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Conservation of Promoter Melting Mechanisms in Divergent Regions of the Single-Subunit RNA Polymerases

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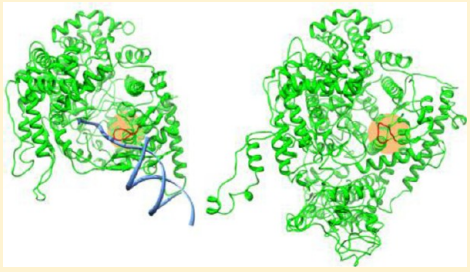
Supporting Information

ABSTRACT: The single-subunit RNA polymerases make up a widespread family of proteins found in phage, mitochondria, and chloroplasts. Unlike the phage RNAPs, the eukaryotic RNAPs require accessory factors to melt their promoters and diverge from the phage RNAPs in the regions where functions associated with promoter melting in the latter have been mapped, suggesting that promoter melting mechanisms in the eukaryotic RNAPs diverge from those in the phage enzymes. However, here we show that an element in the yeast mitochondrial RNAP, identified by sequence alignment with the T7 phage RNAP, fulfills a role in promoter melting similar to that filled by the T7RNAP "intercalating hairpin". The yeast mitochondrial RNAP intercalating hairpin appears to be as important in promoter melting as the mitochondrial transcription factor, MTF1, and both a structurally integral hairpin and MTF1 are required to achieve high levels of transcription on a duplex promoter. Deletions from the hairpin also relieve MTF1 inhibition of promoter escape on premelted promoters, likely because such deletions disrupt interactions with the upstream edge of the transcription bubble. These results are consistent with recent structural and functional studies of human mitochondrial RNAP and further reveal the surprising extent of mechanistic conservation between the eukaryotic and phage-encoded members of the single-subunit RNAP family.



Two large families of RNAPs conduct mRNA, tRNA, and rRNA synthesis in all cells. One is the family of multisubunit RNAPs that includes the eukaryotic pol I, II, and III enzymes, as well as the multisubunit RNAPs of the eubacteria and archaea.[1] The other is the family of RNAPs encoded by many bacteriophage, which also have homologues in mitochondria and chloroplasts and are both nuclear- and plastid-encoded.[2] The latter are usually designated as the single-subunit RNAPs; however, while it is correct that these RNAPs almost invariably function as a single subunit during RNA chain elongation, the mitochondrial and chloroplast RNAPs require an additional factor(s) during transcription initiation, and these factors have been shown to be required for promoter melting.[3,4] The well-studied phage T7RNAP, and its characterized phage RNAP homologues, melt their promoters without accessory factors by using at least two mechanisms: (1) the introduction of a sharp bend in the promoter, centered on the region that is opened,[5−7] and (2) the intercalation of a β-hairpin between the template (T) and nontemplate (NT) strands at the upstream edge of the melted region in the initiation complex.[8−10]

This intercalating hairpin occurs in a region that, at the sequence level, is very poorly conserved in the single-subunit RNAP family,[2] and combined with the differences in factor requirements for promoter melting by the phage versus eukaryotic RNAPs, this would suggest that the phage and eukaryotic enzymes use distinct mechanisms for promoter melting. However, to test this, we created deletions in two regions of the yeast mitochondrial (Mt) RNAP suggested by ambiguous sequence alignments as potential locations of an element that could be functionally and structurally homologous to the phage RNAP intercalating hairpin. We find that deletions in one of these regions generates RNAPs that are transcriptionally active and capable of promoter specific binding but cannot melt a duplex promoter. A region from human MtRNAP that aligns with this putative intercalating hairpin in yeast MtRNAP was recently also shown to be important in allowing initiation from duplex but not premelted templates,[11] suggesting that this promoter melting mechanism is utilized by most, if not all, members of the single-subunit RNAP family.

EXPERIMENTAL PROCEDURES

Mutant RNAP genes were prepared with the Stratagene QuickChange directed mutagenesis kit using vectors described previously.[12] Yeast MtRNAP and MTF1 were expressed in *Escherichia coli* BL21(DE3) and purified as described previously,[12] except that bacterial cultures, following IPTG induction, were transferred to 16 °C for overnight protein expression because we found that low-temperature expression increased the yield of soluble MtRNAP and MTF1.

Transcription reactions (25 μL volume) were conducted at room temperature for 15 min in 4 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.0), 5 mM DTT, and 50 mM NaCl with synthetic

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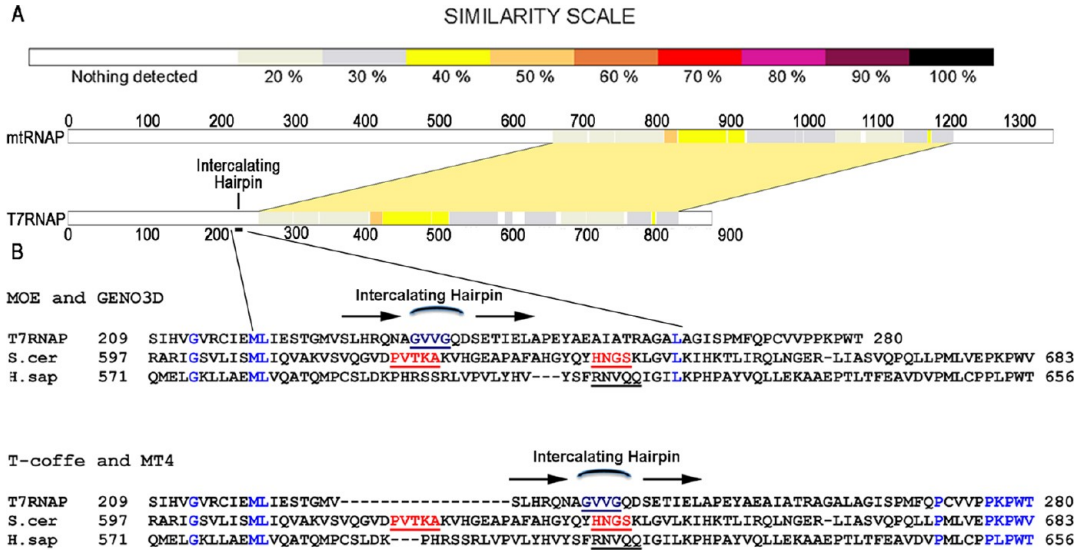

Figure 1. Poor sequence conservation in the N-terminal regions of the single-subunit RNAPs results in ambiguous alignments in these regions. (A) Graphic alignment of the yeast mitochondrial and T7 phage RNAP sequences. The level of sequence identity in C-terminal regions of the RNAPs ranges from 20 to 50% and averages 30%, but in the N-terminal regions, which include the T7RNAP intercalating hairpin, the level of sequence identity is <20%. (B) The alignment of the T7RNAP intercalating hairpin with scMtRNAP or human MtRNAP varies depending on the program used. T-coffee and MT4 align the intercalating hairpin (purple lettering) with scMtRNAP residues 641−644 (red), whereas structural models generated with MOE and GENO3D align the hairpin with residues 621−624.

promoter templates at 2 μM, RNAPs and MTF1 (when present) at 1 μM, NTPs at 0.5 mM, and 0.1 μCi/μL of 3000 Ci/mM [α-^{32}P]ATP to label the transcripts. Transcription reactions were resolved by denaturing polyacrylamide gel electrophoresis (PAGE) and imaged and quantified on a Molecular Dynamics Storm Phosphorimager as described previously.[12] The following synthetic promoter templates were used in these reactions: duplex promoter nontemplate (NT) strand (the underlined base corresponds to position +1; annealed to its complement), AATTCATTTATTTATTAT-TATATAAGTAATAAAGAATAGTTTTATATACTAATAA-TAATATAG; bubble promoter NT strand (annealed to the same T strand as the duplex promoter to create a −4 to +2 mismatch in the bold region), AATTCATTTATTTATTAT-TATAT**GCAGCT**TAAAGAATAGTTTTATATACTAATAA-TAATATAG.

Het1−Het5 promoters were created by annealing a common 33-base T strand to the following 33-base NT strands to generate promoters with mismatches in the −4 to +1 region ranging from 1 to 5 bp in size (underlined base at position +1; bold bases are mismatches): T strand, CGCGTAAAAC-TATTCTTTATTACTTATATCGCG; Het1 NT strand, CGCGATAT**G**AGTAATAAAGAATAGTTTTACGCG; Het2 NT strand, CGCGATAT**GC**TAATAAAGAATAGTTT-TACGCG; Het3 NT strand, CGCGATAT**GCA**TAATAAA-GAATAGTTTTACGCG; Het4 NT strand, CGCGATAT**G-CAG**AATAAAGAATAGTTTTACGCG; Het5 NT strand, CGCGATAT**GCAGC**ATAAAGAATAGTTTTACGCG; Het1D NT strand, CGCGATATAAGTACTAAAGAA-TAGTTTTACGCG; Het2D NT strand, CGCGATA-TAAGT**GC**TAAAGAATAGTTTTACGCG.

Other promoters used in this study were generated by annealing different NT strands, as described in the text, to the T strand shown above.

In MTF1 binding experiments, 1 μM MtRNAPs were mixed with equimolar MTF1 in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 500 mM NaCl in 50 μL volumes and then spun through an Amicon minicon ultrafiltration device with a 100 kDa molecular mass cutoff filter in a minifuge at 8000 rpm for 10 min. Following this, the retentates and filtrates were recovered, and enough buffer was added to the retentate to bring its volume to 50 μL; both retentates and filtrates were analyzed by denaturing PAGE.

Permanganate reactivity experiments were conducted as described previously[9] with the 33 bp duplex promoter template labeled with [γ-^{32}P]ATP at the 5′-end of the T or NT strand and at the same reagent concentrations and buffer conditions used for the transcription reactions but with limiting NTP mixes as specified in individual figure legends.

Electrophoretic mobility shift analysis (EMSA) experiments were conducted with promoters prepared with a 33-base T strand identical to that used to prepare the Het1−Het5 promoters, but labeled with fluoresceine at its 5′-end and with either a fully complementary 33-base NT strand ("duplex" promoter) or an NT strand in which the −4 to +2 region was changed to GCAGCT to create a 6 bp mismatch ("bubble" or "premelted" promoter). Reaction mixtures contained constant amounts of MtRNAP with or without MTF1 and were incubated with varying concentrations of promoter DNA, with promoter always in excess of MtRNAP (specific concentrations are indicated in individual figure legends), in 20 mM Tris-HCl (pH 8.0), 75 mM NaCl, 5 mM DTT, 1 mM EDTA, 10% glycerol, and 0.001% (w/v) bromophenol blue. After a 30 min incubation at room temperature, 10 μL reaction aliquots were resolved on 4 to 15% Native PAGE gels run in 1× TAE buffer and visualized on a Molecular Dynamic Strom imager with excitation at 450 nm and emission detection at 520 nm. K_d values were determined by fitting the fraction of MtRNAP bound to the concentration of free promoter DNA using Origin70 and a Hill equation with the Hill coefficient set to 1.0.

RESULTS

Deletions Centered on scMtRNAP Residues 641 and 642 Disrupt Transcription of the Duplex, but Not Premelted, Templates. The N-terminal regions of distantly related members of the single-subunit RNAP family exhibit only ~20% average sequence identity,[2] making the identification of corresponding functional elements, if present, uncertain; when we aligned the T7RNAP sequence with those of yeast and human mitochondrial RNAPs, the T7RNAP intercalating hairpin (amino acids 228−246) was aligned with residues 616−634 or 633−651 of the yeast enzyme and residues 590−608 or 604−622 of the human enzyme (Figure 1). To determine if either of these alignments identifies an element in the yeast mitochondrial polymerase that corresponds functionally to the T7RNAP intercalating hairpin, we generated deletions in the yeast enzyme and characterized the ability of these deletion mutants to transcribe either fully duplex templates or premelted (bubble) promoter templates in which base pairs from position −4 to +2 were mismatched because of changes in the NT strand versus the consensus. The results were unambiguous: the mutant with residues 621−624 deleted (ΔPVTK) could transcribe both the duplex and bubble templates (Figure 2A, lanes 1, 5, and 13), but the mutants with deletions of residues 641−643 (ΔHNG) or 642−644 (ΔNGS) could transcribe the bubble template (lanes 2, 3, 6, and 7) but not the duplex template (lanes 14 and 15). This suggested that residues around position 642 in the yeast RNAP form a structure with a function analogous to that of the T7RNAP intercalating hairpin, which is required for melting the promoter during initiation.

However, it is also possible that these mutations perturbed the structure of the RNAP and disrupt interactions with the promoter or MTF1, which could also specifically disrupt initiation from duplex promoters. To test this, we conducted EMSA experiments with fluorescently labeled duplex and bubble promoters with the deletion mutants and WT RNAP, with or without MTF1. Addition of yeast mtRNAP to a reaction mixture with a 33 bp fluorescent bubble promoter results in formation of a slowly migrating band on a native 8 to 25% polyacrylamide gel (Figure 2B, lane 1). When MTF1 is added to these reaction mixtures, this band is observed to supershift (lane 2), indicating formation of a promoter−RNAP−MTF1 complex [addition of MTF1 alone at the concentration used in Figure 2 to a labeled bubble or duplex promoter does not result in a shifted band, though when MTF1 is added at a concentration of >5 μM, a slowly migrating but diffuse band is observed, suggesting that MTF1 alone binds DNA weakly (Figure S1 of the Supporting Information)]. A band migrating at the same position is observed with a duplex promoter and with mtRNAP and MTF1 present (lane 3). The ΔHNG and ΔNGS RNAPs also form complexes migrating at the same positions with MTF1 and either bubble or duplex promoters (lanes 5, 6, 8, and 9), but in the absence of MTF1, multiple weak bands are seen in the reactions with the deletion mutants and the bubble promoter (lanes 4 and 7). We have also observed such multiple bands with WT RNAP and bubble promoters when the RNAP is in excess of the promoter and MTF1 is not present (Figure S2 of the Supporting Information; the experiments in Figure 2B were conducted with the promoter in excess of RNAP), suggesting that excess RNAP can form nonspecific complexes with the bubble promoter in the absence of MTFI and that this tendency is exacerbated with the deletion mutants.

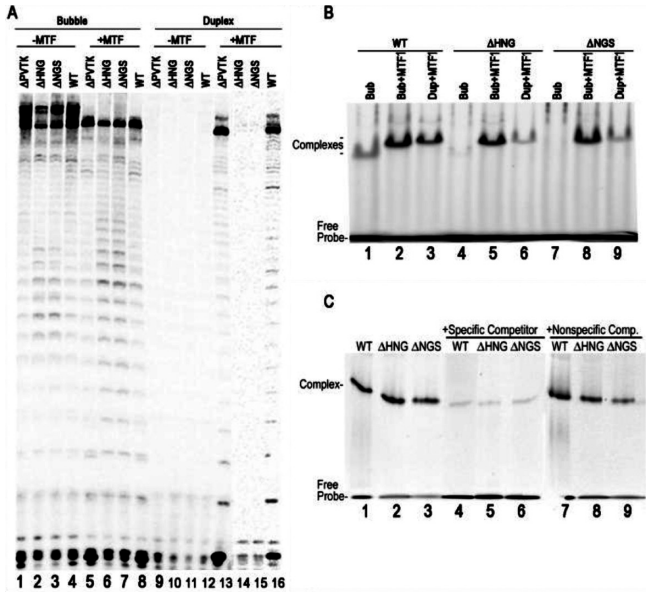

Figure 2. Deletion of scMtRNAP residues 631−633 or 632−634 does not abrogate promoter specific binding but eliminates the ability of the RNAP to transcribe duplex, but not premelted, promoters. (A) Transcription of either a fully duplex (lanes 9−16) or bubble mitochondrial promoter template (containing a heteroduplex in the −4 to +2 segment; lanes 1−8), either without (lanes 1−4 and 9−12) or with added MTF1 (lanes 5−8 and 13−16), shows that all of the RNAPs can transcribe the bubble template, either with or without MTF1, and that none of the RNAPs can transcribe the duplex template in the absence of MTF. However, both WT and ΔPVTK RNAPs can transcribe the duplex template with MTF1, while ΔHNG and ΔNGS RNAPs cannot. (B) An EMSA with fluorescently labeled promoters and WT, ΔHNG, and ΔNGS RNAPs reveals that all three can bind promoter templates and form a ternary complex with the promoter and MTF1: lane 1, WT RNAP with the bubble promoter; lane 2, as in lane 1 but with MTF1 added (note the supershift of the mtRNAP−promoter complex due to MTF1 binding); lane 3, as in lane 2 but with a duplex promoter; lane 4, as in lane 1 but with ΔHNG RNAP; lane 5, as in lane 4 but with MTF1 added; lane 6, as in lane 5 but with a duplex promoter; lane 7, as in lane 1 but with ΔNGS RNAP; lane 8, as in lane 7 but with MTF1 added; lane 9, as in lane 8 but with the duplex promoter. (C) Competition experiment showing promoter binding by ΔHNG and ΔNGS is specific. EMSA experiments were conducted with the bubble promoter and MTF1 and the indicated RNAPs in the absence (lanes 1−3) or presence of a 5-fold excess of specific unlabeled promoter DNA (lanes 4−6) or nonpromoter DNA (lanes 7−9).

We therefore determined whether promoter binding by these mutants is indeed sequence specific in competition experiments. Limiting amounts of WT, ΔHNG, or ΔNGS RNAPs were mixed with a labeled bubble promoter and with either no competitor (Figure 2C, lanes 1−3), a 5-fold excess of an unlabeled bubble promoter (lanes 4−6), or an unlabeled bubble DNA with a nonpromoter sequence (lanes 7−9). For all three RNAPs, the promoter, but not the nonpromoter, DNA was observed to compete for formation of the labeled promoter−RNAP−MTF1 complex. These results indicate that the failure of the ΔHNG or ΔNGS RNAPs to transcribe duplex templates is not due to the general loss of transcriptional activity, or to the loss of promoter specific or MTF1 interaction

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ability, but is instead due specifically to the loss of promoter melting activity.

Deletions Centered on scMtRNAP Residues 641 and 642 Weaken Binding to Duplex, but Not Premelted, Promoters. Though experiments at high RNAP and DNA concentrations indicated that the deletion mutants could form specific complexes with either bubble or duplex promoters and MTF1, we undertook quantitative EMSA experiments to determine if the deletions had any effects on promoter affinity. With a bubble promoter and MTF1, the deletion mutants formed promoter complexes with apparent affinities only 2−3-fold lower than that of the WT enzyme (Figure 3A and Table 1). In the absence of MTF1, the apparent affinity of the WT RNAP for a bubble promoter is decreased by ∼30-fold while the deletion mutants exhibit multiple shifted bands, indicating formation of heterogeneous complexes on this promoter in the absence of the transcription factor (Figure 3B). To estimate the affinity of the promoter for the deletion mutants under these conditions, the amount of promoter present in all the shifted bands was summed and set as the concentration of the bound complex. The apparent affinity calculated in this way was similar for the mutant and WT RNAPs (Table 1). With a duplex promoter and MTF1, the apparent affinity of the WT RNAP was ∼3-fold lower than that with the bubble promoter; however, the apparent affinities of the ΔHNG and ΔNGS mutants were 14- and 28-fold lower, respectively, for the bubble promoter (Figure 3C,D and Table 1). This indicates that the deletions do not markedly affect the affinity for a premelted promoter (though they do form more heterogeneous complexes on such a promoter in the absence of MTF1) but do weaken binding to fully duplex promoters. The EMSA-measured K_d values we obtained for binding of yeast MtRNAP to duplex promoters are higher than those measured recently using fluorescence anisotropy.[13] This may reflect the different methods used to measure these values but may also be due to differences in binding buffer conditions, as we used chloride as the counteranion in our binding buffer and did not have Mg^{2+} present, while the previous study used actetate and glutamate as the counteranions and included Mg^{2+}.

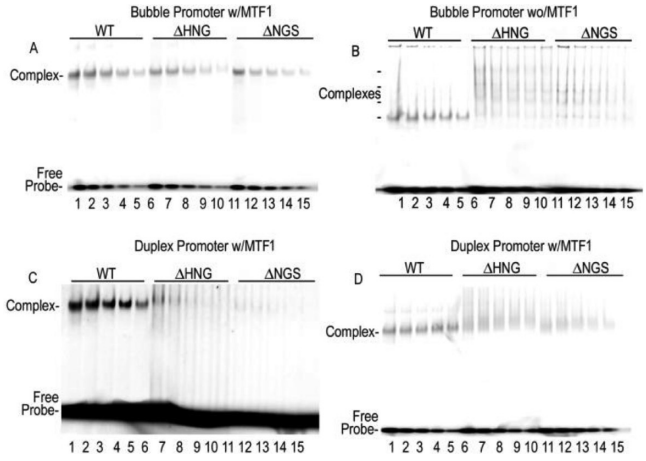

Figure 3. Quantitative EMSA reveals that ΔHNG and ΔNGS RNAPs bind bubble promoters with an affinity comparable to that of WT RNAP but bind duplex promoters more weakly. (A) RNAPs at 10^{-7} M were mixed with 10^{-7} M MTF1 and a fluorescently labeled bubble promoter at concentrations that varied from 10^{-6} M (lanes 1, 6, and 11) to 0.63×10^{-7} M (lanes 5, 10, and 15) in serial 2-fold dilutions and resolved via native 6% PAGE. (B) Like panel A but without MTF1 and with the bubble promoter concentration varied from 10^{-5} M (lanes 1, 6, and 11) to 0.63×10^{-6} M (lanes 5, 10, and 15). (C) Like panel A but with the duplex promoter concentration varying from 4×10^{-6} M (lanes 1, 6, and 11) to 0.25×10^{-6} M (lanes 5, 10, and 15). (D) Like panel C but with RNAPs and MTF1 at 10^{-5} M and the promoter concentration varying from 10^{-4} M (lanes 1, 6, and 11) to 0.63×10^{-5} M (lanes 5, 10, and 15). Note that in panels B and C, gels are shown overexposed to allow visualization of the weak and/or multiple complex bands in the ΔHNG and ΔNGS reactions.

Deletions Centered on scMtRNAP Residues 641 and 642 Abrogate Promoter Melting Ability. To determine directly if the deletions disrupt promoter melting, we used permanganate footprinting at promoter and RNAP concentrations where, as judged from the EMSA experiments, both the WT and deletion mutants should bind the promoter. In complexes formed with the WT enzyme and a duplex promoter, there is minimal permanganate reactivity in the absence of MTF1 (Figure 4, lane 1), but upon addition of

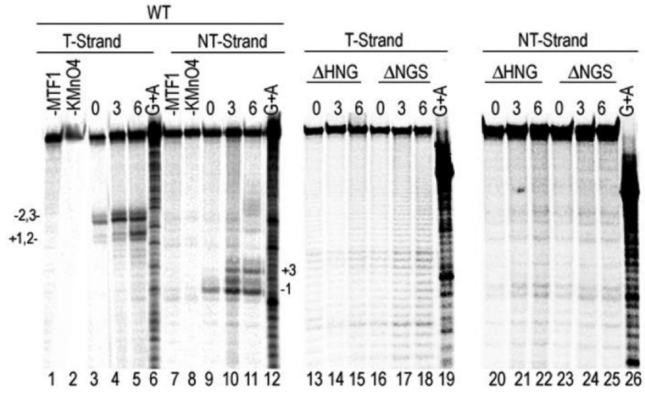

Figure 4. Deletion of scMtRNAP residues 631−633 or 632−634 abrogates the ability of these RNAPs to melt a duplex promoter. The duplex promoter labeled with ^{33}P at the 5′-end of the template (T; lanes 1−6 and 13−19) or nontemplate (NT; lanes 7−12 and 20−26) was mixed with MTF1 and either WT (lanes 3−5 and 9−11), ΔHNG (lanes 13−15 and 20−22), or ΔNGS (lanes 16−18 and 23−25) RNAPs and then treated with $KMnO_4$ in the absence (lanes 3, 9, 13, 16, 20, and 23) or presence of NTPs allowing RNA extension to three (lanes 4, 10, 14, 17, 21, and 24) or six (lanes 5, 11, 15, 18, 22, and 25) bases. The numbering of the bands is relative to the +1 transcription start site, and G+A ladders were run in lanes 6, 12, 19, and 26.

Table 1. Apparent K_d Values for Binding of the Indicated RNAPs to the Indicated Promoter DNAs in the Presence or Absence of MTF1[a]

RNAP	bubble promoter	bubble promoter with MTF	duplex promoter with MTF
WT	$(4.3 \pm 0.8) \times 10^{-6}$	$(1.6 \pm 0.1) \times 10^{-7}$	$(4.8 \pm 0.5) \times 10^{-7}$
AHNG	$(5.3 \pm 1.0) \times 10^{-6}$	$(3.4 \pm 0.4) \times 10^{-7}$	$(4.9 \pm 0.9) \times 10^{-6}$
ANGS	$(8.3 \pm 1.1) \times 10^{-6}$	$(4.3 \pm 0.6) \times 10^{-7}$	$(1.2 \pm 0.4) \times 10^{-5}$

[a]The errors are standard errors (n = 3).

MTF1, reactivity is seen at T strand bases −2 and −3 and weaker reactivity is seen at bases +1 and +2 (lane 3), and on the NT strand around base −1 (lane 9). Addition of NTPs allowing transcript extension to base +3 results in strengthening of cleavage on the T strand (lane 4) and the appearance of reactivity at base +3 on the NT strand (lane 10). Addition of NTPs allowing extension to base +6 enhances T strand reactivity at bases +1 and +2 but does not visibly alter the reactivity on the NT strand (lane 11). In contrast to this, we could observe no permanganate reactivity in complexes formed with either of the deletion mutants (lanes 13−26) under any of these conditions, indicating that the deletions disrupt the ability of the RNAP to melt the promoter.

MTF1 and the Intercalating Hairpin Make Approximately Similar and Synergistic Contributions to Promoter Melting. The results in Figure 2 show that yeast MtRNAP requires both an intact intercalating hairpin and MTF1 to initiate transcription on a fully duplex template but provide no information about the relative contribution of these two elements to promoter melting. To evaluate this, we tested transcription by the WT and deletion mutant RNAPs, in the presence or absence of MTF1, on a series of promoters in which melting is increasingly facilitated by progressive introduction of one to five mismatched base pairs starting at promoter position −4 and extending downstream to position +1. On a promoter with a single mismatch [Het1 (Figure 5A, lane 1)] in the absence of MTF1, runoff transcription by WT RNAP is weak but detectable (approximately one runoff transcript synthesized every 200 min), but runoff transcription by the ΔHNG or ΔNGS RNAPs is undetectable (lanes 6 and 11). A promoter with two mismatches enhances transcription by the WT enzyme by ∼5-fold (lane 2), but transcription by the deletion mutants is ∼3-fold slower (lanes 7 and 12), corresponding to approximately one transcript every 100 min. Upon introduction of three, four, or five mismatches (Het3, Het4, or Het5, respectively), we observe similarly high levels of transcription with all three RNAPs (lanes 3−5, 8−10, and 13−15). Addition of MTF1 to the reaction mixtures with the ΔHNG or ΔNGS RNAPs increases the level of transcription of the Het2 promoter by ∼8-fold (Figure 5B, lanes 7 and 12) and results in detectable transcription of the Het1 promoter (lanes 6 and 11). However, high levels of runoff transcription on the Het1 and Het2 promoters are seen only when both MTF1 and an integral hairpin are present (lanes 1 and 2).

The amount of runoff transcription observed in these assays is a complex function that reflects the efficiency of promoter melting and initiation of transcription, progression through initial (abortive) transcription, promoter escape, elongation, and RNAP cycling following runoff. We therefore also conducted assays in which only ATP was present, to limit the progression of transcription beyond the +2 template position (Figure 6; the promoter used in these experiments initiates AAU). Under such conditions, in addition to synthesizing dimers, the RNAP will make poly-A ladders of indefinite length. In the absence of MTF1, the WT RNAP exhibits low levels of transcription on the Het1 template, which increases as the heteroduplex region is increased in size from 1 to 5 bp (Figure 6A, lanes 1−5). In comparison to WT RNAP, and in the absence of MTF1, the deletion mutants exhibit lower levels of transcription on the Het1 and Het2 promoters and, to a lesser extent, on the Het3 and Het4 promoters, and levels of transcription similar to that of WT are observed only on the Het5 promoter (lanes 5−15). Addition of MTF1 increases the

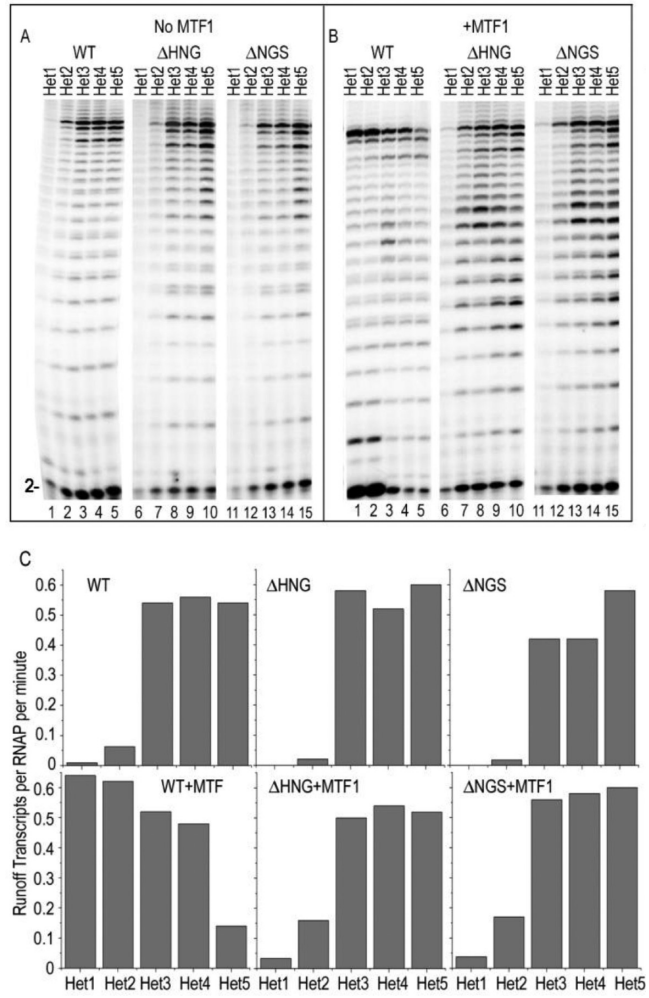

Figure 5. MTF1 and residues 631−634 make approximately equal and synergistic contributions to promoter melting. (A) Promoters containing either one (Het1; lanes 1, 6, and 11), two (Het2; lanes 2, 7, and 12), three (Het3; lanes 3, 8, and 13), four (Het4; lanes 4, 9, and 14), or five (Het5; lanes 5, 10, and 15) mispaired bases extending downstream from position −4 were transcribed with either WT (lanes 1−5), ΔHNG (lanes 6−10), or ΔNGS (lanes 11−15) RNAPs in the absence of MTF1. (B) Like panel A but with MTF1 added. (C) Quantification of the results in panels A and B expressed as the number of runoff transcripts per minute per RNAP. Quantification was done by multiplying the fraction of total radioactivity in a given gel lane (including unincorporated radioactivity) incorporated into runoff (19−21 nucleotides) transcripts by the ATP concentration (500 μM) and dividing by the reaction time, the MtRNAP concentration (1 μM), and the number of A bases[9] in the runoff transcript.

level of transcription 10−20-fold on the Het1 and Het2 promoters for the WT enzyme (Figure 6B, lanes 1 and 2) and by 3-fold on the Het3 promoter (lane 3) and has little effect with the Het4 and Het5 promoters (lanes 4 and 5). With the deletion mutants, MTF1 causes a 2−3-fold increase in the level of transcription on the Het1 and Het2 promoters (lanes 6, 7, 11, and 12) but has little effect on transcription from the Het3−Het5 promoters (lanes 8−10 and 13−15).

Finally, we determined whether the placement of the mismatched base pairs has an effect on transcription. Because promoters with one (Het1) or two (Het2) mismatches at positions −4 and −3 were most sensitive to the effects of a hairpin deletion or MTF1 (promoters with more mismatches

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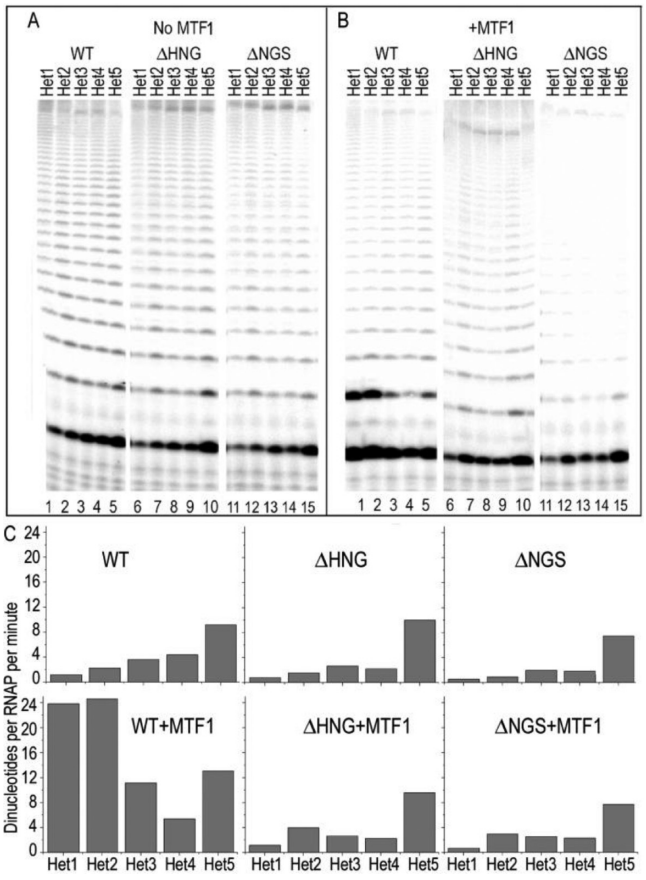

Figure 6. Deletion of scMtRNAP residues 631−633 or 632−634 affects dinucleotide synthesis as well as runoff transcription: (A) Promoters containing either one (Het1; lanes 1, 6, and 11), two (Het2; lanes 2, 7, and 12), three (Het3; lanes 3, 8, and 13), four (Het4; lanes 4, 9, and 14), or five (Het5; lanes 5, 10, and 15) mispaired bases extending downstream from position −4 were transcribed with either WT (lanes 1−5), ΔHNG (lanes 6−10), or ΔNGS (lanes 11−15) RNAPs in the absence of MTF1 and in the presence ATP only. (B) Like panel A but with MTF1 added. (C) Quantification of the results in panels A and B expressed as the number of runoff transcripts per minute per RNAP. Quantification was conducted as described in the legend of Figure 5C after correcting for the number of A bases in a dinucleotide vs runoff transcript.

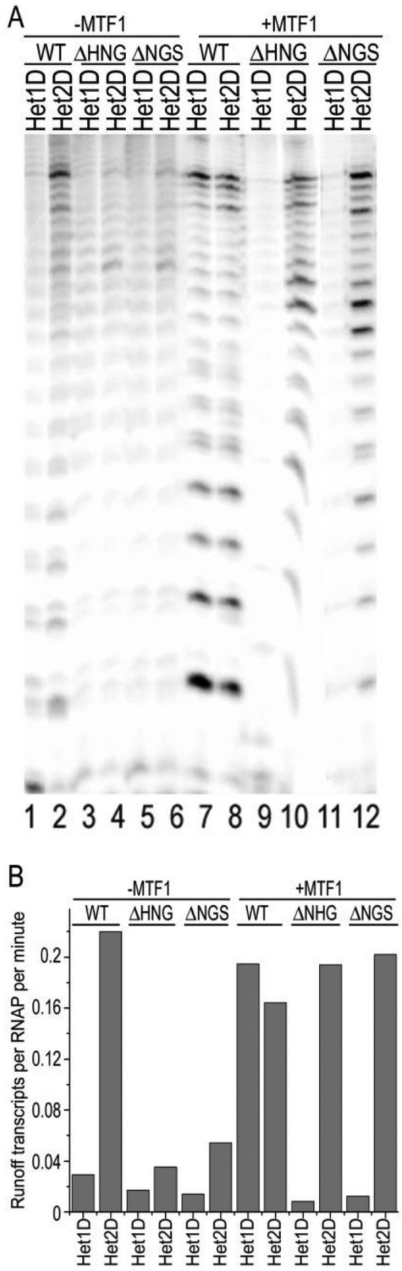

Figure 7. MTF1 and the intercalating hairpin make equal and synergistic contributions to transcription from promoters with mismatches at positions +1 and +2. (A) Promoters containing mismatches at position +2 (Het1D; odd-numbered lanes) or positions +1 and +2 (Het 2D; even-numbered lanes) transcribed with WT (lanes 1, 2, 7, and 8), ΔHNG (lanes 3, 4, 9, and 10), or ΔNGS (lanes 5, 6, 11, and 12) MtRNAPs in either the absence (lanes 1−6) or presence (lanes 7 and 8) of MTF1. (B) Rates of runoff transcription on the indicated promoters and by the indicated MtRNAPs with or without MTF1.

could be transcribed by both deletion mutants in the presence or absence of MTF1), we tested promoters in which either position +2 or both positions +1 and +2 were mismatched (designated Het1D or Het2D, respectively, and corresponding to promoters with one or two mismatches at the downstream, rather than upstream, end of the transcription bubble). In the absence of MTF1, neither the WT MtRNAP nor the deletion mutants showed significant transcription of the Het1D promoter (Figure 7A, lanes 1, 3, and 5). WT MtRNAP transcribed the Het2D promoter at 10-fold higher levels than Het1D (Figure 7A, lane 2, and Figure 7B), but the level of transcription of Het2D by the deletion mutants remained low (Figure 7A, lanes 4 and 6). Addition of MTF1 increased the level of transcription by WT MtRNAP of the Het1D promoter to a level similar to that seen for Het2D (lanes 7 and 8) and similarly increased the level of transcription of Het2D by the deletion mutants (lanes 10 and 12). The level of transcription of the Het1D promoter by the deletion mutants remained low even with MTF1 (lanes 9 and 11).

Overall, these results indicate that (1) both MTF1 and the intercalating hairpin individually contribute to promoter melting. In the absence of MTF1, the WT RNAP transcribes the Het1 or Het2 promoters at ∼3-fold higher levels than the deletion mutants and transcribes the Het2D promoter at ∼6-fold greater levels than the deletion mutants. Addition of MTF1 enhances deletion mutant transcription of the Het1 and Het2 or Het2D promoters by 2−8-fold. (2) The contributions of the hairpin and MTF1 to melting are similar. On the Het1D and

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Het2D promoters, addition of MTF1 to the WT/Het1D reaction mixture increases the level of transcription by ~6-fold, and in the absence of MTF1, the level of transcription of Het2D by the WT MtRNAP is also ~6-fold greater than that by the deletion mutants. With the Het1 and Het2 promoters, the contribution of MTF1 to melting is modesty greater than the contribution of the hairpin because addition of MTF1 to the deletion mutant reactions increases the level of transcription by ~2-fold more than does the use of WT MtRNAP versus ΔHNG or ΔNGS MtRNAPs. (3) The effects of the hairpin and MTF1 are synergistic: high levels of transcription on the Het1 and Het2 or Het1D promoters are seen only when both an integral hairpin and MTF1 are present.

Relief of MTF1 Inhibition of Transcription on Premelted Promoters by ΔHNG and ΔNGS Likely Reflects Disruption of Interactions with NT Nucleotides −2 and −3. Inspection of Figure 5 reveals that the deletions have an effect on transcription patterns beyond the defect in transcription of duplex promoters. With the WT RNAP, it is seen that, in the presence of MTF1, the levels of runoff transcription decrease as the size of the heteroduplex region in the promoter increases (Figure 5B, lanes 1−5). This is not seen with the deletion mutants (Figure 5B, lanes 6−15). The MTF1-dependent decrease in the level of runoff transcription on premelted promoters has been observed previously and attributed to inhibition of promoter escape because of an overly strong interaction with the melted promoter and MTF1.[3,14] It is relieved by deletions in the MtRNAP N-terminal domain that weaken the interaction with MTF1.[3,14] To determine if ΔHNG and ΔNGS also relieved MTF1 inhibition of transcription of escape on bubble templates to an extent as great as that with the much larger N-terminal deletions, we compared transcription on duplex and bubble templates with these mutants to those by RNAPs with residues 1−358 (ΔN1) or 1−264 (ΔN2) deleted. A homology model based on the human MtRNAP structure[11] (Figure S3 of the Supporting Information) reveals that the larger deletion removes the entire N-terminal extension that is absent from T7RNAP, while the smaller deletion removes part of this domain, but that neither removes elements that are structurally similar to T7RNAP. A more extensive deletion of yeast MtRNAP residues 1−380 has been reported to weaken promoter binding and disrupt transcription,[14] and the homology model suggests that this deletion may remove part a region of the MtRNAP that is analogous, and possibly homologous, to the T7RNAP N-terminal domain.

Though yeast MtRNAP N-terminal deletions are reported to weaken MTF1 binding, under the conditions of these assays, MTF1 binds the ΔN1 and ΔN2 initiation complexes as shown by the fact it allows both RNAPs to initiate transcription from duplex templates (compare lanes 7 and 9 to lanes 17 and 19 in Figure 8A) and by the fact that MTF1 binds to ΔN1 and ΔN2 initiation complexes as determined by an EMSA (Figure S4 of the Supporting Information). On the bubble template, the presence of MTF1 reduces the level of transcription by the WT enzyme (compare lanes 2 and 12), and scans of lanes 2 and 12 reveal the large proportionate increase in the level of abortive transcription in the presence of MTF1, indicative of an inhibition of promoter escape. Neither the hairpin nor the N-terminal deletion mutations show such inhibition, and inspection of the transcript patterns (Figure 8A) and scans of the relevant gel lanes (Figure 8B) indicate that, qualitatively, the three-residue hairpin deletions relieve this inhibition as

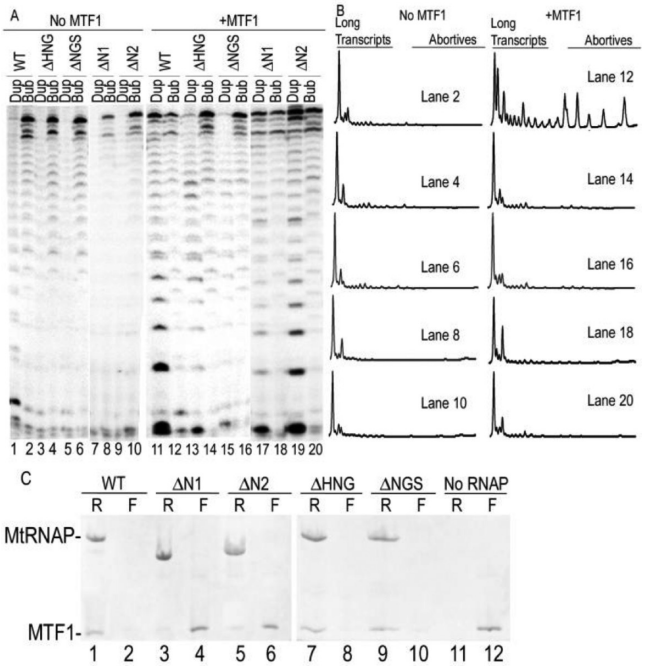

Figure 8. Deletion of scMtRNAP residues 631−633 or 632−634 relieves MTF1 inhibition of transcription on premelted promoters but does not cause defects in MTF1 binding. (A) Duplex (Dup; odd-numbered lanes) or bubble (heteroduplex from position −4 to −2; even-numbered lanes) was transcribed in the absence (lanes 1−10) or presence (lanes 11−20) of MTF1 with either WT (lanes 1, 2, 11, and 12), ΔHNG (lanes 3, 4, 13, and 14), ΔNGS (lanes 5, 6, 15, and 16), ΔN1 (lanes 7, 8, 17, and 18), or ΔN2 (lanes 9, 10, 19, and 20) RNAPs. (B) Scans of the indicated gel lanes from panel A (bubble promoter reactions only). (C) Equimolar mixtures of MTF1 and either WT (lanes 1 and 2), ΔN1 (lanes 3 and 4), ΔN2 (lanes 5 and 6), ΔHNG (lanes 7 and 8), ΔNGS (lanes 9 and 10), or no RNAP (lanes 11 and 12) were filtered through membranes with 100 kDa molecular mass cutoffs. Retentates (R) are shown in odd-numbered lanes and filtrates (F) in even-numbered lanes.

effectively as the large N-terminal deletions. Quantitatively, the proportions of abortive transcripts support this conclusion as MTF1 increased the percent incorporation into 3−8mers on the bubble promoters by 2−3-fold for WT MtRNAP but had no effect on the fractional incorporation into 3−8mers for the four mutant MtRNAPs (Table 2).

We next asked whether relief of MTF1 inhibition by the hairpin deletion enzymes worked through a mechanism similar to that of the N-terminal deletions, by a decrease in affinity for MTF1. Equimolar amounts of MTF1 and MtRNAP were mixed to final concentrations of 1 μM and subjected to filtration through membranes with 100 kDa cutoffs that allowed free MTF1 to flow through, but not MtRNAP or MtRNAP−MTF1 complexes. With WT RNAP, essentially all of the MTF1 was retained with the RNAP (Figure 8C, lanes 1 and 2), while in the absence of any RNAP, all of the MTF1 was found in the filtrate (lanes 11 and 12). The ΔN1 and ΔN2 RNAPs both show reduced levels of retention of MTF1 (lanes 3−6), with the larger ΔN1 deletion having a more severe effect. The ΔHNG and ΔNGS RNAPs, however, show no defect in MTF1 binding and exhibit retention like that of the WT enzyme (lanes 7−10).

Because the hairpin deletions did not affect the apparent affinity of the MtRNAP for MTF1, we asked whether this relief

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Table 2. Percent Incorporation of [α-^{32}P]AMP into Abortive Transcripts on the Indicated Promoters and by the Indicated RNAPs[a]

	bubble (−4 to −2 heteroduplex)		partially single-stranded (−4 NT strand)		partially single-stranded (−2 NT strand)	
MtRNAP	without MTF1	with MTF1	without MTF1	with MTF1	without MTF1	with MTF1
WT	18 ± 1.5	43 ± 3.2	14 ± 1.5	24 ± 1.9	19 ± 4	39 ± 1.5
ΔHNG	8.2 ± 0.9	8.4 ± 1.0	18 ± 1.9	13 ± 0.5	20 ± 0.6	14 ± 2.5
ΔNGS	7.8 ± 0.7	8.1 ± 1.3	15 ± 1.7	10 ± 1.0	18 ± 2.0	14 ± 1.3

[a]Values are shown with their standard deviations (n = 3). Partially single-stranded promoters with −4 and −2 NT strands correspond to promoters with NT strands that extend to position −4 (promoter 1 in Figure 9C) and −2 (promoter 3 in Figure 9C), respectively.

might be due to loss of interactions with the upstream fork of the transcription bubble. We reasoned that, if this were the case, deletion of the relevant DNA elements from a premelted promoter might similarly relax MTF1 inhibition of the WT MtRNAP. We therefore tested transcription of templates in which the complementary NT strand extended from position −12 to −4, −3, or −2 (Figure 9). On templates where the NT strand extended to position −2 or −3, the presence of MTF1 markedly increased the proportion of abortive transcripts in WT reactions, with the increase in abortives being greater when the NT strand was extended to position −2 than position −3 (compare lanes 2 and 3 to lanes 6 and 7 in Figure 9A). However, when the NT strand extended only to position −4, the presence of MTF1 caused almost no increase in abortives (compare lanes 1 and 5), nor was the difference in the effect of MTF1 due to differences in how many base pairs have to be melted with the position −4 versus position −2 NT strand. When we used an NT strand that was complementary to the T strand only from position −12 to −5 but that also had a six-nucleotide noncomplementary tail, we observed MTF1-dependent increases in abortives similar to those seen with the position −2 NT strand (compare lanes 8 and 4 in Figure 9A). In contrast, with ΔHNG or ΔNGS, the proportions of abortives on all promoters were similarly low in the absence or presence of MTF1 (Figure S5 of the Supporting Information). Quantification of transcription on partially single-stranded promoters in which the NT strand extends to either position −4 (PNT-4) or −2 (PNT-2) confirms that MTF1 increases the level of abortive transcription by the WT RNAP on PNT-2 but not PNT-4, and that abortive transcription by the hairpin deletion mutants is unaffected by MTF1 on either promoter (Table 2).

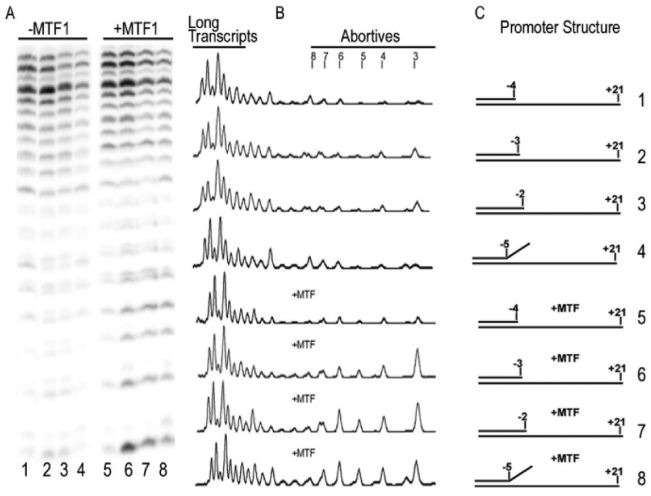

Figure 9. Removal of NT strand nucleotides −2 and −3 relieves MTF1 inhibition of runoff transcription on premelted promoters. (A) Transcription of partially single-stranded promoters in which the NT strand extends from position −4 to −2 (promoter structures as shown in panel C) by WT MtRNAP in the absence (lanes 1−4) or presence (lanes 5−8) of MTF1. (B) Scan of lanes 1−8 from panel A. (C) Structures of promoters used in reactions in panels A and B.

We conclude that removal of NT nucleotides −2 and −3 disrupts interactions responsible for the effect of MTF1 in inhibiting promoter escape and increasing the level of abortive transcription. Because modeling based on the T7RNAP IC structure indicates that the MtRNAP intercalating hairpin would interact with these same nucleotides (Figure S3 of the Supporting Information), this suggests that the relief of MTF1 inhibition by the hairpin deletions is due to a similar mechanism.

DISCUSSION

It is important to define the degree of conservation of structure and mechanism in the single-subunit RNAP family. Initial characterization can suggest a greater degree of mechanistic divergence than is revealed by deeper analysis. For example, the very different sequences and sizes of mitochondrial and phage promoters,[12] the requirement of yeast MtRNAP for an auxiliary factor (MTF1) for transcription initiation, the observation that this factor is released from the RNAP upon transition to elongation,[15] and the detection of weak sequence similarity between MTF1 and *E. coli* σ70 suggested not only that mitochondrial and phage RNAPs used very different promoter recognition mechanisms but also that MTF1 functioned like a sigma factor to endow a core MtRNAP with promoter specificity.[16] In fact, more recent studies have shown that MtRNAPs are capable of promoter specific transcription in the absence of these auxiliary factors,[3,17] and that yeast MtRNAP contains a "promoter recognition loop" similar in size, secondary structure, position, and function to a homologous element in the phage RNAPs.[12] The recently described structure of a human MtRNAP also reveals a similar promoter recognition loop,[11] indicating that such an element may be common to most or all single-subunit RNAPs.

Similarly, poor sequence conservation in the single-subunit RNAPs in the region encompassing the intercalating hairpin and the fact that mitochondrial, but not phage, RNAPs require additional factors for promoter melting would suggest that melting mechanisms are very different in different members of this family. However, our results reveal that the yeast MtRNAP contains an element that appears to be functionally analogous to that hairpin. Homology models of the yeast MtRNAP based on the human MtRNAP structure (Figure S3 of the Supporting Information) show this element superimposes on a disordered loop in the N-terminal region of the human enzyme that is proposed to be similar in structure to the phage intercalating hairpin and is also indicated to play a role in promoter

melting,[11] again indicating that this element may be structurally and functionally conserved in all or most single-subunit RNAPs. Evaluation of transcriptional activity on promoters that are premelted to varying extents by introduction of different numbers of mismatched base pairs into the −4 to +2 region indicates that MTF1 and the intercalating hairpin individually make similar contributions to promoter melting and that both are required for high levels of transcriptional activity on duplex promoters or promoters with only one or two base pair mismatches.

Deletions in the yeast MtRNAP hairpin had another, unexpected, effect on transcription: they eliminated the MTF1-induced inhibition of transcript release observed with full-length MtRNAP on premelted promoters. This inhibition can also be relieved by deletions from the MtRNAP N-terminal domain that weaken MTF1 binding, and this has been interpreted to mean that the combination of a premelted promoter and MTF1 interaction results in an overly stable set of interactions that inhibit escape of the MtRNAP from the promoter (interestingly, human MtRNAP does not exhibit escape inhibition on premelted promoters in the presence of its transcription factor[18,19]). However, we found that the deletions in the intercalating hairpin did not weaken the MtRNAP−MTF1 interaction, at least not to the same extent as the N-terminal deletions, though their effects on relief of MTF1 inhibition were comparable. Instead, we found that MTF1 inhibition could also be relieved by deletion of the −2 and −3 nucleotides of the NT strand, indicating that interactions of MtRNAP with the upstream region of the transcription bubble could also inhibit promoter escape and that the hairpin deletions may relieve this inhibition by disrupting these interactions.

Intriguingly, the mitochondrial RNAP is homologous to phage RNAP, and the catalytic subunit of the mitochondrial DNAP has been shown to be most closely related to the T7 phage DNAP;[20] the human "twinkle" gene has been shown to encode a mitochondrial primase/helicase that is most closely related to the T7 gene 4 primase/helicase[21] (a further functional similarity is that the T7 and mitochondrial RNAPs both prime leading strand DNA replication as well as synthesizing mRNAs[22,23]). To a first approximation, mitochondria may be described as using a DNA transcription and replication apparatus derived from a phage. This may have happened because the structurally simpler phage enzymes are easier to import and assemble into functional complexes in the mitochondrion than would be, for example, a multisubunit bacterial or nuclear RNAP. However, the reason for the conservation of such relatively fine scale structure−function features such as the promoter recognition loop or intercalating hairpin across the evolutionary distances that separate yeast and human mitochondria is surprising and, as yet, without a fully satisfactory adaptive explanation.

■ ASSOCIATED CONTENT

Supporting Information

Low-affinity nonspecific DNA binding by MTF1 alone (Figure S1), multimer formation on bubble promoters with excess WT MtRNAP in the absence of MTF1 (Figure S2), molecular models of yeast MtRNAP showing putative locations of residues deleted in these studies (Figure S3), an EMSA showing association of MTF1 with N-terminal deletion mutant MtRNAP IC (Figure S4), and the effect of MTF on ΔHNG and ΔNGS transcription from partially single-stranded promoters with varying NT strand lengths (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

RNAP, RNA polymerase; Mt, mitochondrial; Mtf1, yeast mitochondrial transcription factor; IC, initiation complex; WT, wild type; T strand, template strand; NT strand, nontemplate strand; EMSA, electrophoretic mobility shift analysis.

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