Intrinsic Promoter Recognition by a “Core” RNA Polymerase*

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Purification of Recombinant Rpo41 and Mtf1—Expression of (His)6-tagged Rpo41 (His-Rpo41) in Escherichia coli was as described previously (10). Purification of His-Rpo41 used for Fig. 1A was by nickel-nitrilotriacetic acid (Qiagen) chromatography as described (13). Purification of His-Rpo41 used for all other figures was by nickel-nitrilotriacetic acid and Mono Q HR 10/10 (Amersham Biosciences) chromatography essentially as described (9). Expression and purification of glutathione S-transferase-tagged Mtf1 was as described (13).

Selective Transcription Assays—Promoter selective transcription reactions were as described previously (10). The reactions contained 100 μM [α-32P]UTP (2500–3000 cpm/pmol UTP). The products of transcription reactions were analyzed on a 6 m urea, 15% polyacrylamide gel for Fig. 1A and 7 m urea, 20% polyacrylamide gels for the other figures. Products were visualized using a PhosphorImager (Amersham Biosciences), and transcript abundance was quantitated using Quantity One software (Bio-Rad).

RESULTS AND DISCUSSION

Rpo41 Alone Can Initiate from a Supercoiled Template—To explore the possibility that Rpo41 might be able to utilize promoters on its own, we used an Mtf1-free, recombinant form of the RNAP (13) in in vitro transcription reactions with two different mitochondrial promoter templates: the consensus 14S rRNA promoter and the variant tRNAcyt promoter as shown in Fig. 1A. We varied the standard reaction conditions (14) by promoter opening, based on the identification of Mtf1 mutations that result in altered promoter utilization in the context of the holo-RNAP, and the ability to correct these defects by supercoiling the DNA template (8, 9). However, our recent description of a mutation in Rpo41 with promoter utilization defects that also can be corrected by supercoiling (10) leaves open the possibility that the interactions of Mtf1 with Rpo41 may act to reveal the intrinsic ability of the core RNAP to recognize and initiate from mitochondrial promoters.

In support of this idea, Rpo41 alone possesses some DNA sequence specificity, recognizing a non-selective template consisting of alternating AT residues but not any homopolymer sequences (11). This simple template has some similarity to the mitochondrial consensus promoter/initiation site, ATATA-AGTA, with the last A representing the +1 nucleotide (nt) of the transcript (12). In this work we have used a highly purified recombinant form of the yeast mitochondrial RNAP to ask whether the core RNAP alone possesses selective transcription activity. We have found that Rpo41 alone is capable of promoter recognition and initiation in the absence of Mtf1, if the template is fully or partially melted open. Mtf1 therefore is critical for open promoter formation but not promoter sequence recognition.

EXPERIMENTAL PROCEDURES

Transcription Templates—Linear and supercoiled plasmid DNA templates containing the 14S rRNA (pJ1305) and tRNAcyt (pJ1110) promoters used in this study were as described previously (9). Linear and bubble 70-bp 14S rRNA and no promoter templates were constructed by annealing the following oligonucleotides (5’ to 3’): 14S rRNA linear, cggaaattcattattattattattttatttttttttattattattattattattattattattattattattttattattattattattattattattattattattattattattattattattattattattattattattattattattattataaactattatattttattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattattat
Promoter Recognition by the Yeast Mitochondrial Core RNA Polymerase

FIG. 1. Rpo41 selectively transcribes the tRNAs\(\text{cys}\) and 14 S rRNA promoters from supercoiled but not linear templates in the absence of Mtf1. A, schematic of 14 S rRNA and tRNAs\(\text{cys}\) promoter-containing DNAs. The template DNA and transcript are represented as straight and zigzag lines, respectively. The transcription start site and the first CTP in the non-template strand are marked as + 1 and C, respectively. In the absence of CTP, transcripts of 107 and 38 nt are made from the 14 S rRNA and the tRNAs\(\text{cys}\) promoter, with the dashed line denoting the untranscribed region downstream of the first C. B, transcripts synthesized in vitro transcription reactions from plasmid vector lacking a promoter (Vector) or containing promoters for 14 S rRNA (107 nt) and tRNAs\(\text{cys}\) (38 nt) were analyzed by gel electrophoresis as described (Ref. 10 and see “Experimental Procedures”). “L” and “S” indicate linear and supercoiled, respectively, forms of the DNA templates. The reactions in lanes 1 and 2 contained 0.38 pmol of Rpo41 and 0.39 pmol of Mtf1, and reactions in lanes 3 and 4 contained 1.9 pmol of Rpo41.

increasing the amount of Rpo41 and the reaction time and testing both linear and supercoiled forms of the DNA template in reactions lacking CTP (−CTP). Transcription by Rpo41 from linear or supercoiled templates required the presence of a functional promoter (Fig. 1B, Vector), and on linear templates, Mtf1 was essential for selective initiation (Fig. 1B, compare lanes 1 and 3). However, core Rpo41 alone was capable of selective initiation from supercoiled but not linear forms of both promoters (Fig. 1B, compare lanes 3 and 4). The appearance of the correct 107- and 38-nt −CTP transcripts from the 14 S rRNA and tRNAs\(\text{cys}\) promoters is proof that Rpo41 is capable of promoter recognition and initiation without Mtf1. Therefore, like its phage relatives, the amino acids of this single polypeptide RNAP can create the contacts necessary for initiation.

Use of a Preopened, Bubble Template Obviates the Need for Mtf1—Because supercoiled templates reduce the energy required for promoter opening (15), the requirement for Mtf1 on linear, but not supercoiled, templates supports the idea that Mtf1 is important for promoter melting (9). However, on supercoiled templates Mtf1 still stimulates transcription by a factor of 5–18 (Fig. 1B, note that there is a factor of 5 more Rpo41 present in the −Mtf1 reactions). To ask if this Mtf1 stimulation reflected the residual problems of Rpo41 with promoter opening, we created a preopened, “bubble” template (mismatched from −4 to +2) based on the 14 S rRNA promoter and used it in vitro transcription reactions with Rpo41 (+ Mtf1) (Fig. 2A). We compared transcription from the open bubble template to that on an equal sized linear template containing the 14 S rRNA promoter (linear) and a similar bubble template lacking any promoter sequence (control). Using equal amounts of Rpo41 in all reactions, we observed no transcription from the control template lacking a promoter or from the linear template in the absence of Mtf1 (Fig. 2B). However, transcription by Rpo41 alone from the bubble template was robust, a factor of 5 greater than the amount from the linear template with Mtf1 (Fig. 2B, compare lanes 1 and 3). We obtained similar results with linear and bubble templates based on the tRNAs\(\text{cys}\) promoter sequence (data not shown). Therefore, Mtf1 is necessary for opening linear templates, and stimulates expression from supercoiled DNA, but is not required for selective recognition of promoters. This behavior is very different from the core bacterial RNAP, which can also initiate from preopened templates, but does not require a promoter sequence for this activity (16).

Mtf1 Inhibits Rpo41 Activity on a Preopened Template—We observed that addition of Mtf1 to Rpo41 on the supercoiled and bubble templates actually appeared to inhibit transcription (Fig. 1B, compare lanes 1 and 2 and Fig. 2B, compare lanes 5 and 6). To explore the mechanism for this inhibition we measured Rpo41 transcription production from the linear and bubble templates in response to increasing amounts of Mtf1. As shown in Fig. 3, increasing Mtf1 stimulated transcription by Rpo41 on the closed, linear form of the 14 S rRNA promoter but repressed transcription from the premelted template. Both effects were maximal at a 1:1 ratio of core RNAP to factor. The inhibition was not caused by nonspecific binding of Mtf1 to DNA; we obtained similar results when the order of addition of components was changed to add Mtf1, rather than Rpo41, to the reaction last (data not shown).

Inhibition by Mtf1 Is Due to a Decrease in Productive Relative to Abortive Transcripts—Inhibition of transcription by Mtf1 on the preopened template could be due to direct effects on initiation at the level of first bond formation or promoter escape. We
found that addition of a dinucleotide primer to the Mtf1-inhibited Rpo41 reaction on the bubble template did not restore transcription (data not shown), indicating that the inhibition does not seem to be at the level of first bond formation. We therefore analyzed both the full-length and truncated reaction products to ask if the inhibitory effect was at the level of promoter escape. Failure to escape leads to the production of short abortive transcripts, in the range of 2–10 nt for the phage and bacterial RNAPs (17, 18). Escape into elongation is accompanied by major conformational changes for both RNAPs, and for the bacterial RNAP, changes in the interaction with sigma factor (19, 20). We have previously shown that Mtf1 is released after a short RNA chain is synthesized (7), presumably also marking this transition. We therefore considered transcripts of <10 nts to be abortive transcripts as shown in Fig. 4A. When Mtf1 was added to Rpo41 on a premelted 14 S rRNA promoter template we observed that the ratio of abortive to full-length transcripts increased by more than a factor of 10 (Fig. 4, A and B). Therefore, when Rpo41 is engaged on a preopened promoter, Mtf1 inhibits the ability of the core RNAP to escape into productive elongation. This could be due to stabilization of the initiating form of Rpo41 or to direct contacts between Mtf1 and DNA increasing the open promoter complex stability and impeding escape.

The role of accessory factors in mitochondrial transcription—These results leave open the role of Mtf1 (or its mTFB homologues in mammalian systems (21, 22)) in mitochondrial transcription. It is clearly important for promoter melting, making it critical in vivo due to the linear physical form of many mitochondrial genomes (23). The promoter melting function of Mtf1 may result from direct contacts with DNA; Mtf1 has been reported to associate relatively nonspecifically with DNA on its own (24). Alternatively, the association of Mtf1 with Rpo41 could cause changes in Rpo41 structure to facilitate promoter opening. In this regard it is interesting that Rpo41 does possess amino acid sequences related to the specificity loop and β-hairpin regions of T7 RNAP used for forming and stabilizing the open promoter (10, 25). In fact, interactions between Mtf1 and Rpo41 actually occur within these same regions of the core RNAP (26). Perhaps these interactions reorganize the structure of Rpo41 to recreate the ability of the core RNAP to open DNA like its phage relative. Mtf1/Rpo41 interactions also occur in the region similar to the RNA exit channel of T7 RNAP (10, 19, 26, 27) in such a way that Mtf1 may, like sigma factor (28), be displaced from the core RNAP by the growing RNA chain. Competition between bound Mtf1 and the exiting RNA may explain the Mtf1-dependent increase in abortive initiation that we observed on premelted templates.

Our results raise another fundamental question: if the core mitochondrial RNAP has retained the intrinsic promoter utilization properties of its phage homologue, why do all the single subunit mitochondrial RNAPs require additional specificity/stimulatory factors? The activity of T7 RNAP is regulated during the phage life cycle through interactions with T7 lysozyme that alter several aspects of initiation including affinity for nucleotides and open complex stability (29, 30). We speculate that similarities between the Rpo41/Mtf1 and the T7 RNAP/T7 lysozyme interactions (10) link the separable factor(s) to an as yet uncharacterized regulation of mitochondrial transcription.
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