555, and ‖Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Primases use single-stranded (ss) DNA as templates to synthesize short oligoribonucleotide primers that initiate lagging strand DNA synthesis or reprime DNA synthesis after replication fork collapse, but the origin of this activity in the mitochondria remains unclear. Herein, we show that the Saccharomyces cerevisiae mitochondrial RNA polymerase (Rpo41) and its transcription factor (Mtf1) is an efficient primase that initiates DNA synthesis on ssDNA coated with the yeast mitochondrial ssDNA-binding protein, Rim1. Both Rpo41 and Rpo41-Mtf1 can synthesize short and long RNAs on ssDNA template and prime DNA synthesis by the yeast mitochondrial DNA polymerase, Mip1. However, the ssDNA-binding protein Rim1 severely inhibits the RNA synthesis activity of Rpo41, but not the Rpo41-Mtf1 complex, which continues to prime DNA synthesis efficiently in the presence of Rim1. We show that RNAs as short as 10–12 nt serve as primers for DNA synthesis. Characterization of the RNA-DNA products shows that Rpo41 and Rpo41-Mtf1 have slightly different priming specificity. However, both prefer to initiate with ATP from short priming sequences such as 3′-TCC, TTC, and TTT, and the consensus sequence is 3′-Pu(Py)2–3. Based on our studies, we propose that Rpo41-Mtf1 is an attractive candidate for serving as the primase to initiate lagging strand DNA synthesis during normal replication and/or to restart stalled replication from downstream ssDNA.

Primases are specialized DNA-dependent RNA polymerases that synthesize short oligoribonucleotides de novo on single-stranded (ss) DNA templates. These RNAs serve as primers for the replicative DNA polymerases during leading and lagging strand synthesis. Interestingly, some systems such as bacteriophage T7 and the human mitochondria utilize their transcriptional machinery for priming DNA synthesis (1–7). In the human mitochondria, replication of both leading and lagging strand is initiated from RNA primers synthesized by the human mitochondrial (mt) RNA polymerase, POLRMT. The RNA primers made at the light strand dsDNA promoter initiate leading strand DNA synthesis, and primers made at a stem loop structure (OriL) in the displaced ssDNA initiate lagging strand synthesis. In addition to the initiation at the OriL, priming has been observed at other sites on the displaced ssDNA (8), which is consistent with the reported primase activity of POLRMT that can transcribe RNA from non-specific ssDNA (9).

Compared with the small size of the human mtDNA genome (16.5 kb), the yeast Saccharomyces cerevisiae mtDNA genome is relatively large (~86 kb); thus, it is unlikely that leading or lagging strand replication would be primed at only one site. For the yeast, both ori-sequence specific and homologous recombination mediated mechanisms have been proposed for mt replication initiation (10–16). The S. cerevisiae mt RNA polymerase Rpo41 and its transcription factor Mtf1 have been shown to prime leading strand synthesis in vitro from dsDNA promoter with ori-sequences (3, 17–19). Similarly, strand invasion and homologous recombination has been proposed to play a role in replication initiation in the Candida albicans yeast mt DNA (15, 16). Both of these modes of replication can initiate leading strand DNA synthesis; however, the mechanism of lagging strand synthesis on the displaced ssDNA is not well understood. Lagging strand initiation would require priming on ssDNA covered with the single strand binding protein (SSB). It has been shown that the S. cerevisiae Rpo41 can transcribe on ssDNA (19, 20). However, Rpo41 associates with Mtf1 (21), and therefore, it is important to determine whether the Rpo41-Mtf1 complex can synthesize RNA on ssDNA and more importantly prime DNA synthesis on ssDNA covered with SSB. Such a priming mechanism would also facilitate the restart of DNA synthesis from ssDNA after replication stalling.

In this study, we investigate the RNA synthesis and priming activities of Rpo41 and Rpo41-Mtf1 on long and short ssDNA templates and determine whether the RNAs made by Rpo41 alone and Rpo41-Mtf1 can prime DNA synthesis by the yeast mitochondrial DNA polymerase Mip1 (ymtDNAP). In vivo, the ssDNA displaced by the replisome catalyzing leading strand synthesis is expected to be coated with the mt ssDNA-binding
protein Rim1 (ymtSSB). Hence, we have compared the effects of ymtSSB on the activities of Rpo41-Mtf1 and Rpo41. Additionally, we use defined ssDNA template to determine the priming start sequences and the length of the minimal primer that can initiate DNA synthesis by the ymtDNAP. Overall, our findings indicate that the complex of the mitochondrial RNA polymerase and transcription initiation factor is an efficient primase under physiologically relevant conditions.

Results

Rpo41 and Rpo41-Mtf1 Can Initiate Transcription on ssDNA Template—The RNA synthesis activity of Rpo41 and Rpo41-Mtf1 on ssDNA template was investigated using M13 ssDNA. The kinetics of RNA synthesis was measured in the presence of all rNTPs spiked with \([\alpha^{32}\text{P}]\)ATP for RNA detection (Fig. 1A). The RNA products were quantified after separating free ATP from the RNA using thin layer chromatography. An example of the TLC image is shown as an inset (Fig. 1A). RNA synthesis is reported as the amount of total nucleotides incorporated into the RNA as a function of time (Fig. 1A). We find that both Rpo41 and Rpo41-Mtf1 are efficient RNA polymerases on ssDNA. The overall kinetics of RNA synthesis by Rpo41 and Rpo41-Mtf1 are comparable; and although Rpo41 makes slightly higher amounts of RNA at the initial time points, RNA synthesis by Rpo41 alone saturates at the later time points, but RNA synthesis by Rpo41-Mtf1 continues...
Rpo41-Mtf1 as a Primase on ssDNA

linearly (Fig. 1A). We also noted that the amount of RNA made at longer times exceeded the amount of the provided template (10 nM M13 ssDNA circle corresponding to ~65 μM nucleotides). This indicates that Rpo41-Mtf1 and Rpo41 are capable of catalyzing rolling circle RNA synthesis on circular M13 ssDNA template.

To assess the size of the RNA products, we analyzed the reactions on polyacrylamide/urea sequencing gels. Previously, it was shown that the lengths of RNAs transcribed by Rpo41 on M13 ssDNA are between 25–100 nt (19). However, we find that the dominant products synthesized by both Rpo41 and Rpo41-Mtf1 are long RNA products; among which some migrated close to the 9 kb marker (in the Rpo41-Mtf1 reaction) and others remained closer to the wells (Fig. 1B). The RNA products running close to the 9 kb DNA marker could be the size of M13 template (~7 kb) and result from a single round of rolling circle RNA synthesis (shown as asterisk mark in Fig. 1B). It was difficult to estimate the exact size of the RNA product due to the length migration of RNA and DNA marker on the polyacrylamide gel. The migration pattern of the long RNAs from rolling circle RNA synthesis did not change when the samples were treated with proteinase K and 1% SDS detergent (data not shown), which indicates that the long RNAs close to the wells are not RNA-protein complexes. The long RNAs were observed within 1 min of reaction time, which indicates that RNA initiation is the rate-limiting step and once initiated the elongation step is much faster; otherwise, we would have observed a progressive increase in RNA length with increasing reaction time. We also observed short RNAs from 10–20 nt in the reactions with Rpo41-Mtf1, and RNAs from 50 nt to 2 kb in the reactions with Rpo41 alone. Thus, our results indicate that Rpo41 and Rpo41-Mtf1 are both efficient RNA polymerases on ssDNA template and this activity makes both short and long RNA products.

Next we investigated the kinetics of RNA synthesis on M13 ssDNA by Rpo41 and Rpo41-Mtf1 in the presence of the ymtDNAP (Mip1). We used both dNTPs and rNTPs nucleotide substrates but spiked the reactions with [α-32P]ATP for RNA detection. The TLC quantitation shows that both Rpo41 and Rpo41-Mtf1 make RNA in the presence of ymtDNAP with similar kinetics (Fig. 1C). However, the amount of RNA synthesized in the presence of ymtDNAP + dNTPs is about 40% lower as compared with RNA synthesis without ymtDNAP (compare Fig. 1, A and C). This is likely due to accompanying DNA synthesis by the ymtDNAP. Analysis of the nucleic acid products on the sequencing gel shows a similar pattern of long and short RNA products as observed in the absence of ymtDNAP, with some differences. There is a decrease in the amount of 10–20 nt and the single round RNA synthesis products in the Rpo41-Mtf1 reactions in the presence of ymtDNAP (Fig. 1D). Analysis on the reaction products on 1% native agarose gel stained with ethidium bromide shows that the long nucleic acid products are >23 kb in sizes (Fig. 1E).

In summary, our results show that Rpo41 and Rpo41-Mtf1 can use ssDNA as a template for RNA synthesis, both in the presence and absence of the ymtDNAP. In contrast to reported studies (19), our results show that RNA synthesis is not limited to short lengths, as both Rpo41 and Rpo41-Mtf1 make very long RNA products, including products from rolling circle RNA synthesis.

Rpo41-Mtf1 Is a Better RNA Polymerase in Comparison to Rpo41 on ssDNA in the Presence of ymtSSB—In vivo, the ssDNA displaced during leading strand synthesis is expected to be coated with the ssDNA binding protein, which in the yeast mitochondria is Rim1 (ymtSSB), a 13-kDa protein that binds ssDNA as a tetramer (22). The ymtSSB is homologous to the Escherichia coli SSB, which binds 35–65 nt of ssDNA/tetramer (23). Based on this information, we calculated that between 1–2 μM of yeast SSB tetramer would be needed to cover the entire M13 ssDNA at 10 nM concentration. Hence, we used 0, 1, 4, and 8 μM tetramer ymtSSB to test its effect on the RNA synthesis activity of Rpo41 and Rpo41-Mtf1, both in the presence and absence of ymtDNAP plus dNTPs. Both the TLC quantitation (Fig. 2A) and polyacrylamide/urea gel (Fig. 2B) show that ymtSSB severely inhibits the RNA synthesis activity of Rpo41. At 1 μM ymtSSB, there is about 80% inhibition, but there are no detectable RNAs at 4 and 8 μM ymtSSB (Fig. 2B). On the other hand, the inhibition of Rpo41-Mtf1 is less severe and there are detectable amounts of RNA made even at 4 and 8 μM ymtSSB.

Next, we checked the RNA synthesis activity of Rpo41 and Rpo41-Mtf1 in the presence of ymtDNAP plus dNTPs and at increasing ymtSSB concentrations. The RNA synthesis activity of Rpo41 in the presence of ymtDNAP remained severely inhibited as observed in the absence of ymtDNAP (Fig. 2, C and D). In contrast, the RNA synthesis activity of Rpo41-Mtf1 was restored to the levels observed in the absence of ymtSSB with ymtDNAP, particularly at 4 and 8 μM ymtSSB concentrations (Fig. 2, C and D).

Thus, our results indicate that Rpo41-Mtf1 acts as a better RNA polymerase as compared with Rpo41 alone on ssDNA template coated with ymtSSB. This could be because Rpo41-Mtf1 has a 4-fold higher affinity for ssDNA as compared with Rpo41 alone (Fig. 3), hence Rpo41-Mtf1 is able to better compete with ymtSSB for binding to the ssDNA and/or is a stronger motor and able to mobilize the ymtSSB whereas Rpo41 alone is unable to carry out these functions.

The Rpo41 and Rpo41-Mtf1 Complex Can Prime DNA Synthesis by ymtDNAP—To determine whether the RNAs made by Rpo41 and Rpo41-Mtf1 can be used for DNA synthesis by the ymtDNAP, we carried out reactions with a mixture of NTPs and dNTPs and spiked the reactions with [α-32P]dGTP for DNA detection. The TLC quantitation shows robust DNA synthesis in reactions containing Rpo41 and Rpo41-Mtf1 (Fig. 4A). Control experiments showed no DNA synthesis without ymtDNAP and negligible synthesis with ymtDNAP alone (Fig. 4B). These results indicate that the ymtDNAP is able to utilize the RNAs generated by Rpo41 and Rpo41-Mtf1 as primers to catalyze DNA synthesis on M13 ssDNA template. The polyacrylamide/urea gel shows that the RNA-DNA products are very long and run close to the wells (Fig. 4C). The agarose gel shows that the nucleic acid products are >23 kb in length (Fig. 4D). In the agarose gel analysis, we treated the nucleic acid products with a mixture of RNaseH and RNase1f and observed that the nucleic acid products running close to the wells disappeared suggesting that these are long RNA products. Interestingly, the sizes of >23 kb products did not change, which suggests that these are
mostly DNA and since there is no change in length, these are primed by short RNAs.

Next, we investigated the RNA-primed DNA synthesis reaction in the presence of ymtSSB. The DNA synthesis reactions were carried out in the presence of NTPs and dNTPs spiked with \([\alpha^{32}\text{P}]dGTP\) at increasing concentrations of ymtSSB (0, 1, 4, and 8 \(\mu\text{M}\) tetramer). The ymtSSB at 1 \(\mu\text{M}\) stimulated the RNA-primed DNA synthesis reactions of Rpo41 and Rpo41-Mtf1 (Fig. 4, E and F). This stimulation is most likely due to increase in the processivity and strand-displacement activities of the DNAP in the presence of ymtSSB, as has been seen in case of human POLRMT and T7 DNAP with their SSB proteins (9, 10).
Rpo41-Mtf1 as a Primase on ssDNA

However, higher ymtSSB concentrations inhibited the RNA-primed DNA synthesis reactions of Rpo41 alone, but not the Rpo41-Mtf1 primed DNA synthesis reaction, which remained stimulated (Fig. 4, E and F). These results indicate that Rpo41-Mtf1 is a better RNA polymerase and a better primase on ymtSSB-coated ssDNA as compared to Rpo41.

FIGURE 3. Equilibrium binding of Rpo41 and Rpo41-Mtf1 to a random 20mer ssDNA by fluorescence anisotropy. The TAMRA-labeled random 20 mer ssDNA was titrated with increasing concentrations of Rpo41 (A) and equimolar mixture of Rpo41-Mtf1 complex (B). Fluorescence anisotropy was measured after excitation at 555 nm and emission at 580 nm. Titration data were fit to Equation 1 to derive the $K_d$ values with standard deviation errors. The $K_d$ value for Rpo41 was observed to be 25 ± 6 nM while that of Rpo41-Mtf1 was 6 ± 1 nM.

FIGURE 4. RNA primers synthesized by Rpo41 and Rpo41-Mtf1 can be extended by the yeast mitochondrial DNA polymerase, Mip1. A, ymtDNAP (100 nM) was incubated with M13 ssDNA (10 nM) in the presence of Rpo41 (100 nM) or Rpo41-Mtf1 (100 nM) and 250 μM of each rNTP and dNTP spiked with [α-32P]dGTP at 30 °C. The reaction products were analyzed by TLC to quantify DNA synthesis. B, control experiments carried out under the same conditions as (A) for 90 min showed no DNA synthesis without ymtDNAP and negligible synthesis with ymtDNAP alone. C, nucleic acid products were analyzed by PAGE (10% acrylamide/7 M urea). D, DNA synthesis was carried out under the same conditions as (A) but with cold NTPs and dNTPs. The agarose gel shows that the nucleic acid products are >23 kb in length. The sizes of >23 kb products did not change upon treatment with RNaseH and RNase1f. E, DNA synthesis was carried out under the same conditions as (A) in the presence of increasing amount of ymtSSB for 90 min. F, nucleic acid products were analyzed by PAGE (10% acrylamide/7 M urea). The error bars depicted are from two independent experiments.
**Rpo41 and Rpo41-Mtf1 Initiate Transcription from Specific Sites (5′-TTC and 5′-TCC) on Nonspecific ssDNA**—To determine if Rpo41 and Rpo41-Mtf1 have any preference for start sites for RNA synthesis on ssDNA, we used a nonspecific 62-nt ssDNA as the template (Fig. 5A). The short template allowed us to map the start sites from the precise lengths of the transcribed RNAs using high-resolution sequencing gels. Reactions were carried out with Rpo41 or Rpo41-Mtf1 in the presence of all NTPs spiked with either [α32P]ATP (Fig. 5B) or [γ32P]ATP (Fig. 5C). As a control, transcription reactions under the same conditions as (B) were carried out using [γ32P]ATP. Start-site mapping of RNA synthesis was done by walking experiments in the presence of 3′-dUTP, 3′-dCTP, and 3′-dGTP. Lane 10 is a prequench control where sample was quenched before the addition of radioactive NTP. D, 30-nt, 41-nt, and 9–18 nt RNAs were quantified and compared for Rpo41 and Rpo41-Mtf1. The error bars are from two independent experiments.

**FIGURE 5. Start-site mapping of RNA synthesis by Rpo41 and Rpo41-Mtf1 on ssDNA template.** A, sequence of the 62-nt nonspecific ssDNA lacking the yeast mtDNA promoter sequence. The start sites used by Rpo41 and Rpo41-Mtf1 for RNA synthesis were determined and are shown in bold. B, RNA synthesis was carried out with 1 μM 62-nt ssDNA, 1 μM Rpo41, 2 μM Mtf1 in the presence of 250 μM of each NTP, spiked with [α32P]ATP. The 24% polyacrylamide urea-sequencing gel shows the 30 and 41-nt RNA products. The 15S rRNA mtDNA promoter makes a 31-nt runoff RNA with AUG3’dC and was used as a control. C, transcription reactions under the same conditions as (B) were carried out using [γ32P]ATP. Start-site mapping of RNA synthesis was done by walking experiments in the presence of 3′-dUTP, 3′-dCTP, and 3′-dGTP. Lane 10 is a prequench control where sample was quenched before the addition of radioactive NTP. D, 30-nt, 41-nt, and 9–18 nt RNAs were quantified and compared for Rpo41 and Rpo41-Mtf1. The error bars are from two independent experiments.
products are runoff products, whereas the 9–18 nt are incomplete RNA products from polymerase stalling or RNA dissociation before reaching the run-off length.

To map the start sites for RNA synthesis, we used mixtures of NTP and 3'-dNTP as nucleotide substrates. In the presence of ATP, GTP, CTP, and 3'-dUTP, RNA synthesis will terminate at the first A-templating base, and based on the sequence of our 62-nt template, this combination of nucleotides will produce run-off products 4-nt shorter in length from the indicated start sites (Fig. 5A). This was indeed the case. In the presence of 3'-dUTP, both the ~30-nt and ~41-nt products migrated faster on the gel, and appeared as single bands (Fig. 5C, compare lanes 2, 3 and 4, 5). The latter result indicates that the multiple bands observed around the ~30-nt RNA product in the absence of 3'-dUTP result from incomplete copying to the end of the template. Interestingly, in the presence of 3'-dUTP, there was no change in the profile of the 9–18 nt RNA products (compare lanes 3 and 5 in Fig. 5C), which indicates that these are not run-off RNA products but aborted or stalled RNA products. From the RNA lengths and knowing that RNA synthesis initiates from a T-templating base, we conclude that there are two major initiation sites on the 62-nt ssDNA template with the sequence TCC and TTC, as marked on the ssDNA template (Fig. 5A). The initiation specificity of Rpo41 and Rpo41-Mtf1 is slightly different. Both make almost equal amounts of the ~41-nt RNA product from polymerase stalling or RNA dissociation but not aborted or stalled RNA products (Fig. 5D).

To further validate these start sites, we carried out walking experiments by using additional terminating nucleotides, 3’-dCTP and 3’-dGTP (Fig. 5C, lanes 6–9). With a mixture of ATP, GTP, UTP, and 3'-dCTP, we observed mainly an 8-nt RNA product with Rpo41 alone (lane 6) and 5-nt and 8-nt product (lane 7) with Rpo41-Mtf1, the expected length products from the proposed start site TTC and TCC, respectively. The intensity of the 8-nt is much higher than that of the 5-nt RNA, which indicates that TTC is a better initiation site for Rpo41-Mtf1 as compared with TCC, which is also consistent with the relative intensities of the 41-nt and 30-nt RNAs (Fig. 5D). With a mixture of ATP, CTP, UTP, and 3’-dGTP, we observed mainly 2-nt RNA (lanes 8 and 9).

In summary, the walking experiments indicate that both Rpo41 and Rpo41-Mtf1 initiate transcription site-specifically from ssDNA, and in the context of this DNA sequence, the preferential initiation site is TTC followed by TCC.

RNA Primers Made by Rpo41 and Rpo41-Mtf1 Support DNA Synthesis—To test whether RNA synthesis by Rpo41 and Rpo41-Mtf1 support DNA synthesis, the reactions were carried out using [α-32P]dATP to detect only the RNA-DNA products. The major RNA-primed DNA product in the Rpo41 reaction is the 41-nt product initiating with TCC (Fig. 6A, lane 1). On the other hand, there are three major RNA-primed DNA products of lengths ~60-nt, 41-nt, and 30-nt in the Rpo41-Mtf1 reactions (Fig. 6A, lane 2), which we determine are from initiation sites CC, TCC, and TCC start sites, respectively (marked on the 62-nt ssDNA sequence).

Next we carried out reactions in the presence of [α-32P]ATP as the spike to detect both the RNA and RNA-DNA products (Fig. 6B). The RNA and RNA-DNA products of the same length appear as doublets: the faster migrating band (Fig. 6B, lane 2, marked with *) corresponds to the RNA-DNA product and the slower migrating bands are the RNAs. As expected, the doublets are not observed when we leave out dNTPs (Fig. 6B, lanes 3 and 4). In addition to the above mentioned products (30-nt, 41-nt, 60-nt), we also observed a ~16-nt band (Fig. 6B, lanes 1 and 3). The ~16-nt appears to be a run-off RNA product from a downstream initiation site of TTT that is not picked up by the ymtDNAP because it is a runoff product.

Overall, our results show that both Rpo41 and Rpo41-Mtf1 initiate RNA synthesis on ssDNA at specific sites that contain a short polypyrime tract. We identify three start sites TTC, TCC and TTT for priming and all contain a purine at the 3'-end, which makes the consensus priming sequence as 3’-Pu(Py)2–3.

Switch from RNA to DNA Synthesis Occurs at 10–12 mer RNA—To determine the length(s) of the RNA primers used by the ymtDNAP for RNA-primed DNA synthesis, the [α-32P]dATP labeled RNA-primed DNA products (Fig. 6A) were excised from the gel and treated with RNases. The RNase If is a single-stranded RNA endonuclease with no documented base preferences for cleavage, and RNase A cleaves after pyrimidine bases (28). Thus, digestion with RNase If/RNaseA provided a reasonably unbiased representation of the RNA to DNA transition points in the DNA synthesis products.

Both the 30-nt and 41-nt products of Rpo41 and 30-nt, 41-nt, and 60-nt products of Rpo41-Mtf1 were analyzed (Fig. 7). After digestion, the length of the RNA primer was estimated by subtracting the length of the DNA product from the total length of the original RNA-DNA product (Fig. 7). The analysis of 30-nt and 41-nt products indicates that the dominant and the shortest primer utilized by ymtDNAP is 10–12 nt in length (Fig. 7, lanes 2, 3 and 5, 6 in both Rpo41 and Rpo41-Mtf1 reactions). We observed faint bands of shorter DNA products, which indicate that longer RNA primers from 12 to 35 nt are also used by the ymtDNAP for DNA synthesis. Similar transition points were observed for the 60-nt RNA-primed DNA product (Fig. 7, lanes 8 and 9 in the Rpo41-Mtf1 reaction).

The results indicate that ymtDNAP uses as short as 10–12 nt RNA transcripts for RNA-primed DNA synthesis on the ssDNA template.

Discussion

The mitochondria lack an apparent primase enzyme, and in human mitochondria, the mitochondrial RNA polymerase POLRMT has been proposed to serve as the primase for both leading and lagging strand DNA synthesis (1, 8, 9). In the yeast, the role of mitochondrial RNA polymerase Rpo41 in priming DNA replication has been less clear. There are multiple promoter-like ori sequences identified on both strands of the yeast mitochondrial DNA, and recent studies have shown that the S. cerevisiae Rpo41-Mtf1 can initiate leading strand synthesis at some of these dsDNA ori-sequences (19). An alternative model has been proposed through studies of the C. albicans yeast, which indicates that replication is initiated by strand invasion and homologous recombination, similar to T4 and T7 phage systems (15, 16). It is possible that, depending on the energy
demands of the cell, both modes of replication initiation are used in the mitochondria, and these modes would explain initiation of leading strand DNA synthesis. Nevertheless, one needs to explain how the ssDNA displaced during leading strand synthesis is replicated. In the recombination dependent replication model, a second recombination event has to occur to replicate the displaced ssDNA strand, which may not be a likely event or may not happen in a timely manner. In the ori-sequence-dependent mechanism, bidirectional replication can occur from the opposite orientations of adjacent replication origins in the yeast mt DNA (10). However, such a mechanism would lead to convergence of two replication forks, potentially leading to disassembly of the replisome. Hence, we propose that both in the ori-dependent and recombination mediated replication initiation, a primase is needed to initiate lagging strand synthesis. We show that Rpo41-Mtf1 is an efficient primase of the ymtDNAP, most strikingly on ssDNA coated with ymtSSB (Rim1), which is a closer mimic of in vivo conditions. Thus, Rpo41-Mtf1 is a prime candidate for a primase enzyme to initiate lagging strand synthesis on ymtSSB-coated ssDNA.

Both Rpo41 and Rpo41-Mtf1 are efficient RNA polymerases on ssDNA and both make short and long RNA products on M13 ssDNA. We estimate that the long RNA products are >23 kb in size and result from rolling circle RNA synthesis on the circular M13 ssDNA. This is in contrast to published results that indicated that Rpo41 makes only short RNAs (25–100 nt) on ssDNA template (19). We show that the RNAs made by Rpo41 and Rpo41-Mtf1, most likely the short ones annealed to the template, prime DNA synthesis by the ymtDNAP, Mip1.

Using defined ssDNA template, we show that both Rpo41 and Rpo41-Mtf1 initiate RNA synthesis at specific sites on ssDNA. Start-site mapping of the priming activity of Rpo41-Mtf1 and Rpo41 on the 62-nt ssDNA template revealed a preference for initiating at short polypyrrimidine tracts. We mapped three

FIGURE 6. Rpo41-Mtf1 makes short transcripts that are utilized by the ymtDNAP Mip1 for DNA synthesis. A, DNA synthesis under the same conditions as in Fig. 5B was carried out on the 62-nt ssDNA template in the presence of Rpo41 or Rpo41-Mtf1, rNTPs, dNTPs, ymtDNAP and spiked with [α-32P]dATP. The 24% polyacrylamide urea-sequencing gel shows the 30, 41, and 60-nt RNA-DNA products shown with asterisk mark. B, transcription reactions under the same conditions as Fig. 5B were carried out in presence and absence of dNTPs spiked with [α-32P]ATP. The faster migrating band corresponds to the RNA-primed DNA product (lane 2, asterisk marked), and the slower band corresponds to the RNA.
priming start sites 3′-TCC, TTC, and TTT and all had a purine at the 3′-end making the consensus sequence as 3′-Pu(Py)2–3. Purine-purine base stacking interactions between the 3′-end purine base in the template and the incoming ATP can increase the binding affinity of the initiating nucleotide, as observed in the transcription initiation complex structure of the bacterial RNAP (29).

Similar pyrimidine trinucleotides have also been identified in initiation sites used by primases including the mouse primase (30), primases purified from simian cells (31) and HeLa cells (32). Eukaryotic primases require the presence of pyrimidines in the ssDNA template for activity and bind polypyrimidine templates more tightly than they do polypurine templates (33, 34). The human mt RNA polymerase POLRMT uses poly(T) tract to initiate at the oriL stem-loop structure during lagging strand synthesis (1). This common characteristic is likely due to the fact that all primases initiate with purine NTPs (31).

As RNA polymerases, both Rpo41 and Rpo41-Mtf1 are equally efficient at catalyzing RNA synthesis on ssDNA. However, a different behavior was observed in the presence of ymtSSB. Although low concentrations of ymtSSB (subsaturating relative to the available binding sites of M13 ssDNA) stimulated the priming activity of both proteins, higher and saturating levels of ymtSSB inhibited Rpo41 but the priming activity of Rpo41-Mtf1 remained stimulated. The higher priming activity of Rpo41-Mtf1 is consistent with the higher RNA synthesis activity in the presence of ymtSSB. Thus, our results are clearly showing that Rpo41-Mtf1 can serve as a primase for DNA synthesis on ssDNA under physiologically relevant conditions. The priming activity on ssDNA would also be needed for events such as the reinitiation of DNA synthesis after problems in DNA replication, such as replication stalling or fork collapse (35). When the replication machinery encounters a lesion, the helicase and polymerase could functionally uncouple generating long stretches of ssDNA likely coated with SSB. Repriming after such replication stalling requires a primase to synthesize a primer while displacing the SSB. In human mitochondria, a

![FIGURE 7. 10–12 nt length RNA products are preferentially used as primers by the ymtDNAP Mip1.](image-url)

The length(s) of the RNA primers used by the ymtDNAP for DNA synthesis were determined by treating the [α-32P]dATP labeled RNA-primed DNA products with RNase A and 1f. The length of the RNA primer was estimated from the total length of the original RNA-DNA product and the length of the DNA product after treatment with RNase If/RNase A. The RNase If/RNaseA digestion profile for the 41-nt product obtained with Rpo41-Mtf1 or Rpo41 shows that RNA primers from 10-nt to 35-nt are used by the ymtDNAP for DNA synthesis. The dominant and the shortest primer utilized by ymtDNAP is 10–12-nt in length for both Rpo41 and Rpo41-Mtf1 (lanes 5 and 6). Similar transition points were observed for the 30-nt (lanes 2 and 3) and the 60-nt RNA-primed DNA products (lane 8 and 9 for Rpo41-Mtf1).
recently described DNA polymerase PrimPol has been suggested to be the primase for repriming DNA synthesis after replication stalling (36–42). However, it was observed that the de novo primer and DNA synthesis activity of PrimPol is suppressed by mtSSB (43). On the other hand, both human (1, 9) and yeast (this work) mtRNAPs can synthesize RNA primers on ssDNA in the presence of the mtSSB and the primers are efficiently extended by the respective DNA polymerases. This suggests that the mtRNAPs can provide the priming function in the event of replication stalling or fork collapse. There is no known PrimPol homolog identified in the yeast suggesting that Rpo41-Mtf1 may serve the role of a primase for repriming stalled forks in the yeast mitochondria.

**Experimental Procedures**

_Nucleic Acid Substrates_—The oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and PAGE purified. M13mp18 ssDNA was purchased from Affymetrix. The sequence of yeast 15S rRNA double-stranded promoter DNA used as a control and a marker is shown below: 5‘-ATAATTTATTTATTTATGATAATATAATTTGTTTTATATAAGAATTTCC-3‘; 3‘-TATTTAATAAATAATATATTCATTATTTATACAAACAAAATATTATTCTTAAAGG-5‘.

The sequence of a random 20nt ssDNA template that lacks the yeast mtDNA consensus nonanucleotide promoter sequence is shown below: 5‘-TAMRAMTAGGTACCTAGCAGGAAT-3‘.

**Proteins**—Rpo41 and Mtf1 were purified as described previously (26, 27). Plasmid pET24d containing the Mip1 gene was a kind gift from Dr. Johan Sedman. Mip1 protein was expressed in _E. coli_ BL21-DE3-RIL (Novagen). Cells were grown in LB medium supplemented with kanamycin (10 μg/ml) and chloramphenicol (34 μg/ml) at 37 °C until OD600 nm reached 0.6. At this point, the temperature was dropped to 25 °C and expression of Mip1 was induced by addition of IPTG (1 mm) and betaine (1 mm) and incubated for 8 h. Harvested cells were resuspended in the lysis buffer (25 mm HEPES, 500 mm NaCl, 100 mm Betaine, 5 mm imidazole, 0.5% Nonidet P-40, 10 mm 2-mercaptoethanol) supplemented with PMSF, E-64, leupeptin, and pepstatin. The suspension was incubated with lysozyme (1.0 mg/ml) for 1 h at 4 °C before being sonicated on ice. The cell lysate was centrifuged at 27,000 rpm for 20 min in a Beckman JLA 25.50 rotor at 4 °C. The cleared lysate was applied to Ni-NTA (Qiagen) affinity column and washed with 20 column volumes of buffer containing 20 mm imidazole, eluted with 10 column volumes of buffer containing 200 mm imidazole and 100 mm NaCl. The protein sample was then loaded onto a DNA-Cellulose column (Worthington) and washed with 20 column volumes of buffer containing 150 mm NaCl. Mip1 was eluted with 10 column volumes of buffer (25 mm HEPES, 500 mm NaCl, 100 mm Betaine, 10 mm 2-mercaptoethanol) and stored at −80 °C. Sample concentration was determined using Nanodrop 2000 (Thermo Scientific). Protein purity was assessed by SDS–PAGE. Rim1 protein was purified as described previously (44).

**Steady State Transcription and DNA Synthesis on M13 ssDNA**—Transcription and DNA synthesis reactions were carried out in reaction buffer A (50 mm Tris acetate pH 7.5, 100 mM potassium glutamate, 1 mm DTT, 0.05% Tween) at 30 °C. The reaction was initiated by combining a pre-incubated complex containing 10 nm M13 ssDNA circle, 100 nm Mip1 (in reactions with ymtDNAP), 100 nm Rpo41, 200 nm Mtf1 with a mixture of 250 μm of each NTP and dNTP, 0–8 μm Rim1 (in reactions with ymtSSB) and 10 mm magnesium acetate spiked with [α32P]ATP (transcription) or [α32P]dGTP (DNA synthesis). The reactions were quenched with 4 M formic acid or 250 mM EDTA. The reactions quenched with formic acid were resolved on a polyethyleneimine thin layer chromatography (TLC) plate to separate the labeled RNA/DNA product and the unincorporated ATP/dGTP. The reactions quenched with EDTA were run on 10% polyacrylamide 7M urea sequencing gel to resolve the individual products. The gel or TLC plate was exposed to a phosphor screen, scanned on Typhoon 9410 phosphorimagery instrument (Molecular Dynamics), and the products were quantitated using the ImageQuant program (GE healthcare).

For agarose gel electrophoresis, reactions were carried out as above with cold NTPs. For RNase treatment, a mixture of RNaseH (NEB) and RNaseIF (NEB) was added to reactions and incubated at 37 °C for 15 min and then the reactions were quenched with EDTA. The samples were mixed with the loading dye with 0.1% SDS and ran on a 1% agarose gel in 1x TBE at 100 V for 3–4 h. The gel was stained with Ethidium bromide.

**Steady State Transcription on Short Synthetic DNA Substrates**—The transcription reactions with short synthetic DNA templates contained 1 μM ssDNA or dsDNA and 1 μM Rpo41, 2 μM Mtf1 and were initiated by adding 250 μM of each NTP, spiked with [γ32P]ATP or [α32P]ATP (Perkin Elmer) at 25 °C. The reactions were quenched with 250 mM EDTA and the RNA products were resolved on a 24% polyacrylamide/4 M urea sequencing gel (Bio-Rad) electrophoresed in 1.5× TBE buffer at 58 Watts. The samples were boiled for 5 min in the presence of the complementary strand to ensure that the resultant RNA product is single stranded. The RNA products were visualized as described above.

**DNA Synthesis and RNase Digestion**—DNA synthesis was carried out in buffer A containing 1 μM each of DNA, Rpo41, and Mip1 and 2 μM of Mtf1 at 25 °C. The reaction was initiated by adding 10 μM magnesium acetate, 250 μM of each NTP and dNTPs spiked with [α32P]dATP. The reactions were quenched after 3 min (unless specified otherwise) with 250 mM EDTA and the products were resolved and visualized as in the transcription reactions.

For the RNase I treatment, the gel was exposed on an x-ray film (Kodak) and the bands were cut out from the gel using the film for guidance. The products from the cut bands were passively eluted in reaction buffer A by incubation at 65 °C for 2 h. 2 μl of neat RNase I (NEB) and RNase A were added to the supernatant and incubated overnight at 37 °C. The RNase
treated samples were resolved on a sequencing gel as described above.

Equilibrium Titration of Protein-DNA Binding—Fluorescence anisotropy measurements were carried out according to the published protocol (45) on a PTI QM-3 spectrophotometer mounted with Glen-Thomson calcite prism polarizers and a thermoelectrically controlled cell holder. Titration was conducted by adding concentrated Rpo41 or equimolar mixture of Rpo41-Mtf1 to a solution of TAMRA-labeled random 20 mer ssDNA in the reaction buffer, and observed anisotropy values (r_{obs}) with excitation at 555 nm and emission at 580 nm were recorded. r_{obs} was plotted against protein concentrations, and was numerically fitted to Equation 1 to obtain the equilibrium dissociation constant ($K_d$),

$$r_{obs} = \frac{(r_0 - r_t)(K_d + D_t + P_t) - \sqrt{(K_d + D_t + P_t)^2 - 4KD_tP_t}}{2D_t} + r_t$$

(Eq. 1)

where $r_{obs}$, $r_0$, $P_t$, $K_d$, $D_t$, $P_t$ denote the observed fluorescence anisotropy due to protein binding to DNA, anisotropy of bound DNA, anisotropy of free DNA, thermodynamic dissociation constant, total amount of DNA, and total amount of protein, respectively.

Author Contributions—A. R. and D. N. designed the study and A. R. generated and analyzed the data. TPL and WY provided purified Mip1 protein. R. R. B. and K. R. provided purified Rim1 protein. A. D. performed fluorescence anisotropy experiment. A. R. generated and analyzed the data. TPL and WY provided purified RNA polymerase, Rpo41-Mtf1 as a Primase on ssDNA treated samples were resolved on a sequencing gel as described above.

Acknowledgments—We thank members of the Patel Laboratory for constructive criticism throughout these studies.

References

1. Fusté, J. M., Wanrooij, S., Jemt, E., Granycome, C. E., Cluett, T. J., Shi, Y., Atanassova, N., Holt, I. J., Gustafsson, C. M., and Falkenberg, M. (2010) Mitochondrial RNA polymerase is needed for activation of the origin of light-strand DNA replication. Mol. Cell 37, 67–78
2. Lee, D. Y., and Clayton, D. A. (1998) Initiation of mitochondrial DNA replication by transcription and R-loop processing. J. Biol. Chem. 273, 30614–30621
3. Xu, B., and Clayton, D. A. (1995) A persistent RNA-DNA hybrid is formed during transcription at a phylogenetically conserved mitochondrial DNA sequence. Mol. Cell. Biol. 15, 580–589
4. Xu, B., and Clayton, D. A. (1996) RNA-DNA hybrid formation at the mitochondrial heavy-strand origin ceases at replication start sites: an implication for RNA-DNA hybrids serving as primers. EMBO J. 15, 3135–3143
5. Fuller, C. W., and Richardson, C. C. (1985) Initiation of DNA replication at the primary origin of bacteriophage T7 by purified proteins. Initiation of bidirectional synthesis. J. Biol. Chem. 260, 3197–3206
6. Fuller, C. W., and Richardson, C. C. (1985) Initiation of DNA replication at the primary origin of bacteriophage T7 by purified proteins. Site and direction of initial DNA synthesis. J. Biol. Chem. 260, 3185–3196
7. Hammond, S. M., and Richardson, C. C. (2009) Motors, switches, and contacts in the replisome. Annu. Rev. Biochem. 78, 205–243
8. Brown, T. A., Cecconi, C., Tkachuk, A. N., Bustamante, C., and Clayton, D. A. (2005) Replication of mitochondrial DNA occurs by strand displacement with alternative light-strand origins, not via a strand-coupled mechanism. Genes Dev. 19, 2466–2476
9. Wanrooij, S., Fusté, J. M., Farge, G., Shi, Y., Gustafsson, C. M., and Falkenberg, M. (2008) Human mitochondrial RNA polymerase primers lagging-strand DNA synthesis in vitro. Proc. Natl. Acad. Sci. USA. 105, 11122–11127
10. Foury, F., Roganti, T., Lecrenier, N., and Purnelle, B. (1998) The complete sequence of the mitochondrial genome of Saccharomyces cerevisiae. FEBS Lett. 440, 325–331
11. Baldacci, G., and Bernardi, G. (1982) Replication origins are associated with transcription initiation sequences in the mitochondrial genome of yeast. EMBO J. 1, 987–994
12. de Zamaroczy, M., Faugeron-Fonty, G., Baldacci, G., Goursot, R., and Bernardi, G. (1984) The ori sequences of the mitochondrial genome of a wild-type yeast strain: number, location, orientation and structure. Gene 32, 439–457
13. Biswas, T. K. (1999) Nucleotide sequences surrounding the nonnucleotide promoter motif influence the activity of yeast mitochondrial promoters. Biochemistry 38, 9693–9703
14. Gerhold, J. M., Aun, A., Sedman, T., Joers, P., and Sedman, J. (2010) Strand invasion structures in the inverted repeat of Candida albicans mitochondrial DNA reveal a role for homologous recombination in replication. Mol. Cell. Biol. 39, 851–861
15. Gerhold, J. M., Sedman, T., Visacka, K., Slezakova, J., Tomaska, L., Nosek, J., and Sedman, J. (2014) Replication intermediates of the linear mitochondrial DNA of Candida parapsilosis suggest a common recombination-based mechanism for yeast mitochondria. J. Biol. Chem. 289, 22659–22670
16. Baldacci, G., Chérief-Zahar, B., and Bernardi, G. (1984) The initiation of DNA replication in the mitochondrial genome of yeast. EMBO J. 3, 2115–2120
17. Graves, T., Dante, M., Eisenhour, L., and Christianson, T. W. (1998) Precise mapping and characterization of the RNA primers of DNA replication for a yeast hypersuppressive petite by in vitro capping with guanylyltransferase. Nucleic Acids Res. 26, 1309–1316
18. Sanchez-Sandoval, E., Diaz-Quezada, C., Velazquez, G., Arroyo-Navarro, L. F., Almanza-Martinez, N., Trasvista-Arenas, C. H., and Brieba, L. G. (2015) Yeast mitochondrial RNA polymerase primes mitochondrial DNA polymerase at origins of replication and promoter sequences. Mitochondrion 24, 22–31
19. Yang, X., Chang, H. R., and Yin, Y. W. (2015) Yeast mitochondrial transcription factor Mtf1 determines the precision of promoter-directed initiation of RNA polymerase Rpo41. PLoS ONE 10, e0136879
20. Mungal, D. A., Jang, S. H., and Jaehning, J. A. (1994) Release of the yeast mitochondrial RNA polymerase specificity factor from transcription complexes. J. Biol. Chem. 269, 26568–26574
21. Van Dyck, E., Foury, F., Stillman, B., and Brill, S. J. (1992) A single-stranded DNA binding protein required for mitochondrial DNA replication in S. cerevisiae is homologous to E. coli SSB. EMBO J. 11, 3421–3430
22. Lohman, T. M., and Overman, L. B. (1985) Two binding modes in Escherichia coli single strand binding protein-single stranded DNA complexes. Modulation by NaCl concentration. J. Biol. Chem. 260, 3594–3603
23. Ghosh, S., Hamdan, S. M., and Richardson, C. C. (2010) Two modes of interaction of the single-stranded DNA-binding protein of bacteriophage T7 with the DNA polymerase-thioredoxin complex. J. Biol. Chem. 285, 18103–18112
24. Ghosh, S., Marintcheva, B., Takahashi, M., and Richardson, C. C. (2009) C-terminal phenylalanine of bacteriophage T7 single-stranded DNA-binding protein is essential for strand displacement synthesis by T7 DNA polymerase at a nick in DNA. J. Biol. Chem. 284, 30339–30349
25. Matsunaga, M., Jang, S. H., and Jaehning, J. A. (2004) Expression and purification of wild type and mutant forms of the yeast mitochondrial core RNA polymerase, Rpo41. Protein Expr. Purif. 35, 126–130
26. Tang, G. Q., Paratkar, S., and Patel, S. S. (2009) Fluorescence mapping of the open complex of yeast mitochondrial RNA polymerase. J. Biol. Chem. 284, 5514–5522
28. W. Nilsen, T. (2014) RNase footprinting to map sites of RNA-protein interactions. *Cold Spring Harb. Protoc.* 10.1101/pdb.prot080788

29. Basu, R. S., Warner, B. A., Molodtsov, V., Pupov, D., Esyunina, D., Fernández-Tornero, C., Kubbachinskiy, A., and Murakami, K. S. (2014) Structural basis of transcription initiation by bacterial RNA polymerase holoenzyme. *J. Biol. Chem.* 289, 24549–24559

30. Davey, S. K., and Faust, E. A. (1990) Murine DNA polymerase. alpha-primase initiates RNA-primed DNA synthesis preferentially upstream of a 3’-CC(C/A)-5’ motif. *J. Biol. Chem.* 265, 3611–3614

31. Yamaguchi, M., Hendrickson, E. A., and DePamphilis, M. L. (1985) DNA primase-DNA polymerase alpha from simian cells: sequence specificity of initiation sites on simian virus 40 DNA. *Mol. Cell. Biol.* 5, 1170–1183

32. Vishwanatha, J. K., Yamaguchi, M., DePamphilis, M. L., and Baril, E. F. (1986) Selection of template initiation sites and the lengths of RNA primers synthesized by DNA primase are strongly affected by its organization in a multiprotein DNA polymerase alpha complex. *Nucleic Acids Res.* 14, 7305–7323

33. Holmes, A. M., Cheriathundam, E., Bollum, F. J., and Chang, I. M. (1985) Initiation of DNA synthesis by the calf thymus DNA polymerase-primase complex. *J. Biol. Chem.* 260, 10840–10846

34. Parker, W. B., and Cheng, Y. C. (1987) Inhibition of DNA primase by nucleoside triphosphates and their arabinofuranosyl analogs. *Mol. Pharmacol.* 31, 146–151

35. Heller, R. C., and Marins, K. J. (2006) Replication fork reactivation downstream of a blocked nascent leading strand. *Nature* 439, 557–562

36. Bianchi, J., Rudd, S. G., Jozwiakowski, S. K., Bailey, L. J., Soura, V., Taylor, E., Stevanovic, I., Green, A. J., Stracker, T. H., Lindsay, H. D., and Doherty, A. J. (2013) PrimPol bypasses UV photoproducts during eukaryotic chromosomal DNA replication. *Mol. Cell* 52, 566–573

37. García-Gómez, S., Reyes, A., Martínez-Jiménez, M. I., Chocrón, E. S., Mourón, S., Terrados, G., Powell, C., Salido, E., Méndez, J., Holt, J. J., and Blanco, L. (2013) PrimPol, an archaic primase/polymerase operating in human cells. *Mol. Cell* 52, 541–553

38. Helleday, T. (2013) PrimPol breaks replication barriers. *Nat. Struct. Mol. Biol.* 20, 1348–1350

39. Im, J. S., Lee, K. Y., Dillon, L. W., and Dutta, A. (2013) Human Primpol: a novel guardian of stalled replication forks. *EMBO Rep.* 14, 1032–1033

40. Keen, B. A., Jozwiakowski, S. K., Bailey, L. J., Bianchi, J., and Doherty, A. J. (2014) Molecular dissection of the domain architecture and catalytic activities of human PrimPol. *Nucleic Acids Res.* 42, 5830–5845

41. Mourón, S., Rodríguez-Acebes, S., Martínez-Jiménez, M. I., García-Gómez, S., Chocrón, E. S., Blanco, L., and Méndez, J. (2013) Repriming of DNA synthesis at stalled replication forks by human PrimPol. *Nat. Struct. Mol. Biol.* 20, 1383–1389

42. Wan, L., Lou, J., Xia, Y., Su, B., Liu, T., Cui, J., Sun, Y., Lou, H., and Huang, J. (2013) hPrimpol/CCDC111 is a human DNA primase-polymerase required for the maintenance of genome integrity. *EMBO Rep.* 14, 1104–1112

43. Guilliam, T. A., Jozwiakowski, S. K., Ehlinger, A., Barnes, R. P., Rudd, S. G., Bailey, L. J., Skehel, J. M., Eckert, K. A., Chazin, W. J., and Doherty, A. J. (2015) Human PrimPol is a highly error-prone polymerase regulated by single-stranded DNA binding proteins. *Nucleic Acids Res.* 43, 1056–1068

44. Ramanagoudr-Bhojappa, R., Blair, L. P., Tackett, A. J., and Raney, K. D. (2013) Physical and functional interaction between yeast Pif1 helicase and Rim1 single-stranded DNA binding protein. *Nucleic Acids Res.* 41, 1029–1046

45. Tang, G. Q., and Patel, S. S. (2006) T7 RNA polymerase-induced bending of promoter DNA is coupled to DNA opening. *Biochemistry* 45, 4936–4946