The N-terminal Domain of the Yeast Mitochondrial RNA Polymerase Regulates Multiple Steps of Transcription*

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Transcription of the yeast (Saccharomyces cerevisiae) mitochondrial (mt) genome is catalyzed by nuclear-encoded proteins that include the core RNA polymerase (RNAP) subunit Rpo41 and the transcription factor Mtf1. Rpo41 is homologous to the single-subunit bacteriophage T7/T3 RNAP. Its ∼80-kDa C-terminal domain is highly conserved among mt RNAPs, but its ∼50-kDa N-terminal domain (NTD) is less conserved and not present in T7/T3 RNAP. To understand the role of the NTD, we have biochemically characterized a series of NTD deletion mutants of Rpo41. Our studies show that NTD regulates multiple steps of transcription initiation. Interestingly, NTD functions in an autoinhibitory manner during initiation, and its partial deletion increases the efficiency of RNA synthesis. Deletion of 1–270 amino acids (DN270) decreases abortive synthesis and increases full-length to abortive RNA ratio relative to full-length (FL) Rpo41. A larger deletion of 1–380 amino acids (DN380), decreases RNA synthesis on duplex but not on premelted promoter. We show that DN380 is defective in promoter opening near the transcription start site. Most strikingly, both DN270 and DN380 catalyze highly processive RNA synthesis on the premelted promoter, and unlike the FL Rpo41, the mutants are not inhibited by Mtf1. Both mutants show weaker interactions with Mtf1, which explains many of our results, and particularly the ability of the mutants to efficiently transition from initiation to elongation. We propose that in vivo the accessory proteins that bind NTD may modulate interactions of Rpo41 with the promoter/Mtf1 to activate and allow timely release from Mtf1 for transition into elongation.

The mitochondrial (mt)2 genome of the yeast (Saccharomyces cerevisiae) is transcribed by nuclear DNA-encoded RNA polymerase (RNAP) subunits that produce ribosomal RNAs, transfer RNA, and mRNAs of the proteins of the oxidative phosphorylation machinery (1). The yeast transcription machinery consists of the core RNAP subunit, a 153-kDa protein called Rpo41, and the transcription factor, a 40-kDa protein called Mtf1 (2–5). Rpo41 is evolutionarily related to the C-terminal domain of Rpo41. A larger deletion of 1–380 amino acids (DN380), decreases RNA synthesis on duplex but not on premelted promoter. We show that DN380 is defective in promoter opening near the transcription start site. Most strikingly, both DN270 and DN380 catalyze highly processive RNA synthesis on the premelted promoter, and unlike the FL Rpo41, the mutants are not inhibited by Mtf1. Both mutants show weaker interactions with Mtf1, which explains many of our results, and particularly the ability of the mutants to efficiently transition from initiation to elongation. We propose that in vivo the accessory proteins that bind NTD may modulate interactions of Rpo41 with the promoter/Mtf1 to activate and allow timely release from Mtf1 for transition into elongation.

The yeast Rpo41 NTD has been implicated previously to play a role in RNA processing and translation (8). However, whether the NTD of the Rpo41 plays a role in transcription related functions has not been determined. Deletion of 1–200 amino acids (aa) causes temperature sensitive petite phenotype in S. cerevisiae and results in mitochondrial genome instability, whereas deletions beyond 200 aa result in the Rpo41 null phenotype (9). Subsequently, it was shown that the region 118–208 NTD of the Rpo41 harbors a binding site for the Nam1p protein, which is involved in RNA processing/translation (10). A model was proposed that the NTD-Nam1p interaction localizes the mt RNAP to the inner mitochondrial membrane, where transcription is coupled efficiently to RNA processing and translation to assure proper gene expression (8). A recent pulldown study has identified several other mt proteins that might associate with Rpo41 (11).

Unlike its phage counterpart, the mt RNAPs require transcription factors to catalyze promoter-specific initiation. The yeast Rpo41 requires only one transcription factor, Mtf1, for promoter-specific transcription. It has been suggested that the NTD of Rpo41 may be involved in interactions with the Mtf1 (12). However, this has not been established. Specific deletions of the N- and C-terminal regions of Rpo41 resulted in a loss of complex formation between the two proteins. Based on these results, it was suggested that multiple regions of Rpo41 are involved in complex formation with Mtf1.

One of the more established functions of Mtf1 is to catalyze promoter-specific melting of the duplex DNA. Fluorescence-based studies have shown that the Rpo41-Mtf1 complex melts the promoter from −4 to +2, but Rpo41 alone does not melt the promoter (13). Recent cross-linking studies indicated that Mtf1 interacts with the promoter DNA via its C-terminal amino acids, and these interactions might aid in promoter opening (14, 15). Earlier studies have shown that although Mtf1 is present in the initiation complex with Rpo41, it is released after 13-mer RNA synthesis (16). Because 8–12-mer RNA synthesis is typically the stage when the RNAP undergoes transition from initiation to elongation (17–20), it was proposed that Mtf1 is released from Rpo41 during the transition into elongation (16). This is analogous to the release of σ factor in the bacterial RNAP (21, 22).
Role of the NTD of Rpo41 in Transcription

To understand the role of the NTD of Rpo41 in transcription, we systematically deleted the N-terminal region of Rpo41 by 100, 270, and 380 amino acids (DN100, DN270, and DN380) (Fig. 1A) and studied the transcriptional properties of the purified truncated Rpo41 proteins. Based on previously reported studies (9, 12), we hypothesized that the deletion of 100 aa from the NTD should minimally affect the transcription activity of Rpo41, but deletions larger than ~200 aa would have significant effects on the transcription activity. Deletion of 380 creates a minimal catalytic domain of Rpo41, within 100 aa of the predicted sequence similarity between Rpo41 and T7 RNAP (14), and therefore, its characterization was particularly interesting. Our detailed biochemical investigation of DN100, DN270, and DN380 proteins shows that the NTD of Rpo41 is autoinhibitory in superior RNAP with increased processivity and the ability to make the transition into elongation with little abortive synthesis. Our studies suggest that the NTD plays a regulatory role during transcription initiation, and its displacement is critical for efficient transition from initiation to elongation.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of NTD Mutants—The following 5’- and 3’-PCR primers were used to create the DNA fragments for the deletion mutants of Rpo41. 5’-primers were as follows: DN100, 5’-ATT TCA GCT AGG CCT TCA GCA GTA ACC TCG ATG ACA AGA; DN270, ATT TCA GCT AGG CCT TCT CCT ACA CCT GTA AAT AAT ATT; and DN380, ATT TCA GCT AGG CCT GAG CAC GAT AAT AAT TCT ATA AAT; the 3’-primer was 5’-GCC AGA TTC GAG CTC TCA CGA GAA AAA ATA TTG ACT GTT.

The PCR fragments were digested with Stul and SacI and inserted into the pJJ1399 vector (3) digested with the same enzymes. The plasmids that showed the correctly sized restricted bands were fully sequenced to confirm the deletion mutants. The expression and purification of the N-terminal deletion mutants of Rpo41 was similar to Rpo41 described previously (13). The molar concentration of the purified proteins was determined from absorbance measurement at 280 nm in the guanidinium HCl buffer and the extinction coefficient of the protein (supplemental Fig. 1B).

Transcription Assays—FL Rpo41 or the NTD mutant was mixed with Mtf1 and promoter DNA, either duplexed (U25D32ds) or premelted (U25D32JB), in buffer containing 50 mM Tris acetate, pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate, 0.05% Tween 20, and 1 mM DTT. The oligonucleotides for the promoter were purchased from Integrated DNA Technologies and PAGE-purified. Transcription reaction at 25 °C was initiated by mixing the protein-DNA complex with 250 μM ATP, UTP, and GTP each, spiked with [γ-32P]ATP. Reactions were stopped at predetermined time periods with 400 mM EDTA and formamide dye (98% formamide, 0.025% bromophenol blue, 10 mM EDTA), heated to 95 °C for 5 min, and analyzed on 23% sequencing gel (19:1 acrylamide to bis-acrylamide) with 4 M urea. The gel was exposed to a phosphor screen overnight and scanned on a Typhoon 9410 PhosphorImager (Amersham Biosciences). The free ATP and RNA bands were quantified using ImageQuant.

Promoter DNA Melting and Binding—Fluorescence measurements were carried out at 25 °C on a Fluoro-Max-4 spectrophluorimeter (Jobin Yvon-Spex Instruments S.A., Inc.). Duplex promoter DNA (200 nM) substituted with 2-aminopurine (2-AP) at position −4 of the NT strand (−4NT), −1 of the T strand (−1T), or +2 of the NT strand (+2NT) (13) was mixed with 200 nM Mtf1 in 50 mM Tris acetate, pH 7.5, 100 mM potassium glutamate, and 10 mM magnesium acetate. To this, 400 nM FL Rpo41, DN100, or DN270, or 600 nM DN380 was added, and the sample was excited with 315 nm light, and the fluorescence intensity was collected from 360 to 380 nm. Protein fluorescence contribution was corrected by conducting experiments with unmodified promoters (without 2-AP). The corrected fluorescence intensity was divided by the corrected fluorescence intensity of free 2-AP DNA (fluorescence intensity of 2-AP DNA minus intensity of non-2-AP DNA) to determine the fold increase in 2-AP fluorescence at each position.

To analyze the binding of Rpo41-Mtf1 with the promoter DNA, fluorescence titrations were performed by adding increasing amounts of Rpo41 to a preformed mixture of 200 nM duplex promoter DNA substituted with 2-AP at −4NT and 200 nM Mtf1. The fluorescence intensity was measured as described above and plotted against total [Rpo41]. Data were fitted to a quadratic equation (19) to determine the equilibrium dissociation constant ($K_d$).

**ATP Binding**—A mixture of 2-AP (−1T) dsDNA (200 nM), Mtf1 (200 nM), and 400 nM FL Rpo41, 400 nM DN100, 400 nM DN270, or 600 nM DN380 in 50 mM Tris acetate, pH 7.5, 100 mM potassium glutamate, and 10 mM magnesium acetate was titrated with increasing [ATP]. Fluorescence was recorded, as described above, after addition of ATP in small aliquots up to a concentration of 600–800 μM. After subtracting the contributions from buffer and protein in experiments carried out in the presence of unmodified DNA, the corrected 2-AP fluorescence intensities between 360 and 380 nm were integrated and plotted as a function of [ATP]. The data were fit to Equation 1 to obtain the ATP $K_d$ values,

$$F_{obs} = F_{max} \times [ATP]/(K_d + [ATP]) + F_o \quad (Eq. 1)$$

where $F_{obs}$ is the observed fluorescence, $F_{max}$ is the final fluorescence intensity, $F_o$ is the background fluorescence, and $K_d$ is the apparent equilibrium dissociation constant of the initially transcribing complex bound to ATP.

Protein-Protein Cross-linking—Dimethyl suberimidate (DMS) solution was prepared freshly by dissolving 10 mg of DMS in 180 μl of 0.15 M ice-cold triethylamine chloride buffer (pH 8.2) and 20 μl of 1 M NaOH to readjust the pH to 8.2. The proteins were preincubated with or without the duplex promoter DNA in triethylamine chloride buffer (pH 8.2) containing 10 mM MgCl₂, 100 mM NaCl, and 1 mM DTT, and the cross-linking reaction was initiated by adding 10 mg/ml DMS at 22 °C. The reaction was quenched after 20 min by adding equal volumes of 1 M glycine. The quenched reactions were mixed with Laemmli dye, heated at 95 °C for 2 min, and analyzed by 4–20% Tris-glycine SDS-PAGE. The proteins were stained with Coomassie dye, and the band intensities were quantified using Alphalager software (Cell Biosciences Corp.). To esti-
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mate the apparent $K_a$ of Rpo41-Mtf1, fraction complex (Equation 2) was plotted against free [Mtf1] and fit to Equation 3,

$$\text{Fractioncomplex} = \frac{C}{(C + R)[\text{Mtf1}]_{\text{free}}} = \frac{[\text{Mtf1}]_{\text{total}}}{K_a} \times (1 - \frac{M}{(M + C)}) \quad \text{(Eq. 2)}$$

$$\text{Fractioncomplex} = \frac{d \times [\text{Mtf1}]_{\text{free}}}{K_a} \times \frac{[\text{Mtf1}]_{\text{free}}}{[\text{Mtf1}]_{\text{free}} + [\text{Mtf1}]_{\text{free}}} \quad \text{(Eq. 3)}$$

where $C$, $M$, and $R$ are band intensities of the Rpo41-Mtf1 complex, uncross-linked Mtf1 and uncross-linked Rpo41, respectively, and where $a$ is the fraction maximum complex.

RESULTS

NTD Deletion Mutants of Rpo41—A series of three N-terminal deletions were created that removed 100, 270, and 380 aa of the Rpo41, and the proteins are designated as DN100, DN270, and DN380, respectively (Fig. 1A). The predicted secondary structure of the NTD of Rpo41 (residues 1–500) was used to choose the deletion sites, such that no predicted helices or $\beta$-sheet regions were disrupted. All of the deletion mutants were bacterially expressed as soluble proteins and purified using the same protocol as the FL Rpo41 (Fig. 1B).

NTD Deletion Mutants of Rpo41 Make Less Abortive Products—Transcription from the promoter was measured using the 57-bp fragment of the yeast 14 S rRNA promoter (U25D32ds) (Fig. 2A). In these experiments, FL Rpo41 or the NTD mutant at 2 $\mu$m was mixed with a slight excess of Mtf1 (2.5 $\mu$m) and U25D32ds (2.5 $\mu$m), and RNA synthesis was measured from 30 s to 5 min in the presence of 250 $\mu$m ATP + [y$^{32}$P]ATP, UTP, and GTP at 25 °C. The RNA products were resolved on a sequencing gel.

Exclusion of CTP from the NTP mix yields a full-length transcript of 30-mer length. Visual inspection of the gel image (Fig. 2B) shows an RNA product of the expected full-length size in FL Rpo41, DN100, and DN270 reactions. A very small amount of the full-length RNA is observed in the DN380 reactions. In addition to the full-length transcript, short RNA transcripts from 2–7-mer are produced over time. These short transcripts are abortive products because they accumulate in large molar excess (10-fold) over the concentration of the DNA-protein complex (2 $\mu$m) (quantitation of the gels shown in supplemental Fig. 2). Interestingly, DN100 and especially DN270 produce lower amounts of these short abortive transcripts as compared with the FL Rpo41.

Thus, Rpo41-Mtf1 shows the typical transcription profile that is observed in all DNA-dependent RNAPs: a distributive phase of initial transcription followed by a processive phase of RNA elongation. The shortest 2-mer is the most abundant abortive RNA product and the longer ones from 3-mer to 7–8-mer are made in progressively decreasing amounts. After 8–12-mer RNA, the synthesis becomes processive, and intermediate RNAs are not visible until 30-mer RNA. These results indicate that similar to phage T7 and bacterial RNAPs (17–20), the mt RNA polymerase may undergo transition from initiation to elongation between 8–12-mer synthesis.

To make a better comparison of the transcriptional properties of the mutants with FL Rpo41, we calculated the abortive to productive ratio ($\mu$M 2–12-mer RNA/$\mu$M full-length RNA) from the molar amounts of abortive and full-length RNA products (supplemental Fig. 2). Longer RNA products >30-mer were observed in FL Rpo41 and to some extent in the DN270 reactions. These longer products appear to be made at the expense of the 30-mer RNA and therefore most likely result from extension after misincorporation and/or from a nontemplated addition to the 30-mer RNA. Therefore, we added up products $\geq$30-mer in our calculation of the full-length RNA.

The abortive to productive RNA synthesis ratio indicates how many abortive events the RNAP goes through before it makes the transition to processive elongation. By our calculation, FL Rpo41 undergoes $\sim$18 abortive events, and DN100 undergoes $\sim$13 for every productive transcription event, but DN270 undergoes only about five abortive events for each productive event (Fig. 2C). These results indicate that DN270 is more efficient than FL Rpo41 or DN100 in making the transition from initiation to elongation.

To determine whether the NTD deletion affects the processivity of initial transcription, we determined what fraction of 2-mer to 7-mer RNA remain unextended from the ratio of $A_N/A_{\leq N}$, where $A$ is the amount of RNA product of length $N$ (Fig. 2D). The unextended fractions are similar for FL Rpo41 and DN100, but $\sim$2 times lower for the DN270 (Fig. 2D), e.g., $\sim$30–40% of 3-mer and $\sim$25% of 4-mer remain unextended in FL Rpo41 and DN100, as compared with 20% of 3-mer and 10% of 4-mer in the DN270 reaction. These calculations indicate that DN270 is more processive during initial transcription and produces less abortive products.
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Rpo41 requires Mtf1 to catalyze promoter-specific transcription initiation on duplex promoters. We investigated whether increasing the concentration of Mtf1 affects the efficiency of initiation. In these experiments, U25D32ds was mixed with FL Rpo41, DN270, or DN380 and Mtf1 (0–12 μM), and transcription activity in 3 min was measured by the gel-based assay. In the absence of Mtf1, very little transcription activity is observed for the proteins, consistent with the requirement of Mtf1 for specific initiation (23). As the Rpo41:Mtf1 ratio approaches 1:1, we see increasing amounts of abortive and full-length RNA products in FL Rpo41 (Fig. 3A, lanes 1–8), and Fig. 3B and C). The products maximized at close to a 1:1 ratio of Rpo41:Mtf1, which is consistent with the proposed 1:1 stoichiometry of the holoenzyme (13, 24). Similar to FL Rpo41, DN270 makes increasing amounts of abortive and full-length RNA with increasing Mtf1 (Fig. 3A, lanes 9–15), but slightly higher amounts of Mtf1 are required to observe maximum full-length synthesis (Fig. 3C). These results suggest that DN270 may interact more weakly with Mtf1.

The abortive to productive ratio provides information on the transition from initiation to elongation, and this remained constant up to 1:1 Mtf1:Rpo41 and then decreased somewhat at higher Mtf1 (Fig. 3D). Overall, the ratio indicates that FL Rpo41 undergoes 20–25 abortive events for every productive RNA synthesis, whereas DN270 is more efficient and undergoes only 5–10 abortive events per productive RNA synthesis, consistent with the results in Fig. 2C. The DN380 is defective in transcribing on the duplex promoter even at high Mtf1 concentrations (Fig. 3A, lanes 16–21).

Overall, the transcription reactions indicate that NTD deletion from 1–270 aa does not affect the catalytic activity of Rpo41. In fact, deletion of this region increases the efficiency of
RNA synthesis and the ability to make the transition from abortive to productive synthesis. The very low activity of DN380 indicates that the NTD region between 270–380 aa plays a critical role in transcription initiation.

**NTD of Rpo41 Interferes with RNA Synthesis on Premelted Promoter**

Rpo41 alone can initiate transcription without requiring Mtf1 if the promoter is premelted from 4 to +2 (25). We tested the transcription activity of FL Rpo41 and NTD mutants on the premelted promoter DNA that contained base changes in the NT strand from 4 to 2 (Fig. 4A). FL Rpo41, DN100, DN270, or DN380 at 2 μM was mixed with 250 μM ATP, UTP, and GTP spiked with [γ-32P]ATP for 3 min at 25 °C. The RNA products were resolved on a 23% polyacrylamide-sequencing gel containing 7 M urea. The ratio of [Mtf1] to [Rpo41] (M:R) is indicated for each lane on the gel. B, molar amounts of abortive products (2–12-mer) were determined from quantification of the RNA products and free ATP and plotted against [Mtf1] for FL Rpo41 (filled circles) and DN270 (blank circles). C, molar amounts of full-length RNAs plotted against [Mtf1] for FL Rpo41 (filled circles) and DN270 (blank circles). D, abortive to productive ratio plotted against [Mtf1] for FL Rpo41 and DN270.

**FIGURE 3. Transcription activity on the duplex promoter at increasing Mtf1 concentrations.** A, FL Rpo41, DN270, or DN380 (3 μM) and U25D32ds (4 μM) were mixed with increasing Mtf1 (0–12 μM) and reacted with 250 μM ATP, UTP, and GTP spiked with [γ-32P]ATP for 3 min at 25 °C. The RNA products were resolved on a 23% polyacrylamide-sequencing gel containing 7 M urea. The ratio of [Mtf1] to [Rpo41] (M:R) is indicated for each lane on the gel. B, molar amounts of abortive products (2–12-mer) were determined from quantification of the RNA products and free ATP and plotted against [Mtf1] for FL Rpo41 (filled circles) and DN270 (blank circles). C, molar amounts of full-length RNAs plotted against [Mtf1] for FL Rpo41 (filled circles) and DN270 (blank circles). D, abortive to productive ratio plotted against [Mtf1] for FL Rpo41 and DN270.

RNA synthesis by FL Rpo41 and to a lesser extent by DN100 is nonprocessive and transcripts of all lengths from 2-mer to 24-mer accumulate in these reactions (Fig. 4B, lanes 1 and 2). On the other hand, transcription by DN270 is highly processive on the premelted promoter, and we observe accumulation of some 2-mer and 11-mer, but mostly the full-length transcript (Fig. 4B, lane 3). Interestingly, DN380, which was defective on the duplex promoter (Figs. 2B and 3A), is quite active on the premelted promoter; DN380 makes some 2-mer but mostly the full-length transcript (Fig. 4B, lane 4). Thus, on a premelted promoter, both DN270 and DN380 are very active, and compared with FL Rpo41, the NTD mutants make full-length RNA with much shorter abortive products.

When Mtf1 was added to the above reactions, RNA synthesis by FL Rpo41 was inhibited severely (Fig. 4B, lane 5), consistent with a previous report (25). The same trend was observed with DN100 (Fig. 4B, lane 6). Both FL Rpo41 and
DN100 makes short RNA products on the pre-melted promoter, but the synthesis of long RNA products is inhibited severely with Mtf1. This inhibition was not observed with DN270 or DN380 (Fig. 4B, lanes 7 and 8). Slightly higher amounts of abortive products were observed in DN270 and DN380 with Mtf1, but the amount of full-length RNA did not decrease.

Transcription was measured on the pre-melted promoter at the increasing Mtf1 concentration. In the absence of Mtf1, FL Rpo41 makes 30-mer RNA and beyond, but RNA synthesis is highly distributive (Fig. 4C, lane 1). Increasing amount of Mtf1 decreases 2–12-mer by 50% (Fig. 4D), but a more severe decrease is observed in full-length RNA products (Fig. 4E).

Thus, at 1:1 ratio of FL Rpo41:Mtf1 and beyond, only short RNA products 2–12-mer are observed (Fig. 4C, lanes 2–7). DN270 and DN380 carry out processive RNA synthesis on the pre-melted promoter, both in the absence and in the presence of Mtf1 (Fig. 4C, lanes 8–14). There is a 2- to 2.5-fold increase in short abortive products in the presence of Mtf1 (Fig. 4D), but the yield of full-length RNA synthesis is not affected (Fig. 4E). An 11-mer RNA accumulates in both DN270 and FL Rpo41 reactions, whose origin is unclear (Fig. 4, B and C). The 14S rRNA promoter is highly AT-rich, except for a few G residues. One of those G residues is at position 11001, and Rpo41 appears to stop before this G residue. Interestingly, DN380 does not show this 11-mer RNA.
The above results indicate that when the promoter is premelted, Mtf1 severely inhibits the synthesis of long RNA products by FL Rpo41, but Mtf1 has little effect on DN270 and DN380. Thus, removal of NTD activates Rpo41, makes it more processive, and allows it to overcome the Mtf1-dependent inhibition of RNA synthesis on premelted promoter. The increase in abortive synthesis with Mtf1 indicates that Mtf1 does associate with the DN270 and DN380 proteins bound to the promoter.

**Deletion of NTD from 1–380 Causes a Defect in Promoter Melting**—DN380 is defective in transcribing RNA on the premelted promoter, but as shown above, it transcribes efficiently from the premelted promoter DNA. This defect of DN380 could be due to a defect in promoter binding, promoter opening, or ATP binding. We therefore investigated these steps of transcription initiation in all of the deletion mutants. We probed promoter melting using 2-AP-modified promoter DNAs. We have previously mapped the melted region of the transcription open complex of mt RNAP using 2-AP-modified promoter DNAs (13). 2-AP is a fluorescent analog of adenine, whose fluorescence intensity is higher in the melted single stranded (ss) DNA state as compared with the duplex (ds) DNA state. To map the melted region of the promoter in the open state, we titrated 2-AP-modified promoter DNAs substituted with 2-AP either at −4NT, −1T, or +2NT with Mtf1 and FL Rpo41, DN100, DN270, or DN380, and 2-AP fluorescence intensity was measured. After correcting the protein fluorescence contribution, the fold increase in fluorescence at each position was determined by dividing the corrected fluorescence intensity of the free 2-AP DNA. Consistent with previous results (13), the highest fluorescence change is observed at position −4NT (Fig. 5A). FL Rpo41 and all of the deletion mutants show a 9–11-fold increase in intensity at −4NT, suggesting that all the deletion mutants can melt the region near the −4 bp. Differences were observed at position −1T and +2NT (Fig. 5A). Whereas FL Rpo41, DN100, and DN270 show a 3–5-fold increase in 2-AP fluorescence intensity at the −1T DNA, DN380 shows only a 2.5-fold increase. At position +2NT, a 3–4-fold increase in intensity is observed with FL Rpo41, DN100, and DN270, but DN380 does not show any increase in fluorescence at +2NT. Additional amounts of Mtf1 did not increase the fluorescence of 2-AP at +2NT (data not shown). These results indicate that the low transcription activity of DN380 on the duplex promoter is due to its inability to melt the promoter near the transcription start site. We conclude that amino acids from 270–380 are important for promoter melting, especially near the transcription start site.

To determine the affinity of the RNAPs for the promoter, fluorescence titrations were performed by adding increasing amounts of Rpo41 to a mixture of Mtf1 and duplex promoter DNA substituted with 2-AP at −4NT (Fig. 5B). The 2-AP fluorescence intensity increased linearly as the concentration of Rpo41 was increased, reaching a plateau with an inflection point at 1:1 Rpo41-Mtf1 DNA (13). All of the proteins excluding DN380 show tight stoichiometric DNA binding, and hence,
the $K_d$ values could not be determined accurately, whereas DN380 showed a $K_d$ of 38 nM. These results indicate that the NTD 1–270 aa are not important for DNA binding, but further deletion to 380 weakens the DNA binding affinity and is detrimental to promoter opening. Thus, DN380 may form a partially melted complex, without melting the duplex region immediately after the start site, that is not the competent preinitiation open complex.

**NTD Mutants Bind Initiating ATP more Weakly**—Previous studies have shown that addition of initiating ATP to Rpo41-Mtf1 DNA increases the 2-AP fluorescence at −1T by ~2-fold (13). A 2.5-fold increase in fluorescence intensity was observed with FL Rpo41, DN100, and DN270, but not with DN380 (supplemental Fig. 3). We used this fluorescence increase as a signal to determine the affinity of ATP for the Rpo41-Mtf1-DNA complex. A mixture of 2-AP(−1T) dsDNA, Mtf1, and FL Rpo41, DN100, or DN270 was titrated with increasing concentration of ATP. The binding data were fit to Equation 1, which provided an ATP $K_d$ of $82 \pm 8 \mu M$ for FL Rpo41 (Fig. 5, C, panel a), which is consistent with previous measurements (13). However, the ATP $K_d$ was 160 ± 10 μM for DN100 (Fig. 5, C, panel b) and 220 ± 15 μM for DN270 (Fig. 5, C, panel c). Thus, deletion of NTD from 1 to 270 decreases the ATP binding affinity by 2–3-fold. These results indicate that the NTD plays a role in stabilizing the initially transcribing complex.

**NTD Mutants Form a Weaker Complex with Mtf1**—To investigate whether the interactions between Rpo41 and Mtf1 may be mediated through the NTD, we investigated complex formation between Rpo41 and Mtf1 using DMS cross-linking. DMS is a homobifunctional cross-linking reagent that can cross-link exposed lysine residues that are within a 11 Å range (26). FL Rpo41 was mixed with Mtf1 and cross-linked with DMS for 30 s to 1 h. Samples were analyzed on 4–20% Tris-glycine gels. A cross-linked complex of FL Rpo41-Mtf1 at the expected size of the 1:1 heterodimer was observed to saturate in intensity by 10–20 min (supplemental Fig. 4, A and B). The cross-linking kinetics could be slower because of the trace amounts of Tris buffer from Rpo41 and Mtf1 preparations. Based on these results, a constant 20 min of cross-linking time was chosen for all subsequent experiments.

A cross-linked Rpo41-Mtf1 band was observed in the absence and in the presence of the promoter DNA in reactions with FL Rpo41, DN100, and DN270 and to a lesser extent with DN380 (Fig. 6A, lanes 1–4 and lanes 5–8). In controls, the Rpo41-Mtf1 band was not observed in reactions that lacked Mtf1 (Fig. 6A, lanes 9 and 11–13) or in reactions that were not treated with DMS (Fig. 6A, lanes 14–17). Some nonspecific higher molecular weight bands were also observed, which appears to arise from cross-linking of lower molecular weight impurities, as these bands are observed at the same place in all the cross-linked reactions and not observed in the uncross-linked reactions. Similarly, in the titrations with Mtf1 (see below), a cross-linked dimer of Mtf1 was observed at very high Mtf1 concentrations. Although Rpo41 appears to form a stoichiometric complex with Mtf1 as indicated by the above biochemical assays, under the cross-linking conditions, a complete shift of Rpo41 into the complex was not observed even with excess Mtf1.

To determine the relative efficiency of binding between Rpo41 and Mtf1, a constant amount of FL Rpo41 or the NTD mutant was titrated with increasing concentration of Mtf1 and cross-linked with DMS (Fig. 6B). The fraction of Rpo41-Mtf1 was quantified and plotted against free [Mtf1]. The data were fit to Equation 3 from which we determined the apparent dissociation constant $K_d$ of 0.6 μM (Fig. 6, C, panel a), both DN270 and DN380 show a ~10-fold increased $K_d$ of 6–8 μM (Fig. 6, C, panels b and c). These results indicate that the NTD deletion from 100 to 380 aa disrupts the interactions of Rpo41 with Mtf1.

We also carried out cross-linking reactions between Rpo41 and Mtf1 in the presence of U25D20 duplex and premelted promoters (supplemental Fig. 5A). The mutants showed a significant improvement in cross-linking to Mtf1 in the presence of the DNA (supplemental Fig. 5, B–D). Plots of cross-linked Rpo41-Mtf1 show apparent $K_d$ values between 0.4–0.8 μM for all the proteins (Fig. 7). These results indicate that Mtf1 binds to DN270-DNA and DN380-DNA complexes, and the affinity is quite tight in the ternary complex.

**DISCUSSION**

The ~80-kDa C-terminal domain of the core subunit of mt RNAP is well conserved among mt RNAPs of yeasts, plants, animals, and humans, and it shows high homology to bacteriophage T7/T3 RNAP (supplemental Table 1). However, the large ~50-kDa NTD of mt RNAPs is less conserved, except between some yeast strains, and it is not present in T7/T3 RNAP. Previous studies have shown that the NTD of yeast Rpo41 interacts with proteins that are involved in RNA processing and translation (8). In this paper, we investigated whether the NTD plays a role in transcription initiation and elongation. We generated a series of NTD deletion mutants lacking 1–100, 1–270, and 1–380 aa and characterized their biochemical properties. Our studies show that the NTD plays a role in transcription initiation, including promoter opening, interactions with Mtf1, and initiating ATP.

**NTD of Rpo41 Is Critical for Preinitiation Open Complex Formation**—DN380, containing the largest deletion of Rpo41, can make the full-length RNA product from a promoter that is premelted from −4 to +2, but otherwise duplex from +2 onward. These results indicate that the C-terminal domain from 380–1351 has all of the elements for promoter-specific initiation of RNA synthesis, as long as the promoter is premelted. The processive nature of RNA elongation indicates that DN380 can efficiently unwind the duplex region beyond the promoter region and may even contain the RNA exit channel. In T7 RNAP, the RNA exit channel is created by the specificity loop and subdomain H after these elements undergo conformational changes when T7 RNAP assumes the elongation structure (27, 28). The 1127–1149 region present in DN380 is, however, severely defective in synthesizing RNA from the duplex promoter. DNA binding studies show that DN380 binds the promoter with a weaker affinity as compared to its presence and in the presence of the promoter DNA in reactions with FL Rpo41, DN100, and DN270 and to a lesser extent with DN380 (Fig. 6A, lanes 1–4 and lanes 5–8). In controls, the Rpo41-Mtf1 band was not observed in reactions that lacked Mtf1 (Fig. 6A, lanes 9 and 11–13) or in reactions that were not treated with DMS (Fig. 6A, lanes 14–17). Some nonspecific higher molecular weight bands were also observed, which appears to arise from cross-linking of lower molecular weight impurities, as these bands are observed at the same place in all the cross-linked reactions and not observed in the uncross-linked reactions. Similarly, in the titrations with Mtf1 (see below), a cross-linked dimer of Mtf1 was observed at very high Mtf1 concentrations. Although Rpo41 appears to form a stoichiometric complex with Mtf1 as indicated by the above biochemical assays, under the cross-linking conditions, a complete shift of Rpo41 into the complex was not observed even with excess Mtf1.
with DN100, DN270, and FL Rpo41. Promoter melting studies with the fluorescent 2-AP-modified DNAs show that DN380 can melt the region of the promoter in the presence of Mtf1, but it is defective specifically in opening the downstream region of the promoter from to . The inability to open the promoter near the transcription start site explains why ATP binding is not observed in DN380 and why it is defective in all of the subsequent steps of the transcription initiation on the duplex promoter. This defect was not observed in DN100 or DN270. Hence, we conclude that 270–380, is involved in promoter opening. Thus, the NTD 1–270 aa is not important for DNA binding, but further deletion to 380 weakens DNA binding and detrimental to promoter opening. DN380 forms a partially melted complex that is not competent for transcription.

DN100 and DN270 are proficient in forming the preinitiation open complex, but both bind the initiating ATP more weakly than FL Rpo41. Thus, NTD from 1–380 is directly or indirectly involved in ATP binding and stabilizing the initially transcribing complex. These results indicate that NTD controls the efficiency of promoter opening and ATP binding.

FIGURE 6. DMS cross-linking of Rpo41 and Mtf1. A, FL Rpo41 or the NTD mutant was mixed with Mtf1 (4 μM each) with or without the duplex promoter DNA (4 μM) and reacted with DMS for 20 min at 22 °C in triethylamine chloride buffer (pH 8.2). Samples were quenched with 1 M glycine, electrophoresed on 4%–20% Tris-glycine gels, and stained with the Coomassie dye. The DNA used here contains the promoter sequence from to of U25D32. B, FL Rpo41 or the NTD mutant (2 μM) was mixed with increasing [Mtf1] and cross-linked with DMS for 20 min. The stained protein bands were quantified, and the fraction of Rpo41-Mtf1 and free [Mtf1] was determined. C, the fraction Rpo41-Mtf1 complex is plotted against free [Mtf1] and fit to Equations 2 and 3 (solid line) to obtain the apparent Kd values of FL Rpo41-Mtf1 (0.6 ± 0.4 μM) (a), DN270-Mtf1 (8 ± 8 μM) (b), and DN380-Mtf1 (6 ± 4 μM) (c). The dashed box highlights the heterocomplex of Rpo41 or NTD mutants with Mtf1. Non-specific cross-linked complexes are denoted by asterisk (*).
Role of the NTD of Rpo41 in Transcription

Abortive product synthesis is a characteristic feature of the conserved mechanism of transcription by DNA-dependent RNAPs (21). Abortive products are typically short RNAs between 2–8-mer that accumulate in solution. The amount of abortive products that are formed relative to the full-length RNA depends on the efficiency of the RNAP transition into elongation (19, 21). Efficient transition to elongation lowers the amount of abortive products and hence gives a lower ratio of abortive to productive RNAs. T7 RNAP, for example, shows about three to four abortive events for every productive event leading to a full-length transcript (19).

Rpo41-Mtf1 displays the typical pattern of transcription initiation. During initial transcription, Rpo41-Mtf1 produces abortive products from 2–8-mer that accumulate in the reaction, after which RNA synthesis becomes processive (Fig. 2). Thus, similar to T7 RNAP, Rpo41-Mtf1 appears to undergo transition from initiation to elongation after ~8-nucleotide RNA synthesis; although, this needs to be established. Deletion of the NTD from 100 to 270 of Rpo41 considerably decreases the amount of abortive products. As compared with FL Rpo41 that shows ~20 abortive events for every productive event, DN270 shows only about five abortive events per productive synthesis. Thus, the ratio of abortive to productive RNA ratio by DN270 is ~4-fold lower than FL Rpo41, which indicates that DN270 is able to make a more efficient transition from initiation to elongation as compared with FL Rpo41. Thus, the 1–270 region appears to be autoinhibitory.

The effect of NTD deletions was more striking when transcription was measured on premelted promoter DNA that contained mismatched bp in the 4 to 2 region. Both FL Rpo41 and DN100 show highly distributive RNA synthesis on the premelted promoter without Mtf1. When Mtf1 is added, full-length RNA synthesis is inhibited severely in FL Rpo41 and DN100, and thus, only short abortive products are observed. This inhibition by Mtf1 was not observed in DN270 and DN380.
Role of the NTD of Rpo41 in Transcription

To make long RNA products, the RNAP needs to release from the promoter and translocate to the downstream region. This happens during the transition from initiation to elongation. On the premelted promoter, Mtf1 appears to block these steps in FL Rpo41 but not when NTD is deleted. There are at least two mechanisms, as discussed below, to explain these results: 1) NTD binds the promoter DNA in the initially transcribing complex and inhibits promoter release, and/or 2) NTD binds Mtf1 and inhibits Mtf1 release, which, in turn, inhibits promoter release and transition into elongation.

Promoter release is triggered in T7 RNAP by initial bubble collapse and a major refolding of the N-terminal domain. In T7 RNAP, the N-terminal domain interacts with the promoter in the initiation complex, and after refolding, specific parts of the N-terminal domain interact with the RNA:DNA hybrid and become part of the RNA exit channel in the elongation complex (27, 28, 30). In multisubunit RNAPs, the transition from initiation to elongation is accompanied by the release of initiation factors that expose the RNA exit channel (22, 31). The mt RNAP appears to be functionally similar to multisubunit RNAP in that the release of Mtf1 has been suggested to accompany the transition from initiation to elongation (16). Thus, the efficiency of Mtf1 release can dictate the efficiency of promoter release and long RNA synthesis. Both DN270 and DN380 are resistant to Mtf1 inhibition and make long RNAs efficiently in the presence of Mtf1, which suggests that Mtf1 release is more facile in these mutant Rpo41 proteins, and the 100–380 NTD might interact with Mtf1.

In T7 RNAP, distributive synthesis on the premelted promoter was attributed to T7 RNAP staying in the initiation conformation and not making the transition into the elongation conformation (19). It was proposed that the inability of the initial bubble in the premelted promoter to collapse inhibited the release of T7 RNAP from the promoter (19, 32), and this could be the case with Rpo41. If the NTD of Rpo41 interacts with the initial bubble region during transcription initiation and because the initial bubble does not collapse in the premelted promoter, Rpo41 stays bound to the initial bubble and cannot escape the promoter. As a consequence, it catalyzes distributive synthesis of RNA, probably by scrunching the DNA template. Both DN270 and DN380 catalyze processive RNA synthesis on the premelted promoter, suggesting that the 100–380 NTD might interact with the initial bubble in the promoter.

Interactions between Rpo41 and Mtf1 Are Partially Mediated via Its NTD—The proposal that the NTD interacts with Mtf1 is supported by our cross-linking studies. The protein–protein chemical cross-linking results show that FL Rpo41 and DN100 form a tight 1:1 complex with Mtf1. This might seem inconsistent with previous studies of gel filtration and glycerol gradient sedimentation that indicated weak interactions in the absence of DNA (33). However, it is possible that the interactions between Rpo41 and Mtf1 are tight but dynamic. That is, Mtf1 has a fast on and off rates from Rpo41; hence, the complex does not survive filtration or sedimentation. Chemical cross-linking will covalently link the proteins and hence able to detect dynamic complexes.

Unlike FL Rpo41, the DN270 and DN380 formed weak complexes with Mtf1. Our quantitation indicates that the deletions lead to a 10-fold increase in the apparent $K_d$ of the Rpo41-Mtf1. In the presence of the promoter DNA, however, we observed strong interactions of both DN270 and DN380 with Mtf1, which suggests that there are additional regions in Rpo41 that interact with Mtf1 in the presence of DNA or the DNA brings the two proteins together in a stable ternary complex. Based on these studies, we conclude that Rpo41-Mtf1 interactions in the absence of DNA are mediated partially via the NTD between 100 and 380.

Overall, our studies indicate that NTD mediates interactions with the promoter, Mtf1, and initiating ATP. We propose that NTD plays a regulatory role in transcription and these interactions allow it to control the efficiency of the various steps of transcription initiation, including the transition from initiation to elongation. The 100–380 NTD mediates initial interactions of Rpo41 with Mtf1 in the absence of the promoter DNA, and we propose that they also control Mtf1 release during the transition from initiation to elongation.

Previous in vivo studies of the NTD deletion mutants of the yeast Rpo41 have shown that accessory proteins such as Nam1p interact with Rpo41 via the NTD (10). NTD is implicated in RNA processing and translation and interaction with Nam1p was proposed to localize the mt RNAP to the inner mitochondrial membrane, where transcription would be efficiently coupled with RNA processing and translation (9). It will be interesting to determine whether Rpo41 is activated in the presence of Nam1p that bind to the NTD. Our studies have shown that NTD is autoinhibitory to transcription. But, perhaps when the NTD is engaged with accessory proteins, Rpo41 is activated in a manner that allows its easy displacement from the promoter and/or Mtf1. We would predict that the transcriptional properties of the activated Rpo41 would be similar to that of the DN270. These hypotheses can be investigated in the future with studies of the Rpo41-Mtf1 with the accessory proteins such as Nam1p.

Comparison to Human mt Transcription Machinery—Recent studies indicate that the human mt RNAP machinery is similar to the yeast mt RNAP (34). The transcription factor TFB2 (a homolog of S. cerevisiae Mtf1) cross-links to the transcription initiation site and to the initiating nucleotide in the transcription initiation complex (35). This result provides compelling evidence for the conservation of the transcription initiation mechanisms in yeast and human mitochondria. However, very little is known about the role of the NTD of the human mt RNAP, POLRMT. The NTD of human POLRMT is distinct from the S. cerevisiae Rpo41. It contains two 35-amino acid pentatractipeptide repeat domains in its NTD. Pentatractipeptide repeat domains in other systems are implicated in RNA editing, processing, and splicing events (36, 37). Recently an intriguing splice variant of POLRMT, in which the N-terminal 262 aa were deleted, was found to localize in the nucleus and transcribe a set of nuclear genes (38). Interestingly, the promoters of these genes were different than the mt genes, suggesting that mechanisms for transcribing nuclear and mt genes may be different. Whether such a variant exists in S. cerevisiae is unknown. This raises very interesting role of the NTD in dictating the specificity of transcription of mt genes.
Role of the NTD of Rpo41 in Transcription

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REFERENCES

1. Lipinski, K. A., Kaniak-Golik, A., and Golik, P. (2010) Biochim. Biophys. Acta 1797, 1086–1098
2. Levens, D., Lustig, A., and Rabinowitz, M. (1981) J. Biol. Chem. 256, 1474–1481
3. Matsunaga, M., Jang, S. H., and Jaehning, J. A. (2004) Protein Expr. Purif 35, 126–130
4. Schubot, F. D., Chen, C. J., Rose, J. P., Dailey, T. A., Dailey, H. A., and Wang, B. C. (2001) Protein Sci. 10, 1980–1988
5. Jang, S. H., and Jaehning, J. A. (1991) J. Biol. Chem. 266, 22671–22677
6. Masters, B. S., Stohl, L. L., and Clayton, D. A. (1987) Cell 51, 89–99
7. Cermakian, N., Ikeda, T. M., Cedergren, R., and Gray, M. W. (1996) Nucleic Acids Res. 24, 648–654
8. Jang, S. H., and Jaehning, J. A. (1991) J. Biol. Chem. 266, 22671–22677
9. Masters, B. S., Stohl, L. L., and Clayton, D. A. (1987) Cell 51, 89–99
10. Cermakian, N., Ikeda, T. M., Cedergren, R., and Gray, M. W. (1996) Nucleic Acids Res. 24, 648–654
11. Schubot, F. D., Chen, C. J., Rose, J. P., Dailey, T. A., Dailey, H. A., and Wang, B. C. (2001) Protein Sci. 10, 1980–1988
12. Jang, S. H., and Jaehning, J. A. (1991) J. Biol. Chem. 266, 22671–22677
13. Masters, B. S., Stohl, L. L., and Clayton, D. A. (1987) Cell 51, 89–99
14. Cermakian, N., Ikeda, T. M., Cedergren, R., and Gray, M. W. (1996) Nucleic Acids Res. 24, 648–654
15. Rodeheffer, M. S., Boone, B. E., Bryan, A. C., and Shadel, G. S. (2001) J. Biol. Chem. 276, 18695–18701
16. Wang, Y., and Shadel, G. S. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 8046–8051
17. Markov, D. A., Savkina, M., Anikin, M., Del Campo, M., Ecker, K., Lambowitz, A. M., De Gnore, J. P., and McAllister, W. T. (2009) Proc. Natl. Acad. Sci. U.S.A. 107, 12133–12138
18. Schinkel, A. H., Koerkamp, M. J., Touw, E. P., and Tabak, H. F. (1987) J. Biol. Chem. 262, 12785–12791
19. Shutt, T. E., Lodeiro, M. F., Cotney, J., Cameron, C. E., and Shadel, G. S. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 12133–12138
20. Sologub, M., Litonin, D., Anikin, M., Mustaev, A., and Temiakov, D. (2009) Cell 139, 934–944
21. Lightowlers, R. N., and Chrzanowska-Lightowlers, Z. M. (2008) Biochem. J. 416, e5–6
22. Hu, L. M. (2002) Biochim. Biophys. Acta 1577, 191–207
23. Mooney, R. A., Darst, S. A., and Landick, R. (2005) Mol. Cell 20, 335–345
24. Ehrenreich, D., and Henning, U. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2018–2021
25. Tahirov, T. H., Temiakov, D., Anikin, M., Patlan, V., McAllister, W. T., Vassilev, D. G., and Yokoyama, S. (2002) Nature 420, 43–50
26. Shutt, T. E., Lodeiro, M. F., Cotney, J., Cameron, C. E., and Shadel, G. S. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 12133–12138
27. Sologub, M., Litonin, D., Anikin, M., Mustaev, A., and Temiakov, D. (2009) Cell 139, 934–944
28. Lightowlers, R. N., and Chrzanowska-Lightowlers, Z. M. (2008) Biochem. J. 416, e5–6
29. Hu, L. M. (2002) Biochim. Biophys. Acta 1577, 191–207
30. Mooney, R. A., Darst, S. A., and Landick, R. (2005) Mol. Cell 20, 335–345
31. Ehrenreich, D., and Henning, U. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2018–2021
32. Tahirov, T. H., Temiakov, D., Anikin, M., Patlan, V., McAllister, W. T., Vassilev, D. G., and Yokoyama, S. (2002) Nature 420, 43–50
33. Schinkel, A. H., Koerkamp, M. J., Touw, E. P., and Tabak, H. F. (1987) J. Biol. Chem. 262, 12785–12791
34. Schinkel, A. H., Koerkamp, M. J., Touw, E. P., and Tabak, H. F. (1987) J. Biol. Chem. 262, 12785–12791
35. Sologub, M., Litonin, D., Anikin, M., Mustaev, A., and Temiakov, D. (2009) Cell 139, 934–944
36. Lightowlers, R. N., and Chrzanowska-Lightowlers, Z. M. (2008) Biochem. J. 416, e5–6
37. Xu, F., Ackerley, C., Maj, M. C., Addis, J. B., Levandovskiy, V., Lee, J., Mackay, N., Cameron, J. M., and Robinson, B. H. (2008) Biochem. J. 416, 15–26
38. Kravchenko, J. E., Rogozin, I. B., Koonin, E. V., and Chumakov, P. M. (2005) Nature 436, 735–739