Assignment of a yeast protein necessary for mitochondrial transcription initiation

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ABSTRACT

Yeast mitochondrial DNA contains multiple promoters that are responsible for expression of its genes. The basic yeast mitochondrial promoter consists of a nonanucleotide consensus sequence [5'-ATATAAGTA(+1)-3'] that must be recognized by transcription proteins, including mitochondrial RNA polymerase and any relevant *trans*-acting factors. Since mitochondrial RNA polymerase alone appeared unable to recognize a mitochondrial promoter, we examined the effects of providing accessory proteins to enable promoter function. After expression in *Escherichia coli* or purification from yeast mitochondria, two proteins were tested; they were ABF2 (a structural homologue of the human mitochondrial transcriptional activator mtTF1) and MTF1 (the gene product of a yeast locus known to exhibit a mitochondrial transcription phenotype). The results show that MTF1 specifies correct transcriptional initiation while ABF2 does not. We conclude that MTF1 is an essential key protein in yeast mitochondrial promoter function. Considering the increasing complexity of the mitochondrial transcription apparatus, we propose a nomenclature system for its components.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* affords several well known advantages for the study of the synthesis and function of both mitochondrial DNA (mtDNA) and mitochondrial RNA (mtRNA). Paramount among these is the ability to use a combined biochemical and genetic approach in a system that is still viable under conditions of loss of mitochondrial function. With regard to transcription, it is known that yeast mtDNA has multiple promoters that are individually utilized for the production of tRNAs, rRNAs and mRNAs. It has also been suggested that a class of yeast mtDNA promoters is involved in priming mtDNA replication (1,2), perhaps in a manner analogous to the mode of leading-strand priming of mtDNA replication in vertebrates (3).

The nature of yeast mtDNA promoters has been extensively examined at the level of *cis*-acting DNA template sequence

requirements. The available data point to the essential requirement of the nonanucleotide sequence 5'-ATATAAGTA(+1)-3' as the basal promoter element (1,4–6). No critical requirements for or effects due to flanking sequences at a distance have yet been observed. However, the identities of *trans*-acting proteins that can recognize and activate yeast mtDNA promoters have remained obscure. Yeast mtRNA polymerase (RPO41) has been sequenced (7) and is most similar to bacteriophages T3, T7 and SP6 among known RNA polymerases; it is likely comprised of a single catalytic polypeptide subunit (8,9).

There are additional proteins that have been suggested to play roles in yeast mitochondrial transcription. These include an ~43-kDa species (10), an ~70-kDa species (11) and a small, basic HMG(high mobility group)-box-containing protein (termed HM [12,13], p19/HM [14] or ABF2 [15]). In particular, the ~43-kDa protein has been shown to confer promoter specificity to a yeast mtRNA polymerase fraction, and it has thus been termed specificity factor (9,10).

Interestingly, a yeast nuclear gene (termed *MTF1* for mitochondrial transcriptional factor 1) has been sequenced that encodes a protein of similar size (39 kDa) to specificity factor, and which appears to be required for mitochondrial transcription (16). Lisowsky and Michaelis (16) cloned the *MTF1* by screening suppressors of *RPO41* temperature sensitive-mutants. MTF1 contains a potential mitochondrial targeting presequence at its amino terminus and disruption of *MTF1* results in loss of mtDNA (16). These data suggest that MTF1 is a mitochondrial protein. The simplest possibility for MTF1 function is that it acts in concert with yeast mtRNA polymerase. In addition, the yeast mitochondrial HMG-box protein ABF2 may be a homologue of a nucleus-encoded human mitochondrial protein (mtTF1) that recognizes and activates human mtDNA promoters (17,18).

Currently the two most obvious candidates for transcription factors participating with yeast mtRNA polymerase in transcriptional initiation are MTF1 and ABF2. In order to test directly for the effects of these proteins in transcription, we expressed the *MTF1* gene in *Escherichia coli* to obtain significant amounts of the protein free of any other yeast protein and purified ABF2 to homogeneity from a yeast mitochondrial extract. The major conclusion derived from *in vitro* transcription assays is

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that MTF1 is a critical protein necessary and sufficient to confer promoter activation when provided with yeast mtRNA polymerase. ABF2, however, is unable to substitute for MTF1 in these reactions.

MATERIALS AND METHODS

Microbial strains and DNA manipulations

Escherichia coli strains were DH5 α and BL21(DE3) (19). Yeast strains were BJ926 (deficient in some major proteases), YAM101 Δ *abf2* (α , *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*, *can1-100*, Δ *abf2::TRP1*), YAM101 *ABF2* (α , *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*, *can1-100*) (provided by Dr. Bruce Stillman, Cold Spring Harbor Laboratories) (15), and DBY2670 (α , *pep4::His3*, *prb1 Δ 1.6R*, *his3- Δ D200*, *ura3-52*, *can1*; provided by Dr. David Botstein, Stanford University). Standard methods were used for restriction fragment isolation, ligation, deletion mutagenesis using exonuclease III, and *E. coli* transformation (20).

In vitro transcription assays

Nonspecific RNA polymerase activity was assayed with the synthetic copolymer poly(dA-dT) as template. The reactions contained 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 100 μ g/ml RNase-free BSA, 400 μ M ATP, 4 μ M UTP, 10 μ g/ml poly(dA-dT) (Sigma), and 0.1 μ M [α -³²P]UTP at a specific activity of 800 Ci/mmol (NEN). Incubation was at 25°C for 30 min. Quantitation of synthesized RNA was carried out as described (21).

Promoter-specific transcription was measured in a run-off assay using either *Cla*I-digested plasmid pYm132 containing 14S rRNA gene (22) or *Ava*II-digested H3ori5 containing a putative yeast mtDNA replication origin and its associated transcriptional promoter (gift of Dr. R.A. Butow, University of Texas Southwestern Medical Center) as template. A 25- μ l transcription reaction contained 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 1 mM DTT, 100 μ g/ml RNase-free BSA, 10–40 mM KCl, 125 μ M each of ATP, CTP, and GTP, 0.5–1 μ M UTP, 10 μ g/ml template DNA, and 0.2–0.5 μ M [α -³²P]UTP at a specific activity of 800 Ci/mmol (NEN). The standard transcription reaction was at 25°C for 30 min. The run-off transcription assay was performed as described (21).

Overexpression of yeast mtRNA polymerase (RPO41)

A schematic diagram of the RPO41 overexpression plasmid is shown in Figure 1. Phage 303, containing ~19-kb of yeast genomic DNA with the gene for yeast mtRNA polymerase (gift of Dr. I. R. Lehman, Stanford University) (23) was digested with *Sal*I. An ~14-kb *Sal*I fragment was isolated and further digested with *Spe*I. The ~7.3-kb *Sal*I-*Spe*I fragment containing the *RPO41* gene was inserted into plasmid Bluescript II KS- to generate plasmid pYMRPO1.1. The *Xba*I-*Xho*I fragment containing the insert of pYMRPO1.1 was subcloned into the yeast expression vector pYES2 (Invitrogen Corporation) which contains a *Ura3* selection marker and an inducible *GAL*I promoter to produce pYMRPO1.2. This plasmid was cleaved with *Sac*I and *Xho*I whose sites are located at the vector DNA 5' to *RPO41* gene, and digested with exonuclease III. The single-stranded DNA of exonuclease III-digested DNA was removed by S1 nuclease and staggered ends were repaired with Klenow fragment. The resulting DNA was circularized by ligation and introduced

into *E. coli* DH5 α . The resulting deletion plasmids were isolated and sequenced by the method of Sanger et al. (24) to determine the end point of each deletion. A plasmid with deletion to -17 with respect to the initial methionine codon of *RPO41* was obtained and designated pYMRPO1.2 Δ 5'-17.

Plasmid pYMRPO1.2 Δ 5'-17 was introduced into *S. cerevisiae* DBY2670 by the lithium acetate method (25). The transformed yeast cells were grown to late log or early stationary stage in the synthetic complete medium without uracil [SCM-Ura (containing 0.67% yeast nitrogen base without amino acids; 2% glucose; 20 mg/liter each of adenine sulfate, tryptophan, histidine, arginine, and methionine; 30 mg/liter each of tyrosine, leucine, isoleucine, and lysine; 50 mg/liter of phenylalanine; 100 mg/liter of aspartic acid; 150 mg/liter of valine; 200 mg/liter of

Fig. 1. Construction of the RPO41 overexpression plasmid. Details of the construction of the plasmid are described in Materials and Methods. The white box represents the *RPO41* coding region; stippled box represents the *GAL*I promoter. Arrows inside boxes indicate the orientation of the gene or the promoter. Abbreviations: A, *Sac*I; B, *Xba*I; P, *Spe*I; S, *Sal*I; X, *Xho*I; Exo III, exonuclease III; S1, S1 nuclease.

threonine)]. This culture was diluted 50-fold into SCMGal-Ura (which used 2% galactose as a carbon source and is otherwise the same as SCM-Ura) to induce the expression of the *RPO41* gene. When the culture reached $OD_{600}=2-3$, the cells were harvested and mitochondria were prepared.

Preparation of mitochondrial S-100 extract

Yeast cells were grown to late logarithmic phase at 30°C in the indicated media, harvested by centrifugation at $3,000 \times g$ for 5 min at 4°C, washed once with deionized water, and subjected to spheroplast preparation according to Gasser (26). Mitochondria were isolated from the spheroplasts as described (27).

Mitochondria from about 100 g of yeast cells were resuspended in 30 ml of ice-cold lysis buffer containing 50 mM Tris-Cl (pH 7.9), 20 mM $MgCl_2$, 0.2 mM EDTA, 2 mM DTT, 40% glycerol, and 2 $\times$ protease inhibitor mixture (1 mM PMSF, 2 μ g/ml each of pepstatin A, antipain, and leupeptin). Detergent lysis of mitochondria and salt extraction of protein were carried out as described for human mitochondria (28). The lysate was centrifuged at 40,000 rpm for 60 min in a Type 75Ti rotor ($100,000 \times g_{av}$), and the supernatant was designated mtS-100.

Purification of ABF2

Three hundred mg protein of mtS-100 prepared from BJ926 grown in 1% yeast extract, 2% bactopectone, 2% galactose, and 0.1% glucose was used to purify ABF2. Heparin Sepharose CL-6B, Blue Sepharose CL-6B, and Phenyl Sepharose CL-4B (Pharmacia) were performed according to Diffley and Stillman (29,30) in buffer A containing 50 mM PIPES (pH 7.25), 20% glycerol, 0.2 mM EDTA, 1 mM DTT, and 1 $\times$ protease inhibitor mixture. In the Heparin-Sepharose chromatography, specific and nonspecific RNA polymerase activities overlapped with their activity peaks at ~ 320 mM $(NH_4)_2SO_4$ and ~ 350 mM $(NH_4)_2SO_4$ respectively, and ABF2 copurified with the nonspecific mtRNA polymerase activity. Fractions containing ABF2 and RNA polymerase activity were combined, adjusted to 300 mM $(NH_4)_2SO_4$ with buffer A, and loaded onto a Blue-Sepharose CL-6B (Pharmacia) column. The flow-through fractions contained specificity factor activity. The column was washed sequentially with 300 mM $(NH_4)_2SO_4$, 1 M NaCl, or 2.5 M NaCl/5% Triton X-100 in buffer A. Fractions containing ABF2 and nonspecific RNA polymerase activity in the final wash were combined and designated Blue Sepharose mtRNA polymerase pool (BS mtRNA pol pool). After Triton X-100 was removed by a small Heparin-Sepharose column, ABF2 pool was subjected to a Phenyl Sepharose chromatography. ABF2-containing fractions were combined, dialyzed against 100 mM NaCl in buffer B [50 mM PIPES (pH 6.5), 10% glycerol, 0.2 mM EDTA, 2 mM DTT, and 1 $\times$ protease inhibitor mixture], and applied to a Mono S HR5/5 column (Pharmacia) equilibrated with 100 mM NaCl in buffer B. The column was washed with 10 ml of 100 mM NaCl in buffer B and eluted with a 20-ml linear gradient from 100 to 600 mM NaCl in buffer B. ABF2 eluted at ~ 330 mM NaCl. Fractions containing ABF2 were pooled, concentrated in a Centricon 10 concentrator (Amicon), and applied directly to a Superose 12 HR10/30 column equilibrated with 500 mM NaCl in buffer B. Active fractions were concentrated in a Centricon 10, dialyzed against enzyme storage buffer [10 mM Tris-HCl (pH 7.9), 50% glycerol, 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, and 1 $\times$ protease inhibitor mixture], and stored at $-20^\circ C$.

Purification of yeast mtRNA polymerase

An mtS-100 (300 mg of protein) prepared from a 30-liter culture of the *RPO41* overexpression strain was dialyzed against buffer C [25 mM HEPES-KOH (pH 7.4), 20% glycerol, 1 mM DTT, 0.1 mM EDTA, 1 $\times$ protease inhibitor mixture] and then applied to a DEAE-Sepharcel (Pharmacia) column (2.5 cm $\times$ 7.1 cm) equilibrated with buffer C. The column was washed with 70 ml of buffer C and then eluted with 95 ml of 150 mM KCl in buffer C. The 150 mM KCl eluent contained both nonspecific and specific RNA polymerase activities. Active fractions were pooled, adjusted to 300 mM KCl, and loaded onto a Blue-Sepharose CL-6B (Pharmacia) column (2.5 cm $\times$ 4.0 cm). The column was washed sequentially with 50 ml each of buffer C containing 300 mM KCl, 800 mM NaCl, or 2.5 M NaCl/5% Triton X-100. Nonspecific RNA polymerase activity was found in last wash. Fractions containing nonspecific activity were combined, dialyzed against buffer C containing 150 mM KCl, and applied to a Heparin-Sepharose column (1.0 cm $\times$ 6.4 cm). Following a wash with 10 ml of buffer C containing 150 mM KCl, the column was eluted with a 50-ml linear gradient from 150 to 500 mM KCl in buffer C. *RPO41* eluted at ~ 310 mM KCl. *RPO41* and ABF2 copurified in above three column chromatographies. *RPO41*-active fractions were pooled, dialyzed against buffer C containing 150 mM KCl, and loaded onto a BioRex-70 (Bio-Rad) column (1.0 cm $\times$ 5.8 cm) equilibrated with buffer C containing 150 mM KCl. After a wash with 9 ml of 150 mM KCl in buffer C, the column was eluted with a 60-ml linear gradient from 150 to 500 mM KCl in buffer C. *RPO41* eluted at ~ 320 mM KCl and was separated from ABF2 whose peak elution was at 360 mM KCl. To concentrate *RPO41*, peak fractions of nonspecific activity were combined, dialyzed against 100 mM KCl in buffer C, applied to a 1-ml BioRex-70 column, and eluted with 5 ml of 1 M KCl in buffer C. Concentrated *RPO41* fractions were

Fig. 2. Dissociation of mtRNA polymerase by Blue-Sepharose chromatography. (A) Blue-Sepharose chromatography was performed as described in the ABF2 purification section of Materials and Methods. The mtRNA polymerase activity pool from the previous column was loaded onto a Blue-Sepharose column and its elution profile is shown. Two μ l of dialyzed fractions were assayed for nonspecific transcription activity of RNA polymerase. If one unit of nonspecific RNA polymerase activity is defined as the incorporation of 1 pmol of UMP into cold, acid-insoluble material in 30 min under the reaction conditions described in Materials and Methods, 2.3×10^4 cpm in this figure is the equivalent of 1 unit of activity. (B) Fractions were assayed for specific mtRNA polymerase activity on the yeast mitochondrial 14S rRNA promoter. Lanes 1–4, 2 μ l each of loading sample, flow-through (Fraction 20), 1 M NaCl eluent (Fraction 52), and 2.5 M NaCl/5% Triton X-100 eluent (Fraction 67); lane 5, 2 μ l of Fraction 67 plus 2 μ l of Fraction 20; lane 6, 2 μ l of Fraction 67 plus 2 μ l of Fraction 52; M, ^{32}P -labeled *HpaII* fragments of pBR322. The 373-nt correctly initiated transcripts are indicated by an arrow.

subjected to gel filtration on a Superose 12 HR10/30 column (Pharmacia) by multiple runs in buffer C containing 100 mM KCl. The elution volume of RPO41 is much larger than estimated from its molecular weight, which might be due to hydrophobic interaction between RPO41 and the column matrix. Fractions containing nonspecific activity of RNA polymerase were combined and ATP was added to a final concentration of 1 mM. This fraction was applied to a single-stranded DNA cellulose (Sigma) column (1 cm×4.4 cm) equilibrated and washed with buffer C containing 100 mM KCl and 1 mM ATP, and eluted with a 60-ml linear gradient from 100 to 500 mM KCl in buffer C plus 1 mM ATP. RPO41 was eluted at about 280 mM KCl. An aliquot of a peak fraction was dialyzed against storage buffer and stored at -20°C for all assays. The protein concentration of this dialyzed fraction was 0.34 mg/ml.

DEAE-Sephacel chromatography of mtS-100 from *ABF2* wild-type strain and *abf2*⁻ deletion mutant

An mtS-100 extract containing 30-mg protein prepared from either YAM101 *ABF2* or YAM101 Δ *abf2*⁻ grown in YPD medium was dialyzed against Tris buffer [50 mM Tris-HCl (pH

7.9), 10% glycerol, 1 mM DTT, 0.1 mM EDTA, 1×protease inhibitor mixture], loaded onto a 3-ml DEAE-Sephacel column, and washed with Tris buffer until the A_{280} reading reached a constant value. The column was eluted with 15 ml of Tris buffer containing 150 mM KCl. No nonspecific RNA polymerase activity was found in the flow-through fraction when *ABF2* wild-type extract was used, but about 70% of the nonspecific activity in the mtS-100 extract of the *abf2*⁻ deletion mutant was in the flow-through fraction. Nonspecific activity of mtRNA polymerase bound to the column was eluted with the 150 mM KCl wash. Peak fractions of nonspecific activity, termed DEAE-mtRNA polymerase, were dialyzed against enzyme storage buffer and stored at -20°C .

NaDodSO₄ (SDS)-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Laemmli (31). Protein was visualized by silver staining (32).

MTF1 expression in *E. coli* and protein isolation

The entire *MTF1* coding region (16) was amplified by PCR on *S. cerevisiae* genomic DNA using a 5' primer with an *EcoRI* site followed by two extra nucleotides GC and a 3' primer with a *HindIII* site. The ~1.05-kb PCR product was purified by electrophoresis onto DEAE-cellulose membrane (Schleicher and Schuell NA-45). The purified DNA fragment was digested with *EcoRI* and *HindIII* and cloned into the same sites of the expression vector pT7-7 (33). Lysogenic *E. coli* strain BL21(DE3), which contains a single copy of the gene for T7 RNA polymerase in the chromosome under control of the inducible *lacUV5* promoter (19), was used to express MTF1. An overnight culture of transformed BL21(DE3) was diluted 1000-fold into a fresh LB-ampicillin medium and grown at 37°C until OD₆₀₀ reached 0.6, when isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce expression of MTF1. The cells were allowed to grow for 4 more hr, after which a 150- μ l culture was pelleted, resuspended in 20- μ l cracking buffer (33), heat denatured, and loaded onto a 10% SDS-polyacrylamide gel. The expressed MTF1 was a fusion protein with 5 extra amino acids (met-ala-arg-ile-arg) at its amino-terminus, and was designated rMTF1 (recombinant MTF1).

Renaturation of rMTF1 from an SDS-polyacrylamide gel was performed according to Hager and Burgess (34). Protein in

Fig. 3. MTF1 expression in *E. coli* and subsequent functional analysis. (A) SDS-polyacrylamide gel analysis of bacterially produced recombinant MTF1 (rMTF1). The cell pellet from a culture was resuspended in cracking buffer, heated, and electrophoresed on a 10% polyacrylamide gel. Lane 1, bacteria containing the pT7-7 alone; lane 2, bacteria containing the *MTF1* gene cloned in pT7-7. The apparent molecular weight in kDa is shown on the left (B) Specificity factor fraction of Blue-Sepharose chromatography can be substituted by rMTF1 in an *in vitro* transcription assay. Protein in cracking buffer from 1 ml of induced culture was separated on a 10% SDS-polyacrylamide gel. The rMTF1 band, and the region corresponding to the rMTF1 band in the lane of bacteria containing pT7-7 alone [bacteria control (b-control)], were excised, eluted, and renatured. Three μ l of rMTF1 or b-control were assayed for transcription activity on 14S rRNA promoter alone, or with addition of 1 μ l of nonspecific RNA polymerase pool of Blue-Sepharose chromatography (BS mtRNA pol pool). Correctly initiated 373-nt transcripts are indicated by the arrow. (C) SDS-polyacrylamide gel analysis of purified RPO41. Ten μ l of a peak fraction of ss-DNA cellulose chromatography was electrophoresed on a 7% polyacrylamide gel in the presence of SDS. This peak fraction was used for *in vitro* transcription assays. (D) One μ l of renatured rMTF1, b-control or purified RPO41 alone (or their combinations as indicated) were assayed for specific transcription activity on the 14S rRNA promoter. M, ³²P-labeled *HpaII* fragments of pBR322. (E) The same as (D) except that *Avall*-digested plasmid H3ori5 containing the *ori5* promoter was used as the template to assay for 228-nt correctly initiated transcripts.

Fig. 4. Titration analysis of RPO41 and rMTF1. Purified RPO41 (0.5 μ l) with addition of different amounts of rMTF1 (A) or 1 μ l of rMTF1 with addition of different amount of RPO41 (B) were assayed for specific transcription activity on a *Clal*-digested pYm132 template. The 373-nt long correctly initiated transcript is indicated by an arrow. M, ³²P-labeled *HpaII* fragments of pBR322.

cracking buffer from a 1-ml culture was electrophoresed in a 10% SDS-polyacrylamide gel. The rMTF1 band and the region corresponding to the rMTF1 band in the lane of pT7-7-alone transformant (bacteria control) were excised. Protein in the gel slices was eluted, precipitated, dissolved in 30 μ l of 6 M guanidine-HCl in dilution buffer [50 mM Tris-HCl (pH 7.9), 20% glycerol, 0.1 mg/ml BSA, 0.15 M NaCl, 1 mM DTT, and 0.1 mM EDTA], and incubated at room temperature for 15 min. The dissolved protein was diluted 50-fold in dilution buffer and allowed to renature for 4 hr at room temperature. The renatured protein was dialyzed against enzyme storage buffer and stored at -20°C . The dialyzed rMTF1 had its concentration of 0.07 mg/ml.

RESULTS

Dissociation of mitochondrial transcription components in Blue-Sepharose chromatography

Fractions containing RNA polymerase activity from Heparin-Sepharose chromatography in the ABF2 purification procedure were capable of initiating transcription accurately on the mitochondrial 14S rRNA promoter to generate the expected 373-nucleotide(nt) transcript (Figure 2B, lane 1). When the RNA polymerase activity pool of Heparin-Sepharose chromatography was subjected to chromatography on a Blue-Sepharose column, mtRNA polymerase was dissociated into two complementary fractions: core mtRNA polymerase containing nonspecific RNA polymerase activity and a specificity factor fraction. The core mtRNA polymerase fraction contained very little accurate transcription initiation activity and instead yielded a heterogeneous array of transcription products when assayed with the 14S rRNA promoter (Figure 2B, lane 4). Core mtRNA polymerase was eluted in the 2.5 M NaCl/5% Triton X-100 wash (Figure 2A).

Fig. 5. Functional analysis of ABF2. (A) SDS-polyacrylamide gel analysis of purified ABF2. Twenty μ l of an ABF2 peak fraction were electrophoresed on a 12% polyacrylamide gel in the presence of SDS. The apparent molecular weight in kDa is shown on the left. (B) Comparison of specific activity of mtRNA polymerase between an ABF2 wild-type strain and an *abf2*⁻ deletion mutant. Mitochondrial extracts prepared from either the ABF2 wild-type strain or the *abf2*⁻ deletion mutant were chromatographed on a DEAE-Sepharose column. A peak fraction of nonspecific RNA polymerase activity was assayed for its specific activity on the 14S rRNA promoter. Lane 1, 0.25 μ l of mtRNA polymerase fraction from the ABF2 wild-type strain; lane 2, 2 μ l of mtRNA polymerase from the *abf2*⁻ deletion mutant. (C) ABF2 does not confer promoter selectivity on RPO41. ABF2 (0.4 μ g), rMTF1 (1 μ l) or RPO41 (1 μ l) alone or in combinations were assayed for their specific transcription activity on the 14S rRNA promoter. The correctly initiated transcript in (B) and (C) is 373 nt.

Specificity factor activity was in the flow-through of the Blue-Sepharose column. When the flow-through fraction was added back to core mtRNA polymerase, accurate transcription initiation activity was restored (Figure 2B, lane 5).

MTF1 is the specificity factor of mtRNA polymerase

We expressed MTF1 in *E. coli* to produce rMTF1 and tested its function using an *in vitro* transcription assay. The expressed rMTF1 was separated from bacterial protein on a SDS-polyacrylamide gel (Figure 3A), eluted from the gel, and allowed to renature. The rMTF1 isolated by this method could substitute for the Blue-Sepharose flow-through fraction containing a specificity factor (Figure 3B). This result demonstrates that MTF1 has a direct role in transcription *in vitro*.

Since the Blue-Sepharose core mtRNA polymerase fraction contains a trace amount of specific transcription initiation activity, this result could be due to MTF1 acting as a general transcriptional activator. To resolve this, we overexpressed core mtRNA polymerase and purified RPO41 to near homogeneity (Figure 3C) and, more importantly, free of a specificity factor (Figure 3D). Purified RPO41 and rMTF1 are capable of initiating transcription accurately on the 14S rRNA promoter (Figure 3D); this demonstrates that MTF1 is the specificity factor of yeast mtRNA polymerase. To ensure that the activity of these two components was not specific to the 14S rRNA promoter, we tested the yeast *ori5* promoter; this promoter is located at a putative mtDNA replication origin (2). The results in Figure 3E demonstrate that these components together activate this promoter in a fashion identical to the 14S rRNA promoter.

In order to demonstrate that the total amount of correctly initiated transcription was related to the amount of rMTF1 present in the reaction, we tested the effect of increasing concentration of rMTF1 in the presence of RPO41 (Figure 4A). The relative abundance of correctly initiated transcription was proportional

Table 1. Terminology for *S. cerevisiae*^a mitochondrial transcription components

Protein	Previous nomenclature	Proposed nomenclature
core mitochondrial RNA polymerase	RPO41 ^b	core sc-mtRNA polymerase
19-kDa basic, HMG-box-containing <i>trans</i> -acting factor	HM ^c , ABF2 ^d , p19/HM ^e	sc-mtTFA
39-kDa <i>trans</i> -acting factor	specificity factor ^f , MTF1 ^g	sc-mtTFB
Additional proteins required for transcriptional initiation	?	sc-mtTFC, sc-mtTFD, etc.

^aThe lower case prefix designates the species of origin: sc, *S. cerevisiae*; sp, *Schizosaccharomyces pombe*; h, human; m, mouse, etc.

^bGreenleaf, A.L., Kelly, J.L. and Lehman, I.R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3391–3394; Masters, B.S., Stohl, L.L. and Clayton, D.A. (1987) *Cell*, **51**, 89–99.

^cCaron, F., Jacq, C. and Rouvière-Yaniv, J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4265–4269.

^dDiffley, J.F.X. and Stillman, B. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 7864–7868.

^eFisher, R.P., Lisowsky, T., Breen, G.A.M. and Clayton, D.A. (1991) *J. Biol. Chem.*, **266**, 9153–9160.

^fSchinkel, A.H., Groot Koerkamp, M.J.A., Touw, E.P.W. and Tabak, H.F. (1987). *J. Biol. Chem.*, **262**, 12785–12791.

^gLisowsky, T. and Michaelis, G. (1989) *Mol. Gen. Genet.*, **219**, 125–128.

to the amount of rMTF1 present. As expected, the amount of accurate transcription initiation was also proportional to the amount of available RPO41 (Figure 4B).

ABF2 is not an alternative specificity factor

ABF2 is similar to human mtTF1 in that both proteins are small, basic, abundant, HMG-box-containing DNA-binding proteins (15,18). Human mtTF1 has the property of conferring promoter selectivity on an intrinsically nonselective or weakly selective RNA polymerase fraction (17,21). It was therefore interesting to test whether ABF2 could be an alternative specificity factor of yeast mtRNA polymerase. We partially purified mtRNA polymerase from the *ABF2* wild-type strain and the *abf2*⁻ deletion mutant by subjecting mitochondrial extracts to chromatography on a DEAE-Sephacel column. Both extracts had specific mtRNA polymerase activity on the 14S rRNA promoter (Figure 5B). This result indicates that ABF2 is not required for maintenance of the basal level of specific activity of the mitochondrial transcription apparatus, as judged by *in vitro* analysis.

However, this result did not exclude the possibility that ABF2 is an alternative specificity factor. We therefore purified ABF2 to homogeneity (Figure 5A), and assayed its intrinsic DNA binding activity as well as its transcription activity with purified RPO41 on the 14S rRNA promoter. Purified ABF2 bound the 14S rRNA promoter DNA (data not shown), indicating that it was in an active form, but RPO41 and ABF2 together did not show specific transcription initiation activity (Figure 5C). This result indicates that ABF2 does not enable yeast mtRNA polymerase to recognize a promoter in the absence of other transcription proteins. However, this result does not exclude a role for ABF2 in altering the efficiency of transcription initiation; we are currently testing this possibility.

DISCUSSION

These experiments suggest that two proteins are necessary and sufficient for accurate activation of yeast mitochondrial promoters. One of these, mtRNA polymerase, has not been purified to absolute homogeneity and the possibility remains that the enzyme we have used could contain a tightly associated additional component.

The second protein is MTF1, the sequence of which was determined by Lisowsky and Michaelis (16) who implicated MTF1 in mitochondrial transcription by genetic and biochemical tests. By *in vitro* transcriptional analyses we have shown that this protein confers promoter specificity in the yeast mitochondrial system. This central conclusion is consistent with the extensive biochemical work of Tabak and co-workers (9,10) who assigned the specificity factor as a protein of 43 kDa.

These results do not preclude the possibility that other proteins will be shown to be important for transcription of yeast mtDNA. In this regard, it will be of interest to learn the role(s) of ABF2 in yeast mitochondria given its apparent structural homology to the documented human mtDNA transcriptional activator mtTF1 (15,18). ABF2 is unable, in the *in vitro* assay system, to substitute for MTF1 and does not enable yeast mtRNA polymerase to recognize a yeast mtDNA promoter. Therefore it may have no role in basal transcription or it may require a more sophisticated analysis (e.g., superhelical closed circular template) to reveal its function. After this manuscript had been completed, Jang and Jaehning (35) reported that the amino-terminal sequence of the

43-kDa protein identified by Schinkel et al. (9,10) is identical to that of *MTF1* gene product (16). From gel retardation and footprinting assays on the yeast mitochondrial 21S rRNA promoter by Schinkel et al. (9), it can be concluded that only MTF1 and RPO41 together can recognize the promoter.

Given the general and overlapping nature of the nomenclature that has been used to date, we propose a simplified system that should be able to be utilized for any mitochondrial species (Table 1). The proteins thus far known to be involved or directly implicated in mitochondrial transcription initiation are listed and additional proteins can be assigned in a straightforward manner. It will be of particular interest to learn if human mitochondrial transcription requires an h-mtTFB protein that would be similar to yeast MTF1 (sc-mtTFB) or whether a significant portion of the human specificity function relies on the interaction of h-mtTFA (mtTF1) and core h-mtRNA polymerase.

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