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

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Primases use single-stranded (ss) DNAs 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)[2] 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

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[2] The abbreviations used are: ss, single-stranded; mt, mitochondrial; nt, nucleotide; NTP, nucleotide triphosphate; RNAP, RNA polymerase; SSB, single stranded binding protein.

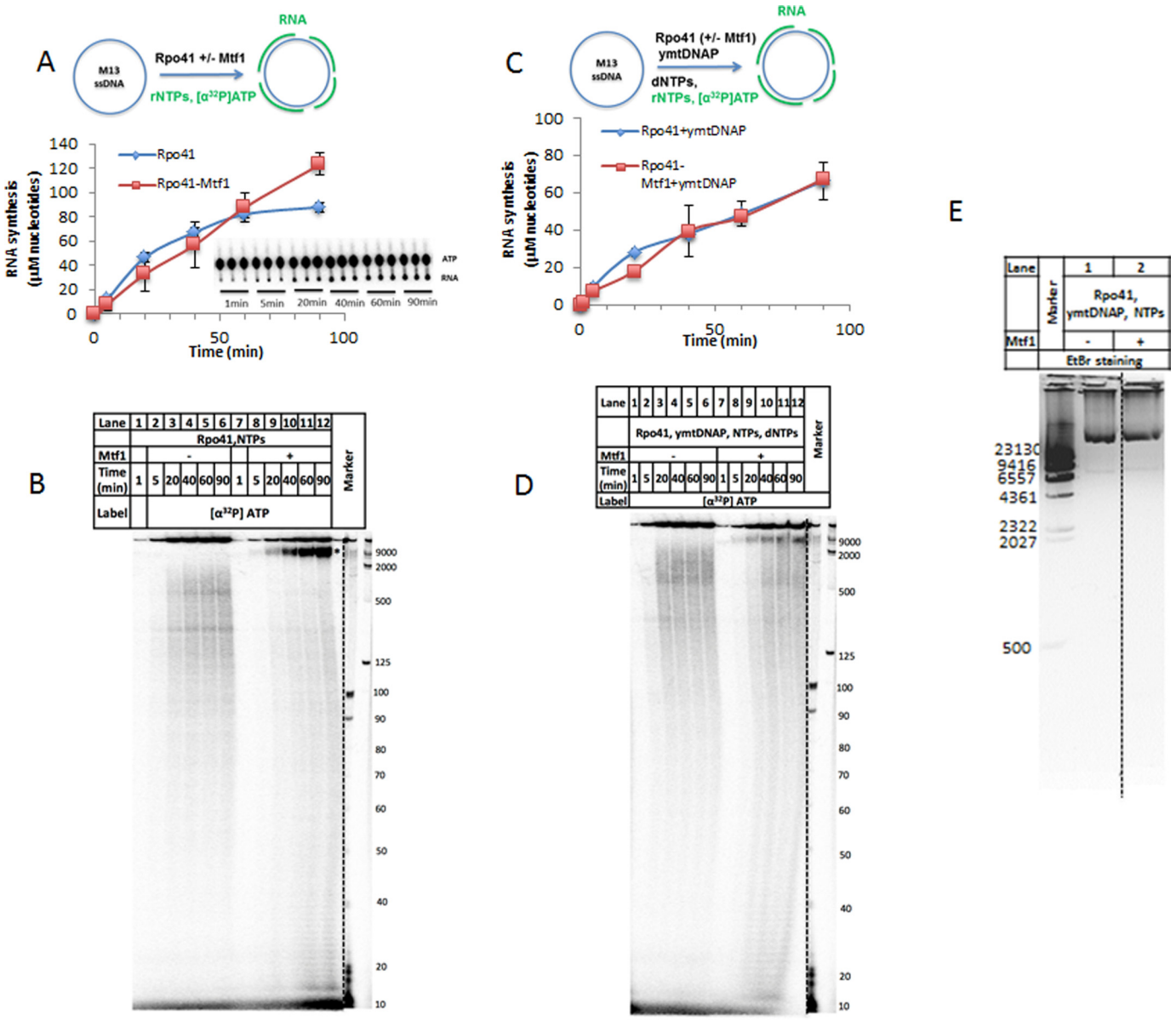

FIGURE 1. **Rpo41 and Rpo41-Mtf1 synthesize RNA on M13 ssDNA template.** *A*, RNA synthesis was carried out using Rpo41 (100 nM) with and without Mtf1 (200 nM), 10 nM M13 ssDNA in the presence of all four rNTPs (250 μM) spiked with [α^{32}P]ATP for varying times (0, 1, 5, 20, 40, 60, and 90 min) at 30 °C. Quantitation of total RNA synthesis as nucleotides incorporated into RNA was obtained from the TLC assay. An example of TLC image is shown as an *inset*. *B*, reaction products were analyzed by PAGE (10% acrylamide/7 M urea). *Asterisk* mark shows the accumulation of single round of rolling circle RNA synthesis product (~7 kb). *C*, RNA synthesis was carried out under the same conditions as *A*, but with added four dNTPs (250 μM) and ymtDNAP (100 nM). The quantitation of total RNA synthesis (*C*) and PAGE analysis (*D*) is shown. *E*, shows the nucleic acid products resolved on the 1% agarose gel stained with ethidium bromide. The *error bars* depicted are from two independent experiments.

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 [α^{32}P]ATP for RNA detection (Fig. 1*A*). 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 in Fig. 1*A*. RNA synthesis is reported as the μM amount of total nucleotides incorporated into the RNA as a function of time (Fig. 1*A*). 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

linearly (Fig. 1*A*). 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. 1*B*). 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. 1*B*). It was difficult to estimate the exact size of the RNA product due to the different 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^{32}P]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. 1*C*). 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. 1*D*). 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. 1*E*).

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. 2*A*) and polyacrylamide/urea gel (Fig. 2*B*) 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. 2*B*). 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^{32}P]dGTP for DNA detection. The TLC quantitation shows robust DNA synthesis in reactions containing Rpo41 and Rpo41-Mtf1 (Fig. 4*A*). Control experiments showed no DNA synthesis without ymtDNAP and negligible synthesis with ymtDNAP alone (Fig. 4*B*). 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. 4*C*). The agarose gel shows that the nucleic acid products are >23 kb in length (Fig. 4*D*). 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 those are long RNA products. Interestingly, the sizes of >23 kb products did not change, which suggests that these are

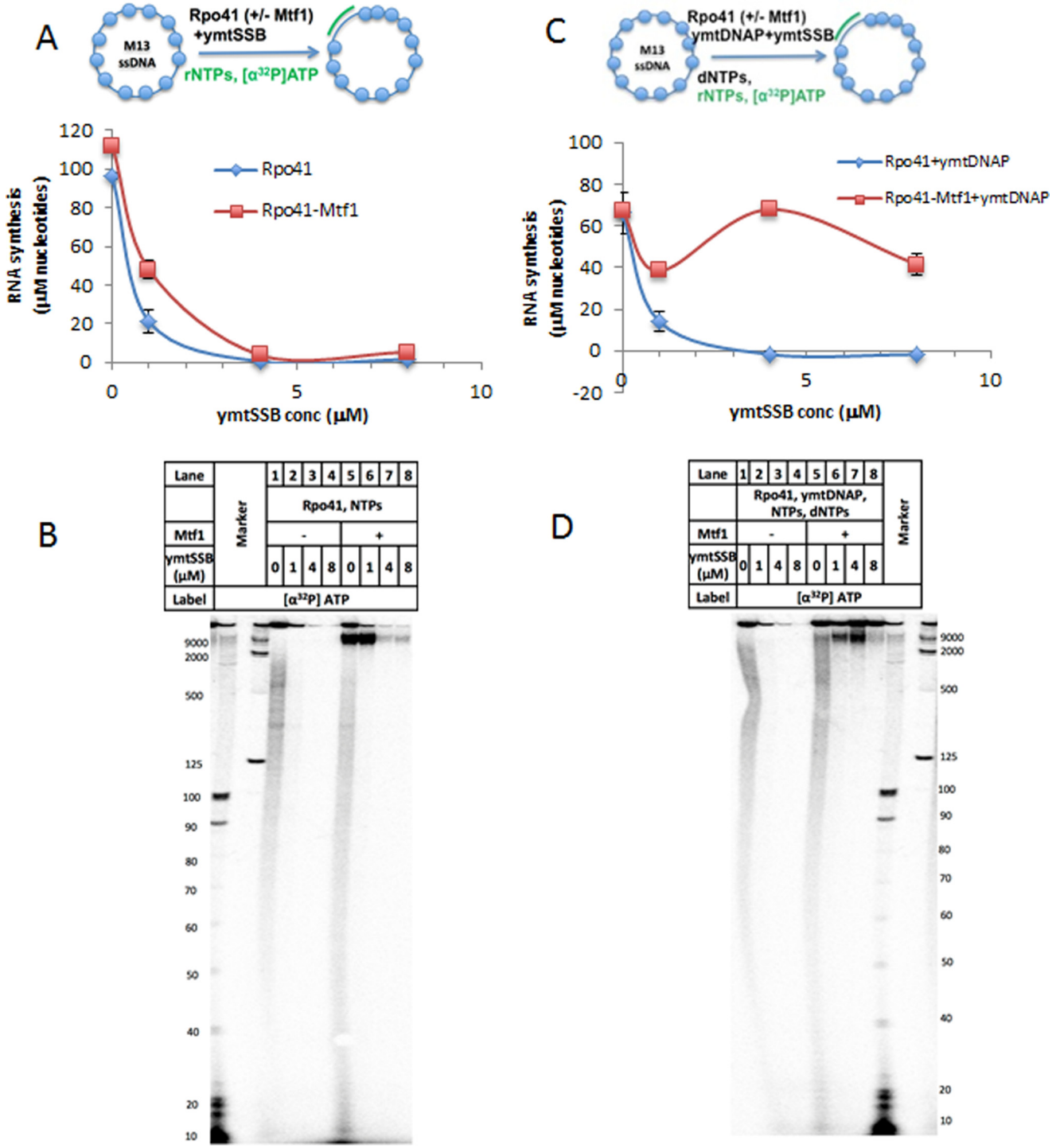

FIGURE 2. **Effect of ymtSSB on the RNA synthesis activity of Rpo41 and Rpo41-Mtf1 on M13 ssDNA template.** *A*, RNA synthesis was carried out under the same conditions as Fig. 1*A*, but in the presence of increasing amount of ymtSSB. Quantitation of total RNA synthesis in the presence of ymtDNAP and ymtSSB obtained from the TLC assay. *B*, reaction products were analyzed by PAGE (10% acrylamide/7 M urea). *C*, RNA synthesis was carried out under the same conditions as Fig. 1*C*, but in the presence of increasing amount of ymtSSB. Quantitation of total RNA synthesis in the presence of ymtDNAP and ymtSSB obtained from the TLC assay. *D*, reaction products were analyzed by PAGE (10% acrylamide/7 M urea). The *error bars* depicted are from two independent experiments.

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 [α^{32}P]dGTP at increasing concentrations of ymtSSB (0, 1, 4, and 8 μM tetramer). The ymtSSB at 1 μM 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,

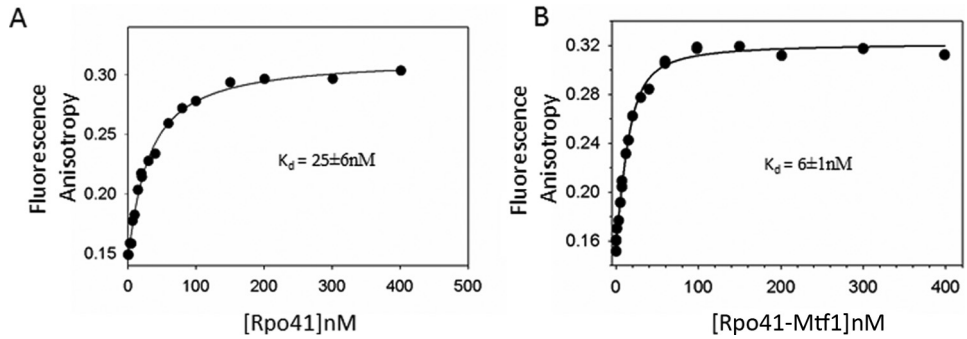

FIGURE 3. **Equilibrium binding of Rpo41 and Rpo41-Mtf1 to a random 20mer ssDNA by fluorescence anisotropy.** The TAMRA-labeled random 20 mer ssDNA was titrated with increasing concentrations of Rpo41 (*A*) and equimolar mixture of Rpo41-Mtf1 complex (*B*). Fluorescence anisotropy was measured after 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.

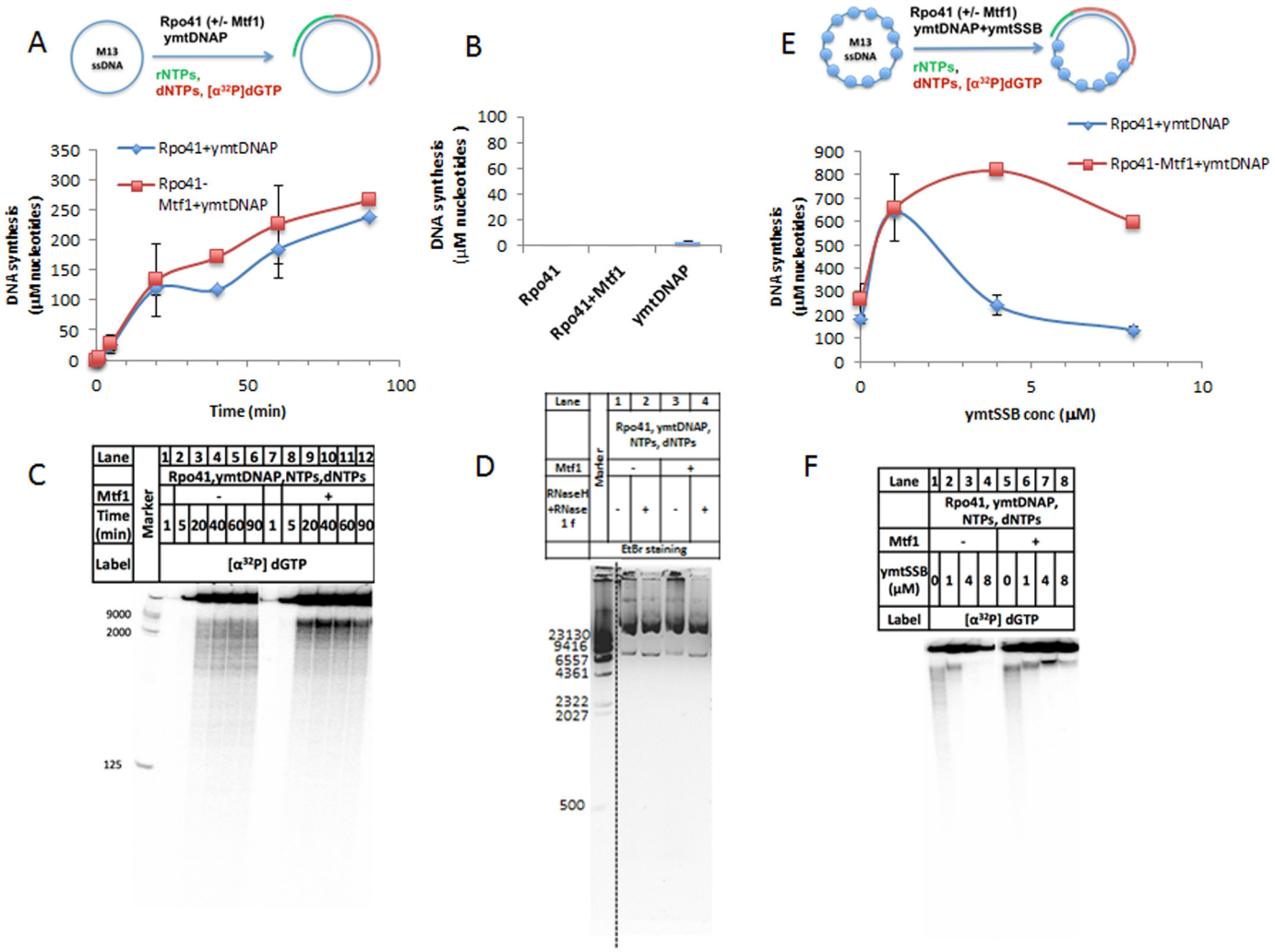

FIGURE 4. **RNA primers synthesized by Rpo41 and Rpo41-Mtf1 can be extended by the yeast mitochondrial DNA polymerase, Mip1.** *A*, ymtDNAP (100 nM) was incubated with M13 ssDNA (10 nM) in the presence of Rpo41 (100 nM) or Rpo41-Mtf1 (100 nM) and 250 μM of each rNTP and dNTP spiked with [α^{32}P]dGTP 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.

24, 25). 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.

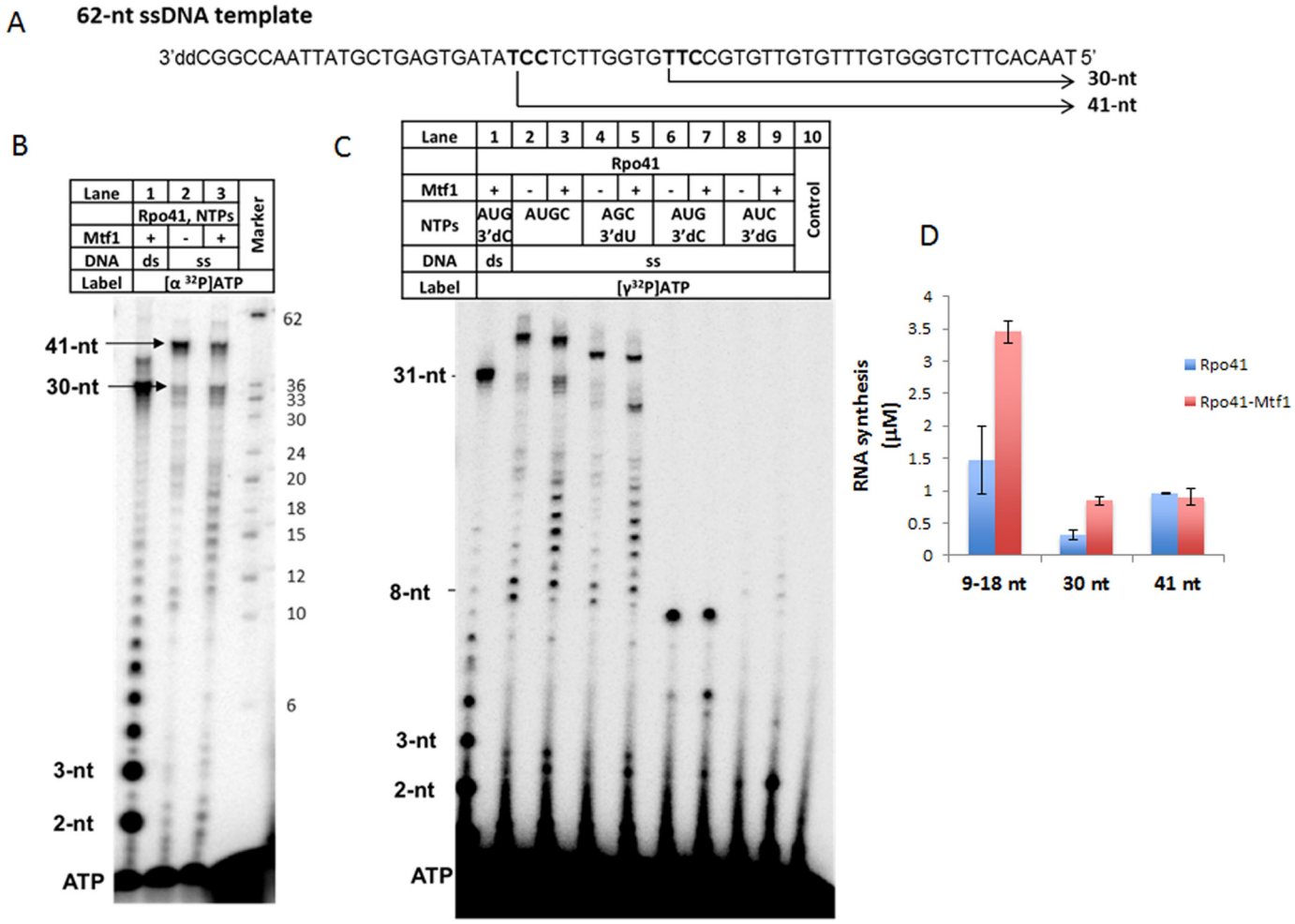

FIGURE 5. **Start-site mapping of RNA synthesis by Rpo41 and Rpo41-Mtf1on ssDNA template.** *A*, sequence of the 62-nt nonspecific ssDNA lacking the yeast mtDNA promoter sequence. The start sites used by Rpo41 and Rpo41-Mtf1 for RNA synthesis were determined and are shown in *bold*. *B*, RNA synthesis 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 [α^{32}P]ATP. The 24% polyacrylamide urea-sequencing gel shows the 30 and 41-nt RNA products. The 15S rRNA ymtDNA 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 [γ^{32}P]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.

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. 5*A*). 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 [α^{32}P]ATP (Fig. 5*B*) or [γ^{32}P]ATP (Fig. 5*C*). As a control and a marker, we used the yeast 15S rRNA double-stranded promoter DNA (sequence under "Experimental Procedures") that makes a 31-nt runoff RNA. Rpo41 alone is not active on the promoter dsDNA (21, 26, 27), but Rpo41-Mtf1 makes short abortive products and the expected 31-mer runoff product from the promoter dsDNA (Fig. 5, *B* and *C*, *lane 1*).

In contrast to the dsDNA promoter, the ssDNA serves as the template for both Rpo41 and Rpo41-Mtf1 for RNA synthesis (Fig. 5, *B* and *C*, *lanes 2* and *3*). Additionally, the RNA products made by both enzymes are of defined lengths, which is an indication that transcription is initiated from specific sites on this nonspecific ssDNA template. If initiation were not sequence specific and randomly initiated on ssDNA, then we would have observed a ladder of RNA products and not the distinct products observed here. The transcription profile was almost indistinguishable when [γ^{32}P]ATP or [α^{32}P]ATP was used as the initiating nucleotide (compare *lanes 2* and *3* in Fig. 5, *B* and *C*), which indicates that Rpo41 and Rpo41-Mtf1 prefer to initiate RNA synthesis on ssDNA using ATP as the initiating nucleotide, which is similar to the preference of initiating with ATP on dsDNA promoter substrates.

There are two major long RNA products made on ssDNA template and several short RNA products between 9 and 18-nt in length (Fig. 5, *B* and *C*). Using molecular markers and the fact that RNA synthesis initiates with ATP (with [γ^{32}P]ATP as spike), we estimate that the lengths of the long RNA products are ~41-nt and ~30-nt initiating from the indicated TCC and TTC sites (Fig. 5*A*). The 31-nt run-off transcription product from the dsDNA promoter substrate is a good marker (Fig. 5, *B* and *C* compare *lanes 1 to lanes 2 and 3*). Further characterization, as detailed below, indicated that the ~30-nt and ~41-nt

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. 5*A*). 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. 5*C*, 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. 5*C*), 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. 5*A*). The initiation specificity of Rpo41 and Rpo41-Mtf1 is slightly different. Both make almost equal amounts of the ~41-nt RNA product from TCC but Rpo41-Mtf1 makes more of the ~30-nt from TTC and more of the shorter 9–18 nt aborted or stalled RNA products (Fig. 5*D*).

To further validate these start sites, we carried out walking experiments by using additional terminating nucleotides, 3′-dCTP and 3′-dGTP (Fig. 5*C*, *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 TCC is a better initiation site for Rpo41-Mtf1 as compared with TTC, which is also consistent with the relative intensities of the 41-nt and 30-nt RNAs (Fig. 5*D*). 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 TCC followed by TTC.

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^{32}P]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. 6*A*, *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. 6*A*, *lane 2*), which we determine are from initiation sites CC, TTC, and TCC start sites, respectively (marked on the 62-nt ssDNA sequence).

Next we carried out reactions in the presence of [α^{32}P]ATP as the spike to detect both the RNA and RNA-DNA products (Fig. 6*B*). The RNA and RNA-DNA products of the same length appear as doublets: the faster migrating band (Fig. 6*B*, *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. 6*B*, *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. 6*B*, *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 polypyrimidine tract. We identify three start sites TCC, TTC 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^{32}P]dATP labeled RNA-primed DNA products (Fig. 6*A*) 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

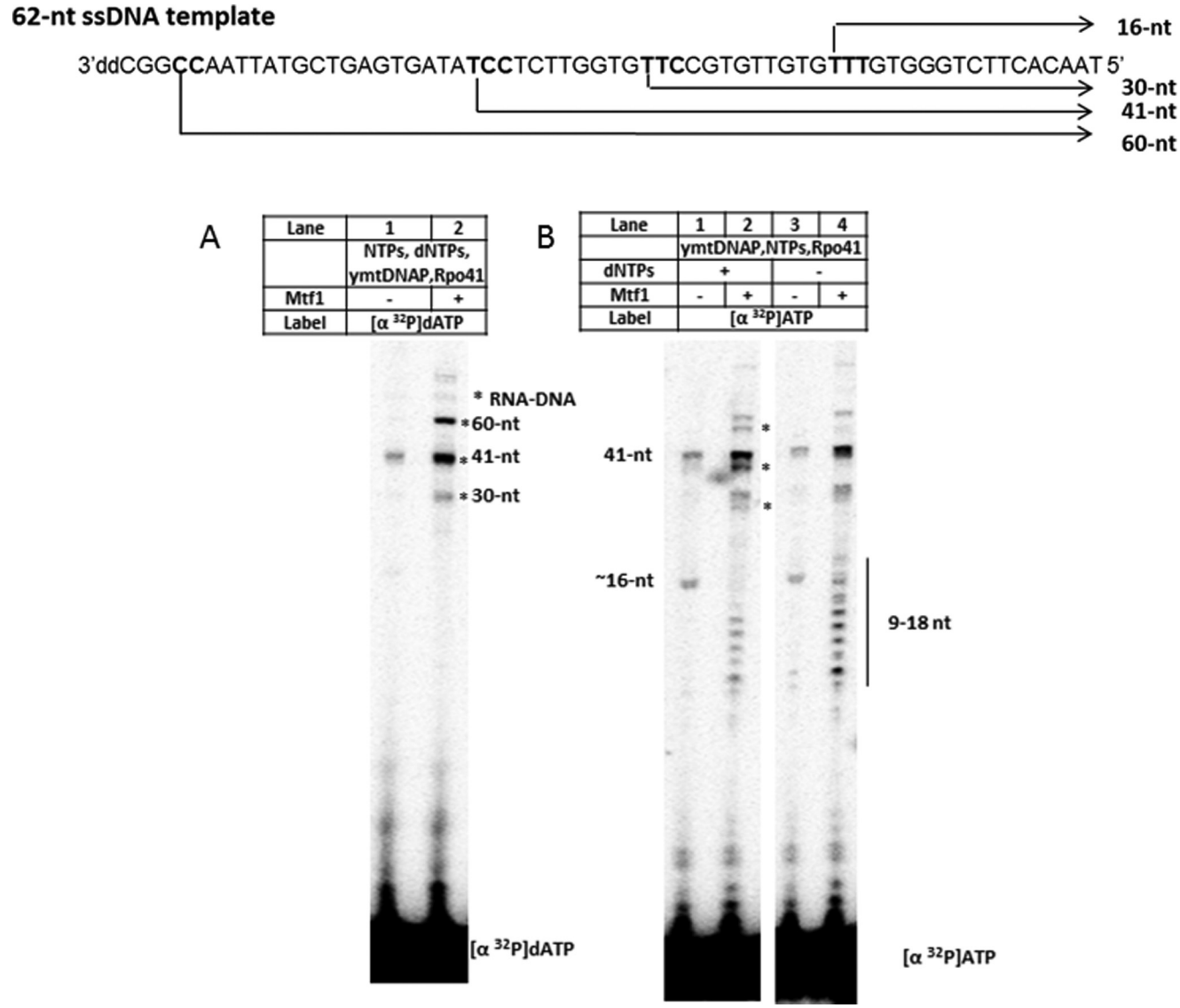

FIGURE 6. **Rpo41-Mtf1 makes short transcripts that are utilized by the ymtDNAP Mip1 for DNA synthesis.** *A*, DNA synthesis under the same conditions as in Fig. 5*B* was carried out on the 62-nt ssDNA template in the presence of Rpo41 or Rpo41-Mtf1, rNTPs, dNTPs, ymtDNAP and spiked with [α^{32}P]dATP. 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. 5*B* were carried out in presence and absence of dNTPs spiked with [α^{32}P]ATP. The faster migrating band corresponds to the RNA-primed DNA product (*lane 2*, *asterisk marked*), and the slower band corresponds to the RNA.

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 polypyrimidine tracts. We mapped three

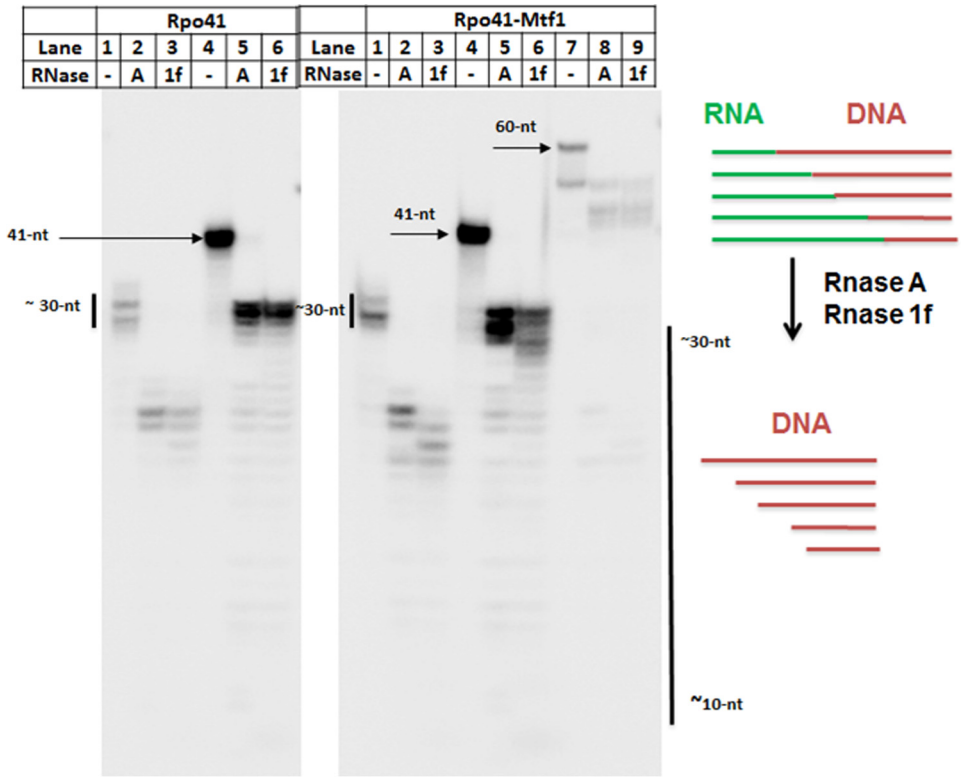

FIGURE 7. **10–12 nt length RNA products are preferentially used as primers by the ymtDNAP Mip1.** The length(s) of the RNA primers used by the ymtDNAP for DNA synthesis were determined by treating the [α^{32}P]dATP labeled RNA-primed DNA products with RNase A and 1f. The length of the RNA primer was 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).

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, this activity is inhibited in the presence of ymtSSB. The inhibition was more severe in the Rpo41 reactions as compared with the Rpo41-Mtf1 reactions, wherein long RNAs were observed even in the presence of the highest 8 μM ymtSSB. Part of the reason for less inhibition of RNA synthesis by Rpo41-Mtf1 is its higher affinity for ssDNA as compared with Rpo41. With higher ssDNA binding affinity, Rpo41-Mtf1 would be able to better compete with ymtSSB for ssDNA binding to enable RNA synthesis. Interestingly, the RNA synthesis activity of Rpo41-Mtf1 was restored to levels observed without ymtSSB when ymtDNAP was present, which was not the case with the activity of Rpo41 alone, which remained severely inhibited. The exact mechanism of stimulation of RNA synthesis activity of Rpo41-Mtf1 by ymtDNAP is not known and will require further studies.

As primases, both Rpo41 and Rpo41-Mtf1 are equally efficient at priming DNA synthesis when ymtSSB was absent. 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

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′-ATAATTTATTTATTATTATATAAGTAATAAATAATTGTTTTATATAATAAGAATTCC-3′; 3′-TATTAAATAAATAATAATATTCATTATTTATTAACAAAATATATTATTCTTAAGG-5′.

The sequence of 62-nt ssDNA template that lacks the yeast mtDNA consensus nonanucleotide promoter sequence is shown below. The dideoxy CTP was placed at the 3′-end of the template to prevent non templated base addition by DNA polymerase: 3′-ddCGGCCAATTATGCTGAGTGATATCCTCTTGGTGTTCCGTGTTGTGTTTGTGGGTCTTCACAAT-5′.

The sequence of a random 20nt ssDNA template used for fluorescence anisotropy studies is shown below: 5′-TAMRA-TAGCGTACTAGCAGCGAAGT-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 $OD_{600\ nm}$ 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. Rim I 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 [α^{32}P]ATP (transcription) or [α^{32}P]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 7 M urea sequencing gel to resolve the individual products. The gel or TLC plate was exposed to a phosphor screen, scanned on Typhoon 9410 phosphorimager 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 RNase1f (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 [γ^{32}P]ATP or [α^{32}P]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 mM magnesium acetate, 250 μM of each NTP and dNTPs spiked with [α^{32}P]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 If (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 spectrofluorimeter 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),

$$r_{\mathrm{obs}} = \frac{(r_{\mathrm{b}} - r_{\mathrm{f}})(K_{\mathrm{d}} + D_{\mathrm{t}} + P_{\mathrm{t}}) - \sqrt{(K_{\mathrm{d}} + D_{\mathrm{t}} + P_{\mathrm{t}})^2 - 4D_{\mathrm{t}}P_{\mathrm{t}}}}{2D_{\mathrm{t}}} + r_{\mathrm{f}} \quad \text{(Eq. 1)}$$

where r_{obs}, r_b, r_f, 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. G. Q. T. performed fluorescence anisotropy experiment. A. R. and S. S. P. wrote the manuscript. A. D. provided critical review of the manuscript. All authors approved the final version of the manuscript.

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