Fluorescence Mapping of the Open Complex of Yeast Mitochondrial RNA Polymerase*

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The mitochondrial RNA polymerase (mtRNAP) of Saccharomyces cerevisiae, consisting of a complex of Rpo41 and Mtf1, is homologous to the phage single polypeptide T7/T3 RNA polymerases. The yeast mtRNAP recognizes a conserved nonanucleotide sequence to initiate specific transcription. In this work, we have defined the region of the nonanucleotide that is melted by the mtRNAP using 2-aminopurine (2AP) fluorescence that is sensitive to changes in base stacking interactions. We show that mtRNAP spontaneously melts the promoter from −4 to +2 forming a bubble around the transcription start site at +1. The location and size of the DNA bubble in this open complex of the mtRNAP closely resembles that of the T7 RNA polymerase. We show that DNA melting requires the simultaneous presence of Rpo41 and Mtf1. Adding the initiating nucleotide ATP does not expand the size of the initially melted DNA, but the initiating nucleotide differentially affects base stacking interactions at −1 and −2. Thus, the promoter structure upstream of the transcription start site is slightly rearranged during early initiation from its structure in the pre-initiation stage. Unlike on the duplex promoter, Rpo41 alone was able to form a competent open complex on a pre-melted promoter. The results indicate that Rpo41 contains the elements for recognizing the melted promoter through interactions with the template strand. We propose that Mtf1 plays a role in base pair disruption during the early stages of open complex formation.

The mitochondrial genome is transcribed by a distinct RNA polymerase, which is simpler in composition when compared with the nuclear RNA polymerases that transcribe the cellular genome. The mitochondrial RNA polymerases (mtRNAP) are highly homologous to the single subunit bacteriophage T7/T3 RNA polymerase (1–4). The transcription machinery of the yeast (Saccharomyces cerevisiae) mitochondria is comprised of a core polymerase, Rpo41, encoded by nuclear RPO41 gene and a transcription factor, Mtf1 (also referred as sc-mtTFB), encoded by nuclear MTF1 gene (1, 5, 6). Both proteins are transported into the mitochondria where they form a heterodimeric Rpo41/Mtf1 complex (mtRNAP) (7). Recent studies have shown that in humans, an alternative transcript of the mtRNAP gene localizes in the nucleus and the truncated protein is responsible for the transcription of select nuclear mRNAs (8).

Unlike the single subunit RNAP of bacteriophages that do not require accessory proteins for transcription initiation, accessory factors are required to initiate specific transcription at the mtRNAP promoters. In yeast, Mtf1 is required in addition to Rpo41 for sequence-specific transcription on linear mtDNA templates (9–11). While the crystal structure of Mtf1 shows unexpected resemblance to ErmC RNA methyltransferase from Bacillus subtilis (12), there is no documented rRNA methyltransferase activity for Mtf1. In mammalian and insect systems, mitochondrial transcription in vitro requires at least three core components including the core RNA polymerase, mtTFA, and mtTFB1 or B2 (4). Efficient mitochondrial transcription in vivo requires mtFB2 while mtFB1 plays a greater role in translation (13–16). The function of Mtf1 in transcription is not completely understood. It is often compared with sigma factors that functions in promoter recognition and DNA melting by bacterial RNA polymerases (11, 17–19), yet primary sequence homology or functional analysis does not support that the two are strongly related (6, 20). It is believed that Mtf1 remains bound to Rpo41 throughout initiation, but is released after the polymerase makes the transition to elongation (11). Recent studies indicate that Rpo41 alone can initiate transcription if the energetic barriers of promoter opening are lowered significantly, e.g. by using supercoiled templates or mismatched bubble (pre-melted) templates (21). These findings led to the proposal that Mtf1 plays a role in facilitating DNA strand separation while Rpo41 contains the elements for promoter recognition and RNA synthesis (21). A similar proposal of intrinsic promoter recognition was also made for the core mtRNAPs of human and mouse due to their own species-specific promoter utilization (3).

The mtRNAP is dedicated to the transcription of respiratory chain proteins, ribosomal RNAs, and transfer RNAs from the circular genome mtDNA (3). In yeast, the promoters that direct the synthesis of these transcripts contain a highly conserved nonanucleotide sequence, [5’-ATAAGTA(+1)] (22, 23). DNase I footprinting studies indicate that yeast mtRNAP occludes the promoter region from −15 to about +15 relative to the transcription start site of +1 (9). During transcription initiation, the RNA polymerase binds to the promoter and melts a specific region of the promoter to form an open complex. In the resulting transcription-competent open complex,
the template strand near the initiation start site (+1) is exposed to incoming nucleotides. The nature or the size of such an initial DNA bubble in the open complex of the yeast mtRNAP is not known.

Fluorescent base analogues, mainly 2-aminopurine (2AP) and to some extent pyrrole-cytosine have been widely used to spectroscopically monitor DNA melting and to map the melted regions of the promoter during transcription initiation and elongation in systems including T7 RNA polymerase and bacterial RNAP (24–31). Compared with the free nucleoside, the fluorescence of 2AP in duplex DNA is greatly quenched by stacking interactions with neighboring bases (32). The fluorescence of 2AP is partly recovered upon DNA melting where base stacking interactions with the 2AP are disrupted. The initial DNA bubble in the open complex of the T7 RNAP was characterized by 2AP mapping experiments and confirmed also by high-resolution crystal structures of initiation complexes (33).

Here, we have used fluorescence methods to define the transcription DNA bubble in the open complex of the yeast mtRNAP. The consensus sequence of the 14S rRNA promoter contains a large number of adenes, which were substituted with 2AP individually. From the observed fluorescence changes, we were able to establish the size and location of the DNA bubble in the open complex of mtRNAP. Using the fluorescence assay, we have investigated also the roles of Mtf1, Rpo41, and the initiating nucleotide in open complex formation. We show that Mtf1 or Rpo41 by itself is incapable of melting the duplex promoter. On the other hand, when mismatches are present in the promoter, Rpo41 is able to form a transcriptionally active open complex. Based on our studies, we propose that Mtf1 is required for the initial melting of the promoter DNA and Rpo41 is required for stabilizing the melted DNA.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of His-Rpo41**—ProEXHTb plasmid containing His-Rpo41 (pJJ1399) was received as a kind gift from Prof. Judith A. Jaehning. The expression protocol and protein induction protocol for His-Rpo41 were carried out as previously described by Matsunaga et al. (34). Briefly, pJJ1399 was transformed into Escherichia coli BL21 DE3 codon plus (RIL) (Stratagene), and cells were induced with isopropyl-1-thio-β-D-galactopyranoside (0.6 mM) for 4.5 h. The cells were harvested at 10,000 × g for 10 min and resuspended in lysis buffer (40 mM Tris-HCl, pH 7.9, 300 mM NaCl, 0.1% Tween 20, 1 mM PMSF, 15% glycerol, 1 mM EDTA, 1 mM DTT, and 1 mg/ml lysozyme, Roche-Complete™ Protease Inhibitor Mixture Tablet). The supernatant from the lysed cells was subjected to polyethyleneimine (PEI) precipitation (0.5 M NaCl and 1/20 of the lysate volume of 10% PEI, pH 7.9). After centrifugation, the supernatant from this step was saturated with 50% ammonium sulfate, and the pellet was collected following a centrifugation at 24,000 × g for 30 min. The ammonium sulfate pellet was dissolved in Buffer N (40 mM Tris-HCl, pH 7.9, including 150 mM NaCl, 0.1% Tween 20, 15% glycerol, 1 mM EDTA, and 1 mM DTT) plus 20 mM imidazole. The solution was applied to GE-Amersham Biosciences Ni Sepharose 6 Fast Flow resin equilibrated with Buffer N + 20 mM imidazole. The column was washed with 50 volumes of Buffer N + 20 mM imidazole and eluted with a linear gradient of imidazole from 20 mM to 350 mM. The peak fractions were pooled and dialyzed against Buffer N and loaded onto a diethylaminoethyl-Sepharose (DEAE) column (Sigma) equilibrated with Buffer N. The flow-through fractions contained the protein, and the pure fractions were pooled and applied to 10 ml of heparin-Sepharose CL-6B resin (GE) equilibrated with Buffer N. The protein was eluted using a linear gradient of 150 mM to 1 mM NaCl in Buffer N. The peak fractions were analyzed for purity by SDS-PAGE and the pure fractions were dialyzed against Buffer N. The protein was concentrated in an Amicon concentrator using a regenerated cellulose ultrafiltration membrane (MWCO 30kDa, Millipore). The concentrated fractions were aliquoted and stored at −80 °C. The purity of the protein was determined using SDS-PAGE, and concentration was determined spectroscopically by measuring A280 in 6 M guanidium hydrochloride using the extinction coefficient 156,900 M⁻¹ cm⁻¹.

**Cloning, Expression, and Purification of N-HisMtf1**—Plasmid DNA containing GST-Mtf1 fusion construct (pJJ1286) was a kind gift from Professor Judith A. Jaehning. By sequencing analysis, we observed that the Mtf1 gene contained 2 mutations, Lys-71 and Ile-76. We re-mutated these two mutations back to their wide-type Arg-71 and Leu-76 using the site-directed mutagenesis kit (Stratagene). This resulting wild-type gene was then cloned out of the plasmid pJJ1286 (WT-pJJ1286). Two restriction sites of NheI and HindIII were engineered on the 5′- and 3′-ends, respectively, of the Mtf1 gene, and the gene was then ligated into the pTrcHisC vector between NheI and HindIII sites using T4 DNA ligase. This plasmid construction allowed the addition of a (His)₆ tag along with a spacer of 6 amino acids to the 5′-end of the coding region of the gene. The DNA sequence was confirmed by sequencing. The resulting plasmid (pTN42) was used to transform E. coli XL1-blue supercompetent cells and the transformed cells were plated on LB-agar plates with ampicillin (100 μg/ml). The plates were incubated at 37 °C for 16 h. Single colonies were selected and inoculated in LB-ampicillin medium for 7 h. 1 liter of LB medium was then inoculated with 10 ml of the inoculum and grown at 37 °C (10 liters total) until the A₆₀₀ reached 0.8, and were then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4.5 h. The cells were harvested by centrifugation at 10,000 × g for 15 min. The pellets were resuspended using lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 0.1 mM PMSF, Roche-Complete™ Protease Inhibitor Mixture Tablet, 10% glycerol, and 1 mg/ml lysozyme). The PEI precipitation and ammonium sulfate fractionation steps were followed as described for His-Rpo41 using Buffer M (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 0.1 mM PMSF, Roche-Protease inhibitors, 10% glycerol). The ammonium sulfate pellet was dissolved in Buffer M and applied to Ni-column as described for His-Rpo41. The protein was eluted with a linear gradient of imidazole from 20 to 400 mM. The peak fractions were analyzed for purity by SDS-PAGE, and the pure fractions were pooled and dialyzed against 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 20% glycerol. The protein was concentrated with an Amicon concentrator. The fractions were aliquoted and stored at −80 °C. Concentration was determined spectroscopically by
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measuring $A_{280}$ in 6 m guanidium hydrochloride using the extinction coefficient 73,590 M$^{-0.1}$ cm$^{-1}$.

**Oligodeoxynucleotides**—All oligodeoxynucleotides with or without 2-aminopurine substitution were custom-synthesized (Integrated DNA Technologies, Coralville, IA) and purified by urea-denatured PAGE before use (35).

**Fluorescence Analysis with 2AP DNA**—Steady-state fluorescence measurements were carried out at 25 °C on a FluoroMax-2 spectrophotometer (Jobin Yvon-Spex Instruments S.A., Inc.). Fluorescence spectra of 200 nM 2AP incorporated double-stranded (ds) promoter or mismatched bubble (pre-melted) promoter were collected from 350 to 420 nm (6-nm bandwidth) with excitation at 315 nm (2-nm bandwidth) in the absence or presence of Rpo41 and Mtf1 up to 800 nM. After subtracting the contributions from buffer and proteins in the presence of unmodified DNA, the corrected 2AP fluorescence intensities between 360 and 380 nm were integrated for comparison. The fluorescence measurements of duplex promoter in the presence of initiating nucleotide ATP up to 600–800 μM were carried out in the same manner. The buffer was 50 mM Tris-acetate, pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate. The ATP concentration dependence of 2AP fluorescence intensities were analyzed by using the Michaelis-Menten Equation to obtain apparent affinity ($K_d$).

**Steady State RNA Synthesis**—Transcription assays were carried out at room temperature (22–25 °C) in 10 μl of reaction buffer: 50 mM Tris-acetate, pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate, 0.1% Tween 20, 1 mM DTT). To start RNA synthesis, nucleotides mixture of ATP, GTP, and UTP (50 μM each) with [γ-32P]ATP (GE Healthcare) were added to a pre-mixed solution of equimolar Rpo41 and Mtf1 (0.5 μM each) with excessive promoter DNA (1 μM). After 3 min, the reaction was quenched by adding 100 mM EDTA and formamide dye (98%, 0.025% bromphenol blue, 10 mM EDTA). Each sample was then boiled at 95 °C for 5 min prior to being loaded on an 18% sequencing gel (19:1 acrylamide to bis-acrylamide) with 7 M urea to resolve RNA products. After electrophoresis, the gel was exposed to a phosphor screen, scanned on Typhoon 9410 PhosphorImager instrument (Amersham Biosciences) to quantify RNA products.

**RESULTS**

2-Aminopurine-modified Promoters—To map the melted region of the promoter in the open complex, we substituted adenines in the non-templated (NT) and the template (T) strands of the active 14S rRNA promoter of the yeast mitochondria with the fluorescent probe 2AP (Fig. 1). There are 12 adenines in the region from −9 to +4 of the promoter and each was replaced with 2AP individually, except the adenines at −8 and −7 (Fig. 1B). Transcription activity of the individual 2AP modified promoters was tested in the presence of ATP, UTP and GTP spiked with [γ-32P]ATP. Abortive RNA products (2-mer to 11-mer) and the 20-mer run-off RNA product were observed with the unmodified promoter (Fig. 2A). The 2AP-substituted DNAs contained a C:G bp at +19 (Fig. 1A, left panel); therefore, the runoff product was 18-mer in the modified DNAs (Fig. 2A). Normal transcription was observed when adenines in the region −6 to −1, or at +2 or +4 were substituted with 2AP.

Quantitative analysis of the transcription reactions showed similar ratios of the run-off product to the 2–11-mer abortive products in unmodified and 2AP-modified promoters (Fig. 2B, FIGURE 1. Sequences of the 14S rRNA promoter DNAs. A, 14S rRNA promoter fragment used in the transcription assays. The arrow represents the direction of transcription starting from position +1. The conserved non-templated sequence from −8 to +1 are in bold. B, positions of 2-aminopurine substitution on the NT or T strand are shown in bold. The guanine at −1 NT (underlined) was also substituted with 2AP.

![Image](FEBRUARY 27, 2009)

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A. The yeast mitochondrial 14S rRNA promoter

![Image](5B)

B. Positions of 2AP substitution on the 14S rRNA promoter

![Image](5C)

FIGURE 2. Effects of 2AP modification on the transcription activity of the yeast mtRNAP. A, gel assay of the transcription activity of Rpo41/Mtf1 on promoters with 2AP substitution at specific positions. 1 μM promoter DNA was pre-mixed with 0.5 μM equimolar Rpo41 and Mtf1 in 10 μl of reaction buffer (50 mM Tris-acetate, pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate, 0.1% Tween 20, 1 mM DTT). RNA synthesis was initiated by adding a nucleotide mixture of ATP, GTP, and UTP (50 μM each) spiked with [γ-32P]ATP at room temperature (22–25 °C). After 3 min, the reaction was quenched by adding 100 mM EDTA and gel loading buffer containing 98% formamide. RNA products were resolved by 18% polyacrylamide gel with 7 M urea.

Lane WT: the unmodified DNA allows synthesis up to 20-mer RNA upon adding ATP, UTP, and GTP. In all other lanes with 2AP modification, the sequence only allows RNA synthesis up to 18-mer RNA synthesis upon adding ATP, UTP, and GTP because of the insertion of C:G at +19 (Fig. 1, A and B for sequence differences). In Lane PreQ, 100 mM EDTA and the gel loading solution were present in the solution of mtRNAP and promoter prior to adding nucleotides substrates. The right panel compares the transcription on the bubble promoter (−4GCACGT+2 on the NT strand) by Rpo41 with or without Mtf1. B, quantification of the ratio of run-off RNA to 2–11-mer abortive RNA products from the duplex promoter (left panel) or from the bubble promoter (right panel). The error bars represent a range of the data from two to three measurements.
Interestingly, when the adenine at +1NT (2APNT+1) or +3T (2APT+3) was substituted with 2AP, only short abortive products, 2–6-mer for 2APNT+1 and 2–4-mer for 2AP T+3, were observed (Fig. 2A), indicating that these changes affect steps required in the elongation of the 2-mer and 3-mer RNA products. Because short RNA products were observed, it is likely that these changes do not affect steps in the formation of the preinitiation open complex.

We also carried out transcription reactions on a pre-melted promoter (a stretch of mismatches from −4 to +2 on the NT strand: GCAGCT) with and without Mtf1 (Fig. 2A, right panel). Quantitative analysis (Fig. 2B, right panel) indicated that the ratio of the run-off product to the 2–11-mer abortive products was 2.5 times greater when transcription was catalyzed by Rpo41 alone than by Rpo41/Mtf1 (Fig. 2B, right panel). These results are consistent with published results that showed that Rpo41 on its own is capable of specifically transcribing the pre-melted promoter and the presence of Mtf1 results in the accumulation of shorter abortive products (21).

**Fluorescence Mapping of DNA Melting by Yeast mtRNAP—**
To map the melted DNA region, 2AP fluorescence of each of the modified promoters was measured. Fig. 3A shows the corrected fluorescence spectrum of the double-stranded (ds) promoter with 2AP at −4NT (2APNT−4) and the spectrum of the ds promoter in the presence of Rpo41/Mtf1. The fluorescence of 2AP was almost undetectable in the ds promoter. Upon addition of Rpo41/Mtf1, the fluorescence was greatly enhanced. Such measurements were carried out with the other 2AP-modified ds promoters. The fluorescence was measured in three types of samples: (a) 2AP-containing single-stranded (ss-DNA), (b) 2AP-modified ds promoter (ds-DNA), and (c) 2AP-modified ds promoter with Rpo41/Mtf1. Typical concentration of 2AP-substituted ss- or ds-DNA was 200 nM, and the maximum fluorescence change was determined from the titration of each of the 2AP ds-DNA with increasing concentration of a mixture of equimolar Rpo41 and Mtf1. Shown in Fig. 3B are the corrected maximal intensities of 2AP fluorescence, typically achieved when the Rpo41/Mtf1 concentrations were above 200 nM. The 2AP fluorescence intensities were corrected for background buffer signal and protein fluorescence by including unmodified promoter instead of the 2AP-modified promoter. Inner filter effect at the excitation and emission wavelengths from the presence of mtRNAP and DNA was less than 5%. To minimize errors from fluctuations in the fluorescence intensity when measured at a single emission wavelength, e.g. 370 nm, the 2AP fluorescence intensities from 360 to 380 nm were summed.

The results summarized in Fig. 3B, show that the fluorescence of 2AP in ss-DNA is generally higher than in ds-DNA (Fig. 3B). The fluorescence of dsDNA increased upon addition of Rpo41/Mtf1 when 2AP was present at −4, −3, +1 or +2 in the NT strand or at −1 in the T strand. Replacement of the guanine at −2NT with 2AP (that would make a 2AP:C base pair) also increased the 2AP fluorescence upon addition of Rpo41/Mtf1. The highest fluorescence signal was observed with 2AP at −4 NT followed by −3 NT. No significant increase in fluorescence was observed when 2AP was present at −9, −5, or +3 on the T strand, or −6 or +4 on the NT strand (Fig. 3B).

**Role of Mtf1 in Promoter Melting—** Studies in the past have suggested that Mtf1 might facilitate DNA strand separation during open complex formation (21). To test whether Mtf1 alone or Rpo41 alone has the ability to melt the promoter DNA, selected 2AP-modified ds promoters were titrated with increasing concentrations of Mtf1 or Rpo41. No fluorescence
increase was observed at any of the positions even when the concentration of Mtf1 or Rpo41 was in excess of the promoter (Fig. 3C). Similarly, no fluorescence changes were observed when the initiating nucleotide ATP was added with each of the proteins (data not shown). These results demonstrate that Rpo41 alone or Mtf1 alone is incapable of opening the promoter. Thus, Rpo41 and Mtf1 together are required to effectively melt the duplex promoter DNA and to form the open complex.

Stoichiometry of the Pre-initiation Open Complex—To determine the stoichiometry of the Rpo41 and Mtf1 proteins in the open complex, fluorescence titrations were carried out by adding increasing amounts of Rpo41 to a pre-formed mixture of 2APNT_4 and Mtf1 at 200 nM each. No fluorescence increase was observed when the DNA was titrated with Mtf1 alone (Fig. 4A). But when the DNA and Mtf1 mixture was titrated with Rpo41, a linear increase in 2AP fluorescence intensity was observed as Rpo41 concentration was increased in the reaction, until a plateau was reached with an inflection point around 200 nM Rpo41 (Fig. 4A). These results indicate a 1:1 stoichiometry of Rpo41 and Mtf1 in the open complex. This stoichiometry was confirmed in a reverse titration experiment where 200 nM 2APNT_4 promoter and 200 nM Rpo41 were titrated with increasing amounts of Mtf1. Maximal 2AP fluorescence change was observed when Mtf1 concentration reached 200 nm and above (data not shown).

To determine the stoichiometry of mtRNAP and promoter DNA in the open complex, 2APNT_4 ds promoter (200 nM) was titrated with mtRNAP (equimolar mixture of Rpo41 and Mtf1). 2AP fluorescence increased linearly up to 200 nM mtRNAP concentration (Fig. 4B) after which the fluorescence intensity reached a plateau. These results demonstrate that the stoichiometry between the promoter and the mtRNAP is 1:1; that is an equimolar amount of Rpo41, Mtf1, and ds promoter is required to form the open complex. Titrations with other 2AP-modified DNAs provided the same results. The stoichiometric nature of these titration curves at 200 nM DNA indicates very tight binding of Rpo41/Mtf1 to the ds promoter (K_d < 10 nM). Taken together, these results are consistent with the model shown in Fig. 4C in which the 14S rRNA promoter is melted by Rpo41/Mtf1 starting from −4 and ending at +2.

Effect of the Initiating Nucleotide on DNA Melting—Next, we asked whether adding the initiating nucleotide, ATP, would affect the size of the DNA bubble and/or the base unstacking interactions at individual positions in the open complex. We addressed this question by comparing the 2AP fluorescence at individual positions from to +4 of the open complex (36). ATP had no effect on the 2AP fluorescence of the 2APNT_4 promoter when the initiating nucleotide ATP was added with each of the proteins (data not shown). These results demonstrate that Rpo41 alone or Mtf1 alone is incapable of opening the promoter. Thus, Rpo41 and Mtf1 together are required to effectively melt the duplex promoter DNA and to form the open complex.

FIGURE 4. The 1:1 complex of Rpo41 and Mtf1 opens the promoter DNA. A, 200 nM 2APNT_4 promoter DNA was titrated with increasing concentrations of Rpo41 (triangles). A mixture of 200 nM 2APNT_4 promoter and 200 nM Mtf1 was titrated with increasing concentrations of Rpo41 (circles). B, 200 nM 2APNT_4 promoter was titrated with increasing concentrations of a 1:1 mixture of Rpo41 and Mtf1. The linear increase of 2AP fluorescence reaches a plateau at about 270 nM Rpo41/Mtf1, demonstrating the stoichiometry of Rpo41/Mtf1 and promoter DNA. The 2AP fluorescence between 350 and 420 nm was monitored after excitation at 315 nm. The fluorescence data in both panels were corrected by subtracting the contributions from buffer and protein. C, scheme shows the minimum size and the location of DNA bubble in the open complex. The location of disrupted base pairing and base stacking interactions from −4 to +2 on the 14S rRNA promoter by mtRNAP are mapped based on the results of the 2AP fluorescence changes. Proposed contacts of the opened promoter with the mtRNAP components are drawn.
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It has been shown that Rpo41 catalyzes specific transcription without requiring Mtf1 when the promoter is pre-melted (21). We created a pre-melted promoter by mutating six bases in the NT strand from –4 to +2 to create mismatches at these positions (Fig. 7A). This promoter is capable of supporting sequence-specific transcription by Rpo41 alone (Fig. 2). To monitor the formation of open complex, the adenine at −1T was substituted with 2AP. The fluorescence of 2AP in the pre-melted promoter was about 4 times more intense than in the ds promoter (Fig. 7B). Upon addition of Rpo41, the 2AP fluorescence of the pre-melted promoter increased, reaching a level slightly higher than the fluorescence of 2AP in the ds promoter with Rpo41 alone (Fig. 7B). We make a comparison with Rpo41/Mtf1 because the ds promoter had not shown any increase in fluorescence with Rpo41 alone. Similar increases in fluorescence of the pre-melted promoter were observed upon addition of Rpo41/Mtf1 (Fig. 7B). Thus, the structure of the open complex with this pre-melted promoter (with base changes in the NT strand) appears to be similar to the open complex formed with the ds promoter. The results indicate that once the base pairing near the transcription initiation site is broken (e.g. by pre-melting the DNA), Rpo41 is able to bind to the promoter without Mtf1 to form a transcriptionally active open complex.

The pre-melted promoter was titrated with increasing concentration of Rpo41 or the equimolar mixture of Rpo41/Mtf1. The titration curves with Rpo41 or Rpo41/Mtf1 were indistinguishable up to 300 nM protein concentration (Fig. 7C), beyond which point further addition of Rpo41 did not increase the 2AP fluorescence while adding Rpo41/Mtf1 caused an additional ~10% increase prior to reaching a plateau. Thus, the final base unstacking conformation of 2AP at −1 is very similar in the presence of Rpo41 or Rpo41/Mtf1. The affinity of the pre-

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**FIGURE 5.** 2AP fluorescence mapping of DNA melting by yeast mtRNAP in the presence of ATP. A, effect of ATP on the fluorescence of 2AP at various positions on the ds promoter with Rpo41/Mtf1 complex. 200 nM 2AP-DNA was equilibrated with 400 nM Rpo41/Mtf1 (1:1), and 400 μM ATP (final) was added to the enzyme-promoter mixture. Although the 2AP fluorescence signals were measured at 1 min, they remained stable for 5 min. Error bars reflect the range of data from at least two measurements. B, fluorescence of 200 nM promoter with 2AP at −1T with 400 nM Rpo41/Mtf1 was enhanced upon adding ATP. C, fluorescence of 200 nM promoter with 2AP at −2NT and 400 nM Rpo41/Mtf1 decreases with increasing concentrations of ATP.

ds promoter when only Rpo41 alone or Mtf1 alone was present (data not shown).

Taking advantage of the significant increase of 2AP fluorescence at −2NT and −1T upon the addition of ATP, we used fluorimetric titrations to measure the affinity of the initiating nucleotide ATP for the Rpo41/Mtf1 and ds promoter complex. As ATP concentration was increased, there was a significant increase in the 2AP fluorescence at −1T and a significant decrease in the 2AP fluorescence at −2NT. Titration with increasing ATP concentrations provided the ATP \( K_d \) values of 89 μM for the 2AP at −1T, ds promoter and 135 μM for the 2AP at −2NT, ds promoter. These values of ATP \( K_d \) are comparable to the reported 49–75 μM \( K_m \) of ATP determined from analysis of run-off product formation under steady-state transcription conditions (37, 38).

The titration of 2AP at −1T promoter with increasing concentration of mtRNAP closely resembled that of 2AP at −2NT (Fig. 6A versus Fig. 4B), suggesting that its defect in transcription is beyond the step of pre-initiation open complex formation, consistent with the observation that abortive products were made with this modified promoter. A new promoter construct with dual 2AP substitutions on +3T and −2NT (2APNT_{−2}/2AP_{−3}) showed similar fluorescence titration curves to those of 2APNT_{−2} without or with the initiating nucleotide ATP (Fig. 6, B and C versus Fig. 5C), suggesting that 2AP modification on −3T does not disrupt open complex formation. An ATP \( K_d \) value of 110 μM for the 2APNT_{−2}/2AP_{−3} promoter was derived from the titration, which is consistent with the ATP \( K_d \) of 135 μM for the 2APNT_{−2} promoter.

**Rpo41 Forms an Open Complex with a Pre-melted Promoter**

It has been shown that Rpo41 catalyzes specific transcription without requiring Mtf1 when the promoter is pre-melted (21). We created a pre-melted promoter by mutating six bases in the NT strand from –4 to +2 to create mismatches at these positions (Fig. 7A). This promoter is capable of supporting sequence-specific transcription by Rpo41 alone (Fig. 2). To monitor the formation of open complex, the adenine at −1T was substituted with 2AP. The fluorescence of 2AP in the pre-melted promoter was about 4 times more intense than in the ds promoter (Fig. 7B). Upon addition of Rpo41, the 2AP fluorescence of the pre-melted promoter increased, reaching a level slightly higher than the fluorescence of 2AP in the ds promoter with Rpo41 alone (Fig. 7B). We make a comparison with Rpo41/Mtf1 because the ds promoter had not shown any increase in fluorescence with Rpo41 alone. Similar increases in fluorescence of the pre-melted promoter were observed upon addition of Rpo41/Mtf1 (Fig. 7B). Thus, the structure of the open complex with this pre-melted promoter (with base changes in the NT strand) appears to be similar to the open complex formed with the ds promoter. The results indicate that once the base pairing near the transcription initiation site is broken (e.g. by pre-melting the DNA), Rpo41 is able to bind to the promoter without Mtf1 to form a transcriptionally active open complex.

The pre-melted promoter was titrated with increasing concentration of Rpo41 or the equimolar mixture of Rpo41/Mtf1. The titration curves with Rpo41 or Rpo41/Mtf1 were indistinguishable up to 300 nM protein concentration (Fig. 7C), beyond which point further addition of Rpo41 did not increase the 2AP fluorescence while adding Rpo41/Mtf1 caused an additional ~10% increase prior to reaching a plateau. Thus, the final base unstacking conformation of 2AP at −1 is very similar in the presence of Rpo41 or Rpo41/Mtf1. The affinity of the pre-
melted promoter for Rpo41 appears slightly higher than for Rpo41/Mtf1 under such conditions. Thus, the presence of the Mtf1 does not increase the binding affinity of Rpo41 for the pre-melted promoter.

Adding ATP to the complex of the pre-melted promoter and Rpo41 alone or Rpo41/Mtf1 caused noticeable increases in the 2AP fluorescence at −1T (Fig. 7B). Analysis of the titration curves with increasing ATP showed a higher affinity of ATP for Rpo41/Mtf1 (K<sub>d</sub> = 63 μM) than for Rpo41 alone (K<sub>d</sub> = 131 μM) (Fig. 7D). These results indicate that Mtf1 might be involved in interactions with the initiating nucleotide. Alternatively, the Rpo41 conformation might be altered by Mtf1 leading to changes in the interactions between the polymerase and ATP.

**DISCUSSION**

Melting of the duplex promoter by the RNA polymerase to form a transcriptionally competent open complex is a critical step in transcription. This process makes the template strand accessible to the incoming nucleoside triphosphates that are substrates of the RNA polymerization reaction. In addition to the biochemical approaches such as potassium permanganate (KMnO<sub>4</sub>) and diethylpyrocarbonate (DEPC) footprinting (39), the use of highly specific fluorescent footprinting assays with 2AP over the past decade has revealed detailed transcription-dependent promoter structures and dynamics with a single base pair resolution (24, 26–28, 31, 40). Here, we have used 2AP fluorescence footprinting to examine the in vitro changes in the 14S rRNA promoter structure of the yeast mitochondria that accompany the formation of the preinitiation open complex.

Fluorescence studies of yeast mitochondrial promoters modified with 2AP at specific positions in the consensus region allowed us to establish the minimum size of the DNA bubble, the stoichiometry of the Rpo41/Mtf1 complex, and the role of Mtf1 and initiating nucleotide ATP in open complex formation. The nonanucleotide sequence, 5'-(-8)ATATAAGTA(+1) serves as the minimal promoter sequence in yeast mitochondria (22, 23, 41). Individual adenines in the nonanucleotide sequence were substituted with the fluorescent analog 2AP. Based on the observed 2AP fluorescence changes, the size of the DNA bubble was established from −4 to +2 in the open complex of Rpo41/Mtf1. The melted DNA region in the open complex of yeast mRNAP is strikingly similar to that of T7 RNAP (27, 28, 33), and much smaller than that of the E. coli σ<sup>70</sup>-RNAP, where the DNA bubble is 14 bp in size and open from −12 (42, 43). The fluorescence intensity changes indicated that 2AP at −4 and −3 positions in the open complex of the yeast mRNAP adopt an unstacked configuration, which is similar to the conformation of bases at analogous positions in the open complex of the T7

![Image](image-url)
RNAP. In the open complex of T7 RNAP, the highest fluorescence was observed at ∼4, which was attributed to complete unstacking of that base from a neighboring strongly quenching guanine (27, 33). Similarly, here the 2AP at −3NT showed a large increase in fluorescence upon formation of the Rpo41/Mtf1 complex from its extremely low initial level in the ds promoter. This increase is likely a result of the 2AP base unstacking from the neighboring guanine nucleotide at its 3’-side. The 2AP at −4NT does not have a neighboring guanine, and its high fluorescence intensity indicates that it adopts an extrahelical configuration in the open complex.

Unlike its closely related T7 RNAP that spontaneously melts the promoter on its own, our results show that Rpo41 requires the presence of Mtf1 to initiate DNA melting. However, Mtf1 alone does not melt the promoter DNA. Our results show that a 1:1 complex of Rpo41 and Mtf1 is the functional unit that is required to form the open complex, and the apparent dissociation constant of Rpo41/Mtf1 to 2AP modified 14S rRNA promoters is less than 10 nm. It has been suggested that Rpo41 and Mtf1 proteins form a complex (11), but we find that the extent of DNA melting was not affected by the order of Rpo41 or Mtf1 addition or by adding a pre-mixed Rpo41/Mtf1. Unlike T7 RNAP that requires the initiating NTP (GTP) to fully melt the promoter DNA (27), adding ATP to the mtRNAP pre-initiation open complex did not change the extent of DNA melting. For T7 RNAP, the addition of initiating GTP to its preinitiation open complex causes a shift to the open form from the closed form in the binary complex (27, 35). The initial bubble size was maintained constant in the mtRNAP open complex upon addition ATP, consistent with the DNase I footprinting results that did not detect downstream extension of DNA contacts upon adding the first nucleotide (9). Our studies showed that adding ATP did bring about differential changes in the base unstacking interactions in the region close to the transcription start site, from −2 to −1, leaving other positions essentially undisturbed. These results indicate that the promoter structure, specifically the conformation of bases upstream of the transcription start site, is slightly rearranged during the early initiation stage from its structure in the preinitiation stage.

Mtf1 is required by Rpo41 for sequence specific initiation of transcription on ds promoters (9,10). On pre-melted promoter, however, Rpo41 can catalyze specific RNA synthesis without Mtf1 (21). Consistent with these results, we find that Rpo41 forms an open complex with a promoter that contains mismatches in the region from −4 to +2. These results indicate a role of Mtf1 in the initial stages of DNA melting. The mechanism by which Mtf1 catalyzes initial DNA melting is not known. Mtf1 may act directly by contacting the DNA in complex with Rpo41 to initiate DNA melting or Mtf1 may act allosterically and induce conformational changes in Rpo41 to promote DNA melting (12). The requirement of both Rpo41 and Mtf1 in the formation of the open complex and in initiating sequence-specific transcription suggests that the two steps are closely coupled and both components of the mtRNAP may contribute to promoter recognition and melting. Once the DNA region near the transcription state site is melted, the opened DNA can interact with Rpo41. In T7 RNAP, the promoter is recognized primarily by the specificity loop and the AT-rich recognition loop, and DNA melting is initiated by the insertion of the intercalating β-hairpin at the −4/−5 junction (33). Modeling of Rpo41 based on its sequence homology to T7 RNAP has predicted the presence of regions corresponding to the specificity loop and intercalating β-hairpin of T7 RNAP (44). Because Rpo41 recognizes a pre-melted promoter with mutated NT strand around the transcription start site and initiates specific transcription on this promoter (21), the sequence-specific interactions of Rpo41 with the promoter must arise from its interactions with the template strand. Similarly, if Mtf1 in the complex makes physical contacts with the DNA, it likely interacts with the NT strand surrounding the transcription start site.

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