A *Saccharomyces cerevisiae* Mitochondrial Transcription Factor, sc-mtTFB, Shares Features with Sigma Factors but Is Functionally Distinct

GERALD S. SHADEL AND DAVID A. CLAYTON*

Department of Developmental Biology, Stanford University School
of Medicine, Stanford, California 94305-5427

Received 4 November 1994/Returned for modification 19 December 1994/Accepted 24 January 1995

In *Saccharomyces cerevisiae* mitochondria, sc-mtTFB is a 341-amino-acid transcription factor required for initiation of transcription from mitochondrial DNA promoters. Specific transcription in vitro requires only sc-mtTFB and the bacteriophage-related core sc-mtRNA polymerase. Mutational analysis of sc-mtTFB has defined two regions of the protein that are important for normal function both in vivo and in vitro. These regions overlap portions of the protein that exhibit similarity to conserved region 2 of bacterial sigma factors. One mutation in this region of sc-mtTFB (tyrosine 108 to arginine [Y108R]) has a defective phenotype that matches that observed for mutations in the corresponding residue of *Bacillus subtilis* σ^A and σ^E proteins. However, mutations in the sigma 2.4-like region, including a 5-amino-acid deletion corresponding to crucial promoter-contacting amino acids of sigma factors, did not eliminate the ability of sc-mtTFB to initiate transcription specifically in vitro. This suggests a mechanism of promoter recognition for sc-mtRNA polymerase different from that used by bacterial RNA polymerases. Two mutations in a basic region of sc-mtTFB resulted in defective proteins that were virtually dependent on supercoiled DNA templates in vitro. These mutations may have disrupted a DNA-unwinding function of sc-mtTFB that is only manifested in vitro and is partially rescued by DNA supercoiling.

Transcription of *Saccharomyces cerevisiae* mitochondrial DNA (mtDNA) initiates from a simple nonanucleotide promoter sequence present at multiple locations in the circular mitochondrial genome (3, 29). Individual promoters precede mitochondrial genes encoding proteins, rRNAs, and tRNAs and are also found associated with putative yeast mitochondrial replication origin sequences (*ori* or *rep*) (22). Transcription initiation from a yeast mitochondrial promoter in vitro requires only two essential protein components, a 150-kDa mitochondrial RNA polymerase (sc-mtRNA polymerase) and a 39-kDa specificity factor (sc-mtTFB) (13, 37). Cloning and characterization of the gene encoding sc-mtRNA polymerase (*RPO41*) revealed that the enzyme is homologous to the single-subunit bacteriophage RNA polymerases from T7, T3, and SP6 (21). Early biochemical studies demonstrated that sc-mtTFB was required for specific initiation of transcription from a yeast mitochondrial promoter by sc-mtRNA polymerase (27). Footprinting experiments showed that sc-mtRNA polymerase and sc-mtTFB, when together, will protect the promoter region from DNase I digestion, while neither protein alone had specific DNA-binding activity (28). However, sc-mtTFB does bind DNA in a nonspecific manner (25). Little is known about how these two proteins interact at the promoter to form a specific closed initiation complex, except that formation of this complex is rate limiting in vitro (2).

Promoters in human mtDNA are more complex than those of *S. cerevisiae* mtDNA and are functionally dependent on the HMG-box transcription factor h-mtTFA (4, 23). The yeast homolog of h-mtTFA, sc-mtTFA, is an abundant protein in yeast mitochondria and is composed almost entirely of two HMG-box domains (7). However, unlike h-mtTFA, sc-mtTFA is not critical for transcription initiation and stimulates tran-

scription initiation only modestly in the presence of sc-mtTFB and sc-mtRNA polymerase (24). The difference in transcriptional activation capability of the two mtTFA homologs is due to the absence in sc-mtTFA of additional basic charged regions found in h-mtTFA. These charged regions stabilize specific DNA binding by the HMG-box domains of h-mtTFA to upstream regions of the promoter, thereby allowing activation of transcription (5). Thus, the yeast mitochondrial transcription system is clearly distinguished from that of humans in that the primary transcription factor in yeast mitochondria is not the HMG-box protein sc-mtTFA, but rather sc-mtTFB (29).

The gene for sc-mtTFB (*MTF1*) was initially isolated as a high-copy suppressor of a temperature-sensitive strain carrying a mutation in the *RPO41* gene (18). Disruption of the *MTF1* gene in *S. cerevisiae* leads to a stable petite phenotype and loss of mtDNA, consistent with a role in mtDNA transcription and replication. Inspection of the predicted amino acid sequence of sc-mtTFB revealed regions that exhibit limited similarity to the conserved regions 2 and 3 of bacterial sigma factors (13) and a region near the center of the primary sequence that is rich in basic amino acids (18). Specific subregions have been defined within the conserved region 2 of bacterial sigma factors (subregions 2.1 to 2.4). Subregion 2.4 is involved in specific recognition of the -10 DNA element of the bacterial promoter (6, 8, 10, 14, 30, 34, 35, 39), and subregion 2.3 has been implicated in facilitating the unwinding of DNA during transcription initiation (1, 15, 26, 36). Deletions in subregion 2.1 (17) or region 3 (38) of sigma factors apparently reduce the affinity of sigma factors for the core RNA polymerase, suggesting that these regions are necessary for this interaction. Subregion 2.2 is the most highly conserved region in the sigma factors (19), implying that it has a critical, but yet to be defined, function.

We have generated a total of 20 mutations in the sc-mtTFB protein and subsequently analyzed the effects of these mutations both in vivo and in an in vitro transcription assay with all

* Corresponding author. Phone: (415) 723-6230. Fax: (415) 725-7739.

recombinant components. This analysis has permitted the identification of important regions of sc-mtTFB based on both in vivo and in vitro criteria. In addition, two of the mutations resulted in a strong temperature-sensitive petite phenotype, which should be useful in future analysis of the function of sc-mtTFB in the cell.

MATERIALS AND METHODS

Enzymes and reagents. Restriction enzymes, T4 DNA polymerase, T4 DNA ligase, T4 polynucleotide kinase, and Klenow fragment of *Escherichia coli* DNA polymerase I were obtained from Boehringer Mannheim. The T4 gene 32 protein was from New England Biolabs. Sequenase was from United States Biochemicals. Radioactive nucleotides were from Dupont, New England Nuclear. 5-Fluoro-orotic acid (5-FOA) was obtained from PCR Incorporated. Reagents for yeast media were from Difco. All other reagents were of the highest quality commercially available.

Construction of bacterial and yeast expression plasmids. The plasmid pE1-1 was obtained from a yeast genomic library constructed in the shuttle vector λ -Yes-R and contains a 2.9-kb *XhoI* restriction fragment harboring the *MTF1* gene and flanking sequences. This plasmid contains a yeast *URA3* selectable marker and the *CEN4/ARS1* replication origin.

The plasmid pGS345 was constructed by insertion of the *MTF1*-containing *XhoI* fragment from pE1-1 into the shuttle vector pRS314 (32). This plasmid contains a *TRP1* selectable marker and the *CEN6/ARS4* replication origin and was used to introduce site-directed mutations in the *MTF1* gene with subsequent phenotypic analysis by plasmid shuffle.

To allow expression of sc-mtTFB in *E. coli*, the *MTF1* coding region was inserted into the plasmid pT7-7 (33). This was accomplished by using two complementary oligonucleotides that create *NdeI* and *BamHI* ends when annealed and which contain the 5' 34 nucleotides (nt) of the *MTF1* coding region. The oligonucleotides were ligated into the *NdeI* and *BamHI* sites of pT7-7. This portion of the *MTF1* gene also contains a naturally occurring *EcoRV* site. The remainder of the *MTF1* coding region was obtained from the plasmid pE1-1 on an *EcoRV*-*HindIII* restriction fragment that was ligated into the pT7-7 plasmid containing the synthetic 5' portion of the *MTF1* gene to create the plasmid pGS344. For expression of mutated forms of sc-mtTFB, appropriate restriction fragments from the mutated pGS345 plasmids were used to replace the wild-type sequences in pGS344.

To permit expression of sc-mtRNA polymerase in *E. coli*, the *RPO41* gene was inserted into the plasmid pT7-7 in a fashion similar to that described above for sc-mtTFB. Here the *RPO41* gene was engineered so that the ATG start codon of pT7-7 preceded the codon for amino acid 27 of the *RPO41* DNA sequence. The resulting recombinant protein produced from this plasmid (pGS317) should closely resemble the mitochondrial form of the enzyme which is processed at amino acid 29 upon import into mitochondria (2a).

Site-directed mutagenesis. *E. coli* BW313 (*dut ung*) was transformed with the plasmid pGS345. Single-stranded uracil-containing DNA was prepared by infecting a culture of this strain with the helper phage M13K07. The mutagenesis reaction was done essentially as described previously (16) except that the total reaction volume was 50 μ l and 1 $\times$ One-Phor-All buffer (Pharmacia) was used instead of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer.

Yeast strains and media. The basic techniques used for the growth and genetic manipulation of yeast strains have been described previously (11). The yeast strain GS102 (*MATa ura3-52 leu2-3,112 trp1 Δ MTF1::LEU2 Δ his3- Δ 200 ade2 pE1-1[λ -YESR::MTF1 URA3 CEN4/ARS1]*), used for the *MTF1* plasmid shuffle experiments, was created as follows. The *MTF1* null strain, BX104 (*MATa ura3-52 leu2 trp1 Δ MTF1::LEU2*), contains a *LEU2* insertion in the *MTF1* gene described by Lisowsky and Michaelis (18). The yeast strain DBY2006 (*MATa ura3-52 leu2-3,112 trp1- Δ 1 Δ his3- Δ 200 ade2*) was transformed with the plasmid pE1-1, a *CEN* plasmid containing the *MTF1* gene and a *URA3* selectable marker, to generate the strain GS100. To obtain a yeast strain with functional mitochondria that are dependent on the plasmid-borne copy of the *MTF1* gene, BX104 and GS100 were mated and sporulated. Spores that were *LEU*⁺ *URA*⁺ *trp*⁻ *his*⁻ *ade*⁻ were picked for further analysis. These spores were then tested for their ability to grow on glycerol medium (YPG) and for lack of growth on glycerol medium plus 5-FOA (YPG-FOA). A spore having the above phenotype was designated GS102 and used in the plasmid shuffle experiments. The GS105 (*MATa ura3-52 leu2-3,112 trp1 Δ MTF1::LEU2 Δ his3- Δ 200 ade2 pGS345 [pRS314::MTF1 TRP1 CEN4/ARS6]*) and GS106 (*MATa ura3-52 leu2-3,112 trp1 Δ MTF1::LEU2 Δ his3- Δ 200 ade2 pRS314*) strains were derived by transforming GS102 with pGS345 and pRS314, respectively, and then carrying these strains through the plasmid shuffle.

Plasmid shuffle strategy for phenotypic analysis of mutations in the *MTF1* gene. The plasmid shuffle strategy was essentially as described previously (31). The yeast strain GS102 contains a plasmid-borne copy of the *MTF1* gene linked to the *URA3* selectable marker and is the only wild-type copy of the gene present. Thus, this strain requires the presence of this plasmid (pE1-1) to maintain functional mitochondria. The GS102 strain was transformed with pGS345 deriv-

atives harboring the mutation of interest, and *TRP*⁺ transformants were selected. Transformants were streaked onto synthetic complete media containing 5-FOA. Colonies from the 5-FOA plates were then examined for growth on YPD and YPG at 18, 30, and 37°C to assess their mitochondrial phenotype.

Isolation of recombinant proteins. The sc-mtRNA polymerase, sc-mtTFB, and all of the mutated sc-mtTFBs were expressed in *E. coli* BL21(DE3) with the pT7-7 overexpression system (33). Liquid cultures (5 ml) of BL21(DE3) containing the plasmid of interest (pGS317 for sc-mtRNA polymerase, pGS344 for sc-mtTFB, or pGS344 harboring the mutation of interest) were grown overnight at 30°C in a rotary shaker. The culture was then diluted 1/100 into 5 ml of fresh Luria-Bertani medium, and the culture was grown at 30°C to an optical density at 600 nm (OD_{600}) of 0.8. Expression of the recombinant proteins was induced by the addition of IPTG (isopropyl- β -D-thiogalactopyranoside) to 0.4 mM, and culture growth was continued for 4 h. After induction, the cultures were placed on ice and the OD_{600} for each was determined. To allow comparison of the various mutations, samples were taken from induced cultures so that ($OD_{600} \times \text{ml}$) = 2.5. To facilitate the analysis of a large number of mutations, we chose to isolate the abundant insoluble fraction of the recombinant protein and take advantage of the documented ability to denature and renature sc-mtTFB and sc-mtRNA polymerase (27). A better yield of sc-mtRNA polymerase was obtained by performing the growth and induction steps at 37°C.

To isolate the insoluble fraction, the bacteria were collected by centrifugation in a microcentrifuge (16,000 $\times$ g, 30 s), washed once with 0.5 ml of lysis buffer (50 mM Tris-Cl [pH 8.0], 100 mM KCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride), and resuspended in 0.4 ml of lysis buffer. Lysozyme was added to the samples at a final concentration of 0.4 mg/ml, and the samples were incubated at room temperature for 15 min with occasional inversion. The samples were centrifuged in a microcentrifuge (16,000 $\times$ g, 15 min, 4°C). The supernatant was removed, and the pellet was resuspended in 0.4 ml of lysis buffer containing Triton X-100 (0.5%). The samples were left at room temperature for 5 min, and the above centrifugation was repeated. The supernatant was again removed, and the pellet was washed once with 0.4 ml of lysis buffer (pH 7.9, without Triton X-100). The pellet was then washed with ice-cold acetone and dried.

The denaturation and renaturation of the insoluble protein were done essentially as described previously (12) except that bovine serum albumin (BSA) was not added to the dilution buffer. The dried pellet was resuspended in 50 μ l of dilution buffer (0.05 M Tris-HCl [pH 8.0], 20% glycerol, 0.15 M KCl, 1 mM dithiothreitol, and 0.1 mM EDTA) containing 6 M guanidine hydrochloride and incubated at room temperature for approximately 1 h with occasional mixing to ensure that the protein was in solution. The sample was then diluted 50-fold with dilution buffer (first 50 μ l into 1,000 μ l and then 400 μ l into 1,000 μ l) and allowed to renature overnight (8 to 12 h) at room temperature. The samples were then used directly in transcription reactions or diluted twofold with 80% glycerol and stored at -20°C.

Transcription in vitro. DNA template used in these assays was the plasmid p14SGS, which contains the mitochondrial 14S rRNA promoter (a standard nonanucleotide promoter) cloned into pBSIISK⁺ (Stratagene) on a 150-bp restriction fragment. The template was used undigested for negatively supercoiled template or digested with *EcoRI* to generate a linear template. Except where stated in figure legends, transcription was performed in the absence of rCTP. This allows a specific transcript of 109 nt to be produced because of the absence of a template-encoded guanosine until position +110. This permitted a specific transcript to be observed from the supercoiled template and reduced the background of transcription observed from the contaminating T7 RNA polymerase in the protein fractions. All transcription reactions were in a total volume of 25 μ l. Template DNA (1 μ l of a 0.25- μ g/ μ l stock solution), 12.5 μ l of 2 $\times$ transcription buffer (20 mM Tris-HCl [pH 7.9], 20 mM MgCl₂, 20 mM KCl, 2 mM dithiothreitol, and 200 μ g of BSA per ml), 2 μ l of distilled water, 3 μ l of recombinant sc-mtRNA polymerase, and 3 μ l of recombinant sc-mtTFB were mixed and incubated on ice for 10 min. Then 0.5 to 1.0 μ l of [α -³²P]UTP (5 to 10 μ Ci) and 2.5 μ l of 10 $\times$ nucleoside triphosphate mix (1.25 mM [each] GTP and ATP and 10 μ M UTP) were added, and the transcription reaction was performed in a 30°C water bath for 12 min. The samples were then placed on ice, stopped by the addition of 87 μ l of stop solution (0.4 M sodium acetate [pH 5.2], 10 mM EDTA, and 10 μ g of *E. coli* tRNA), and precipitated by adding 300 μ l of ice-cold ethanol and incubating on dry ice for 30 min. The samples were then centrifuged in a microcentrifuge (16,000 $\times$ g, 15 min, 4°C), washed with 0.5 ml of 70% ethanol, and dried. The RNA pellet was resuspended in formamide gel-loading buffer, heated to 95°C, and loaded onto a 8% denaturing polyacrylamide gel. After electrophoresis, the products were analyzed by autoradiography.

Western (immunoblot) analysis. Yeast whole-cell protein extracts were prepared from cultures grown in YPG medium as described in the legend to Fig. 2. The proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through a 12% polyacrylamide gel. The proteins were then transferred to a supported nitrocellulose membrane (Schleicher & Schuell; BA-S) with an electroblotting apparatus. The sc-mtTFB protein was detected with rabbit anti-sc-mtTFB antibody (1/1,000 dilution) and the ECL Western blotting detection reagents (Amersham) precisely as described by the manufacturer.

FIG. 1. Mitochondrial phenotype of sc-mtTFB mutations. (A) Growth of four sc-mtTFB mutants on YPD and YPG at 30°C and YPG at 37°C are shown. The mutants are labeled on the left. The 1-291 and 1-268 mutations are the result of nonsense codons introduced at codons 292 and 269, respectively. Δ115-119 is a 5-amino-acid deletion of residues 115 to 119. I117S is a change of isoleucine 117 to serine (this designation is used throughout the text). (B) Growth of three temperature-sensitive strains. Liquid cultures of the five strains were diluted to the same OD₆₀₀, and 10-fold serial dilutions were plated from left to right. The GS105 strain is the wild-type *MTF1* plasmid shuffle strain, GS106 is the *MTF1* null strain, and the three point mutants (Y42R, Y108R, and D52A) are temperature sensitive for growth on glycerol.

RESULTS

Mutagenesis of sc-mtTFB and assessment of mitochondrial phenotypes. We employed a plasmid shuffle strategy to determine the mitochondrial phenotypes of mutations in the *MTF1* gene encoding the 341-amino-acid sc-mtTFB. Mutations were introduced by site-directed mutagenesis into the *MTF1* gene located on a *TRP1* plasmid (pGS345). This mutated plasmid was then used to transform the yeast strain GS102 carrying the *MTF1* gene on a *URA3* plasmid (see Materials and Methods). Growth of the transformed strain on 5-FOA requires loss of the wild-type *MTF1* gene on the *URA3* plasmid and forces the strain to become dependent on the mutated *MTF1* gene on pGS345. To test the plasmid shuffle assay, two nonsense mutations were introduced into the *MTF1* gene at codons 292 and 269 so that truncated proteins containing amino acids 1 to 291 and 1 to 268, respectively, would be synthesized *in vivo*. Growth of these two strains on dextrose medium (YPD) and glycerol medium (YPG) at 30 and 37°C is shown in Fig. 1. A plasmid shuffle with the 269 nonsense mutation (1-268) resulted in a strain that was unable to grow on YPG at any temperature (petite phenotype). The 292 nonsense mutation (1-291) yielded a strain that was able to grow on YPD and YPG at 30°C but which was unable to grow on YPG at 37°C (temperature-sensitive petite phenotype). For comparison, the

FIG. 2. Schematic representation of the sc-mtTFB protein and location of the 20 mutations analyzed in this study. At the top of the figure is the sc-mtTFB protein shown with regions (shaded boxes) that have limited similarity to the bacterial sigma factor regions 2 (subregions 2.1 to 2.4 are indicated) and 3 and the basic region near the center of the protein (box with plus signs). The numbers below the diagram are the amino acid positions of sc-mtTFB. These regions are shown in more detail in the expanded portion of the figure along with the actual amino acid sequence of sc-mtTFB written below each. Single point mutations are designated by a single arrow below the altered residue. Multiple point mutations are indicated by interconnected arrows. Solid arrows indicate mutations at residues that are highly conserved between sc-mtTFB and the sigma factor family of proteins. The white box underneath the 2.4 region indicates a 5-amino-acid deletion (Δ5) of residues 115 to 119. The plus signs indicate the location of basic residues in the basic region of sc-mtTFB. All of the mutations change the indicated residue to alanine unless otherwise specified by the amino acid below the arrow in the diagram. For example, in the 2.1 region the one mutated protein has aspartic acid (D) 34 and lysine (K) 35 each changed to alanine (mutant D34A-K35A) and another mutated protein has tyrosine (Y) 42 changed to arginine (R) (mutant Y42R).

MTF1 plasmid-shuffle wild-type strain (GS105) and null strain (GS106) are shown in Fig. 1B. GS105 is viable on YPD and YPG at all temperatures while GS106 is unable to grow on YPG (petite). The ability of the 1-291 mutant to grow on glycerol at the permissive temperature demonstrates that the C-terminal 50 amino acids are dispensable for normal activity. However, removal of these amino acids results in loss of function at 37°C. On the basis of these two deletions, the amino acids between 268 and 291 encompass a potentially important region of sc-mtTFB.

Twenty additional mutations were introduced into the *MTF1* gene that resulted in amino acid changes throughout the sc-mtTFB polypeptide (Fig. 2). The C-terminal 50 amino acids were not targeted for mutagenesis since they are apparently dispensable for activity on the basis of the nonsense mutations described above. Eleven of these mutations were alanine-scan-type mutations that changed clustered charged residues to alanine, eight were point mutations that changed amino acids in proposed sigma factor-like regions, and one was a 5-amino-

FIG. 3. Western analysis of temperature-sensitive strains harboring the Y42R and Y108R mutations in sc-mtTFB. Cultures of GS105 (wild-type sc-mtTFB) and the two mutant strains were grown to mid-log phase at 30°C in YPG medium and then shifted to 37°C. Samples were removed prior to the temperature shift (lanes A) and at 5 h (lanes B), 9 h (lanes C), and 21 h (lanes D) after the temperature shift to 37°C. Yeast whole-cell protein extracts were prepared from each sample for Western analysis as described in Materials and Methods. Molecular mass markers are indicated to the left of the figure, and the 40-kDa sc-mtTFB protein is indicated by the arrow.

acid deletion in the sigma factor 2.4-like region of sc-mtTFB. All mutations were analyzed for mitochondrial phenotypes through the plasmid shuffle strategy. Those mutations that exhibited a mitochondrial phenotype are shown in Fig. 1. Only the 5-amino-acid deletion (Δ 115-119) displayed a nonconditional petite phenotype (Fig. 1A). The tyrosine 42-to-arginine (Y42R), Y108R, and D52A mutations all exhibited a temperature-sensitive petite phenotype (Fig. 1B). The Y42R and Y108R mutants were more temperature sensitive than D52A as demonstrated by serial dilution of the strains on selective media. The remainder of the mutations did not result in a mitochondrial phenotype as judged by growth on YPG medium. A typical mutation of this class is I117S, shown in Fig. 1A. Growth was observed on YPD and YPG at all temperatures. None of the mutants exhibited cold sensitivity on glycerol at 18°C.

To ensure that the temperature-sensitive defects were due to loss of the transcriptional activation capability of sc-mtTFB and not a result of the instability of the mutant proteins at 37°C, Western analysis was performed on extracts of yeast cultures that had been grown for various times at the nonpermissive temperature (Fig. 3). As with the wild-type strain (GS105), the levels of sc-mtTFB in the two most severe temperature-sensitive strains (Y42R and Y108R) were not decreased even after growth for 21 h at 37°C. Similar results were obtained with the strain harboring the D52A mutation (data not shown).

Reconstitution of mitochondrial transcription initiation with recombinant proteins. Expression of both the sc-mtRNA polymerase (RPO41) and sc-mtTFB proteins with the pT7-7 system resulted in more than half of the protein residing in the insoluble fraction of the cell lysate. Since both of these proteins have been successfully renatured after isolation from SDS-PAGE gels and denaturation by guanidine hydrochloride, we reasoned that such a denaturation-renaturation strategy could be used with the crude insoluble fraction to assay transcription activity of these proteins easily in vitro. Both sc-mtRNA polymerase and sc-mtTFB activities were isolated by this method and tested for transcription capacity in vitro with the mitochondrial 14S rRNA promoter (Fig. 4). Addition of renatured sc-mtTFB to the renatured sc-mtRNA polymerase in a transcription reaction resulted in a specific transcript from the mitochondrial 14S rRNA promoter. No transcript was observed when renatured sc-mtRNA polymerase was added to the reaction alone or together with a renatured extract from *E. coli* harboring pT7-7 alone (no sc-mtTFB). This demonstrated

FIG. 4. Transcription from a yeast mitochondrial promoter in vitro with recombinant proteins. Transcription reactions and the preparation of recombinant proteins were done as described in Materials and Methods. In these reactions, all four ribonucleoside triphosphates were added so that a 129-nt runoff transcript (indicated by the arrow) was produced from the linear p14SGS template. The protein fractions added to each reaction are indicated at the top of the figure as follows: sc-mtRNA polymerase purified from yeast cells (yeast RPO41), sc-mtRNA polymerase purified from *E. coli* (r-RPO41), sc-mtTFB purified from *E. coli* (r-sc-mtTFB), r-sc-mtTFB that was purified by native chromatographies (native r-sc-mtTFB), and a control extract from *E. coli* that was identical to r-sc-mtTFB, but devoid of sc-mtTFB (pT7-7).

that the reaction was dependent on the addition of sc-mtTFB. The observed transcript was the same length as a control transcript synthesized with sc-mtRNA polymerase purified from yeast cells and recombinant sc-mtTFB purified by native purification methods (37) (Fig. 4).

Activity of mutated sc-mtTFB proteins in vitro. The denaturation-renaturation method described above was used to analyze all of the mutated sc-mtTFB proteins in vitro. Eleven of the mutations had no significant effect on sc-mtTFB function in vitro. All of these mutated proteins exhibited between 25 and 100% of the activity of wild-type sc-mtTFB in a transcription reaction with a linear DNA template (Fig. 5). The remaining nine mutations resulted in proteins that were severely defective in the transcription assay; we tested these proteins with both linear and supercoiled DNA templates. Seven of the nine defective proteins (Y42R, E46A-E47A-K49A, D52A, Y108R, D248A-D251A, D260A-R264A, and Δ 115-119) exhibited low activity on both types of templates in vitro. Transcription reactions with these mutated sc-mtTFB proteins (except for the Δ 115-119 mutant) on a supercoiled DNA template are shown in Fig. 6. Proteins harboring the mutation Y42R or Y108R (the two strongest temperature-sensitive mutations) sponsored the lowest levels of transcription initiation in vitro and could be detected only upon longer exposure. The remaining two in vitro-defective mutated proteins, R178A-K179A and H187A-R189A, showed a marked dependence for a supercoiled DNA template (Fig. 7). The transcription reaction with the Δ 115-119 mutated sc-mtTFB protein was included in Fig. 7 as a supercoiling-independent control since it was representative of the former group of in vitro-defective mutated proteins. Since tem-

FIG. 5. Transcription in vitro of mutated sc-mtTFB proteins that retained substantial activity. All proteins were isolated from *E. coli* by the denaturation-renaturation method described in Materials and Methods. Transcription reactions were performed on linear p14SGS template in the absence of rCTP so that a 109-nt transcript (arrow) was produced (see Materials and Methods). All of the reactions contained r-RPO41 plus wild-type sc-mtTFB (r-sc-mtTFB) or mutated sc-mtTFB proteins as indicated at the top of the figure.

plate topology and preference could be affected (both in vivo and in vitro) by sc-mtTFA, which is known to bend and wrap DNA (9), we tested the ability of sc-mtTFA to complement or alter mutated sc-mtTFB function in vitro on both linear and supercoiled templates. None of the in vitro-defective mutated proteins were rescued by the addition of sc-mtTFA to the reaction (data not shown).

To ensure that nine mutations that displayed a phenotype in vitro were synthesized at the same level as the wild-type protein and did not result in unstable proteins, whole-cell *E. coli* extracts containing these mutated proteins were subjected to SDS-PAGE (data not shown). All of the mutated proteins were synthesized at nearly identical levels as wild-type sc-mtTFB. This suggests that the in vitro defects were most likely not due to lower levels of the mutated protein in the reaction. In addition, dilution experiments with sc-mtTFB revealed that the protein is in 10- to 100-fold excess in the in vitro reaction and has to be diluted 1,000- to 10,000-fold to achieve the low level of transcription observed with the in vitro-defective proteins (data not shown). For these reasons, we do not believe that the

FIG. 6. Transcription in vitro of mutated sc-mtTFB proteins that exhibited low levels of activity. All proteins were isolated from *E. coli* by the denaturation-renaturation method described in Materials and Methods. Transcription reactions were performed on supercoiled p14SGS template in the absence of rCTP such that a 109-nt transcript (arrow) was produced (see Materials and Methods). All of the reactions contained r-RPO41 plus wild-type sc-mtTFB (r-sc-mtTFB) or mutated sc-mtTFB proteins as indicated at the top of the figure.

FIG. 7. Two mutated sc-mtTFB proteins are dependent on supercoiled templates. All proteins were isolated from *E. coli* by the denaturation-renaturation method described in Materials and Methods. Transcription reactions were performed either on linear (L) or on supercoiled (SC) p14SGS template (as indicated) in the absence of rCTP such that a 109-nt transcript (arrow) was produced (see Materials and Methods). All of the reactions contained r-RPO41 plus wild-type sc-mtTFB (r-sc-mtTFB) or the mutated sc-mtTFB proteins indicated at the top of the figure. The $\Delta 115-119$ protein was included as a supercoiling-independent control.

observed in vitro defects are the result of small differences in the ability of the mutant proteins to fold properly or of loss of protein during the renaturation procedure. Nonetheless, in the absence of corroborating defects in vivo, mutations resulting in defects only in vitro have to be interpreted with caution.

Correlation of in vivo and in vitro phenotypes. A summary of the in vivo and in vitro phenotypes of the 20 mutations is provided in Table 1. For 15 of the mutations, the phenotype in vivo correlated with the transcription activity in vitro. Those mutations that resulted in proteins with wild-type activity in vitro displayed no mitochondrial phenotype, and the four mutations that displayed a petite phenotype were defective in the transcription assay in vitro. In addition, the three temperature-sensitive mutations exhibited relative activities in vitro that reflected the severity of the mitochondrial phenotype. The Y42R and Y108R mutated proteins had more severe transcriptional defects in vitro than the D52A protein, consistent with their greater temperature sensitivity in vivo (Fig. 1B).

There were several noteworthy differences between the in vitro activity of five of the mutated sc-mtTFB proteins and the observed phenotypes in *S. cerevisiae*. Five of the mutations (E46A-E47A-K49A, R178A-K179A, H187A-R189A, D248A-D251A, and D260A-R264A) were defective in the in vitro assay, yet wild type in vivo. Two of these are the basic region mutations that resulted in proteins that were dependent on template supercoiling. It is possible that either supercoiling of mtDNA or the presence of other protein factors is able to compensate for the low activity of these mutated proteins in vivo. Finally, the $\Delta 115-119$ sc-mtTFB protein was still capable of significant levels of transcription in vitro (comparable to the two supercoiling-dependent proteins [Fig. 7]). This suggests that this mutation does not impair the ability of the holoenzyme to recognize the yeast mitochondrial promoter but disrupts another critical function of the protein.

TABLE 1. Summary of sc-mtTFB mutant phenotypes

Mutant sc-mtTFB	Protein region ^a	In vivo phenotype ^b	In vitro phenotype ^c
D34A-K35A	Sigma 2.1-like	WT	WT
D37A-K40A	Sigma 2.1-like	WT	WT
Y42R	Sigma 2.1-like	TS petite	Defective sc-independent
E46A-E47A-K49A		WT	Defective sc-independent
L51A	Sigma 2.2-like	WT	WT
D52A	Sigma 2.2-like	TS petite	Defective sc-independent
I59A	Sigma 2.2-like	WT	WT
K89A-E90A		WT	WT
S106A-T107A	Sigma 2.3-like	WT	WT
Y108R	Sigma 2.3-like	TS petite	Defective sc-independent
E115A	Sigma 2.4-like	WT	WT
I117A	Sigma 2.4-like	WT	WT
Δ115-119	Sigma 2.4-like	Petite	Defective sc-independent
D130A-K13A	Sigma 2.4-like	WT	WT
R178A-K179A	Basic	WT	Defective sc-dependent
H187A-R189A	Basic	WT	Defective sc-dependent
E212A-K214A		WT	WT
E222A-E223A-D225A	Sigma 3.0-like	WT	WT
D248A-D251A	Sigma 3.0-like	WT	Defective sc-independent
D260A-R264A	Sigma 3.0-like	WT	Defective sc-independent

^a Designations in this column refer to the location of mutations in putative functional regions of sc-mtTFB postulated on the basis of the predicted amino acid sequence (13, 18).

^b The in vivo phenotypes are as follows: wild type (WT), no detectable growth inhibition on YPG medium at any temperature; petite, lack of growth on YPG at all temperatures; temperature-sensitive (TS) petite, lack of or slow growth on YPG at 37°C.

^c The in vitro phenotypes are as follows: wild type (WT), 25 to 100% of specific transcription initiation activity of renatured, recombinant wild-type sc-mtTFB; defective sc-independent, less than 25% activity on both linear and supercoiled (sc) templates; defective sc-dependent, substantial transcription initiation observed only on supercoiled (sc) templates.

DISCUSSION

The sc-mtTFB protein is essential for specific recognition of a yeast mitochondrial promoter. In fact, it is the only protein component other than the single-subunit core sc-mtRNA polymerase (RPO41) necessary to achieve specific transcription initiation from a mitochondrial promoter in vitro. Given its important role in promoter recognition, functional comparisons have been drawn between sc-mtTFB and sigma factors (2, 13, 20), which are the critical protein determinants of promoter specificity in bacteria. As a result, the sc-mtTFB amino acid sequence has been examined in detail for potential similarity, with inferred homology, to this diverse family of proteins. One such search identified regions within sc-mtTFB that exhibit some similarity to the conserved regions 2 and 3 of sigma factors (13). These regions of sigma factors have been proposed to be involved in recognition of the bacterial promoter (6, 8, 10, 14, 30, 34, 35, 39), DNA unwinding (1, 15, 26, 36), and interactions between the sigma factors and the core RNA polymerase (17, 38). The significance of these matches has been questioned (19) and is difficult to evaluate for several reasons. First, a potentially important degree of identity is found only when sc-mtTFB is compared with the entire group of bacterial sigma factors, a group that within itself is quite divergent (19). Second, mutagenesis of several sigma factors revealed that many of the highly conserved residues in these regions are extremely tolerant of amino acid changes (14, 36), making an assessment of the relevance of conserved residues difficult even amongst known sigma factors. Finally, there has been no systematic genetic analysis of sc-mtTFB to begin to validate any hypotheses about the structure or function of the protein based on its amino acid sequence. Thus, we present here the results of a mutagenesis study of sc-mtTFB that has allowed the initial identification of important regions of the polypeptide (Fig. 8) and has provided some insights into how this protein might function to promote specific transcription initiation from a yeast mitochondrial promoter.

Mutational analysis defines two regions of sc-mtTFB that potentially correspond to those in conserved region 2.0 of sigma factors. Of the 20 mutated sc-mtTFB proteins that were analyzed in this study, four (Y42R, D52A, Y108R, and Δ115-119) were defective both in vivo and in vitro (Table 1). These mutations define two regions of the sc-mtTFB protein that overlap portions of the sigma 2.0-like region of sc-mtTFB (Fig. 8, black boxes). The importance of the region between residues 42 and 52 is strengthened further by the E46A-E47A-K49A mutation which results in a protein that is defective in the in vitro assay. In addition, mutations that alter a conserved tyrosine residue of region 2.3 of the *Bacillus subtilis* σ^A and σ^E sigma factors have severe defects both in vivo and in vitro (14, 26). This matches the phenotype of the Y108R mutation in sc-mtTFB (the corresponding tyrosine residue), which results in a temperature-sensitive petite phenotype in vivo and a virtually inactive protein in vitro. Together, these results support the hypothesis that the 2.0-like region of sc-mtTFB and the conserved region 2.0 of bacterial sigma factors are homolo-

FIG. 8. Genetically defined regions of sc-mtTFB. A linear representation of sc-mtTFB is presented. The black boxes denote regions of the protein that were defined by mutations that had effects both in vivo and in vitro. The potentially important region spanning residues 178 to 189 (gray box) overlaps the basic region and contains both of the supercoiling-dependent mutations. The amino acid positions are given below the diagram along with the relative locations of the sequence-defined regions (putative sigma factor homologies and the basic region).

gous. However, as will be discussed in the following section, functional differences in the 2.4-like region of sc-mtTFB suggest that, while the 2.0-like region of sc-mtTFB may be homologous to that of sigma factors, these regions may have diverged considerably. Such divergence might also explain why several mutations that changed highly conserved residues in the putative sigma 2.0-like domain had no effect on the activity of sc-mtTFB (i.e., K40A, L51A, I59A, and S106A-T107A).

The subregion 2.4-like sequences of sc-mtTFB are not essential for specific promoter recognition by sc-mtRNA polymerase. The 2.4 region of sigma factors has been studied extensively, and it is well established that this region of the protein plays a critical role in binding and recognizing the -10 DNA element of bacterial promoters. Four mutations characterized in this study were in the region postulated to be similar to region 2.4 of sigma factors (Fig. 2; Table 1). Of these, only the $\Delta 115-119$ deletion mutation had deleterious effects. This mutation had the most severe effects in vivo, resulting in a nonconditional petite phenotype, and its activity was also compromised in vitro. The 5-amino-acid deletion ($\Delta 115-119$) and E115A were constructed because the corresponding residues in a variety of sigma factors have been implicated by genetic methods in contacting directly critical nucleotides of the bacterial promoter. The I117S mutation was made because it corresponds to a highly conserved residue in sigma factors and was the only residue conserved when this region is aligned with sc-mtTFB (13). Neither the E115A nor the I117S mutation had deleterious effects on sc-mtTFB function in vivo or in vitro. While the $\Delta 115-119$ protein was clearly defective both in vivo and in vitro, it was still capable of significant levels of specific transcription initiation in vitro (Fig. 7). These results are inconsistent with a role for this region of the protein in determining promoter specificity. We thus believe that the mechanism of promoter recognition by yeast mtRNA polymerase is fundamentally different from that of bacterial RNA polymerases and does not involve the sigma 2.4-like region of sc-mtTFB in the same capacity as in its use by the sigma factors. An alternative possibility, consistent with all data concerning promoter recognition in yeast mitochondria, is that the specificity determinants of promoter recognition reside on the sc-mtRNA polymerase subunit. In this regard, it is interesting to note the similarity between sc-mtRNA polymerase and the monomeric RNA polymerases of bacteriophages T7, T3, and SP6 (21), which are all capable of recognizing promoters without the aid of additional proteins. The amount of transcription initiation observed in vitro with the $\Delta 115-119$ protein is similar to that observed for other mutations that have a wild-type phenotype in vivo (i.e., R178A-K179A and H187A-R189A [Fig. 7 and Table 1]). This suggests that the $\Delta 115-119$ mutation disrupts a critical function of sc-mtTFB, distinct from promoter recognition, resulting in a petite phenotype.

Two closely linked mutations in sc-mtTFB result in dependence on supercoiled DNA templates in vitro. The remaining mutations that merit discussion had discernible effects only in vitro. Two of the in vitro-defective mutations, R178A-K179A and H187A-R189A, partially overlap the basic region of the sc-mtTFB protein originally described by Lisowsky and Michaelis (18) (Fig. 2 and 8). Both of these mutations resulted in severely defective proteins in vitro. Interestingly, the activity of these two mutations was dramatically enhanced when a supercoiled DNA template was used (Fig. 7). Indeed, there was a virtual dependence on having a supercoiled template in vitro. Similar supercoiling-dependent mutations have been observed in bacterial sigma factors and have been interpreted as defects in the ability of these proteins to unwind DNA during transcription initiation (1, 15). These mutations are located in

region 2.3 of the bacterial sigma factors. It is interesting that mutations in the basic region of sc-mtTFB display this phenotype, rather than mutations in the 2.3-like region. These results raise the possibility that sc-mtTFB may play a role in unwinding DNA during transcription initiation and that the basic region mutations disrupt this capacity of the protein in vitro. The reason that these mutations have no effect in vivo could result from a significant population (or localized regions) of yeast mtDNA existing in negatively supercoiled form, effectively rendering these mutations silent.

Two in vitro-defective sc-mtTFB mutations (D248A-D251A and D260A-R264A) overlap with the C-terminal portion of the region of sc-mtTFB that exhibits some similarity to region 3 of the sigma factors (13). This region of sc-mtTFB (residues 248 to 264) is rich in aspartic acid and hydrophobic residues (6 of 17 residues are aspartic acid, and 6 of 17 are hydrophobic), and these two in vitro-defective mutations replace three of these aspartic acid residues with alanine. This suggests that the acidic nature of this region may be an important feature. The E222A-E223A-D225A mutation, which simultaneously changes two amino acids that would correspond to two adjacent highly conserved residues in region 3 of the sigma factors, has no effect on sc-mtTFB function. The results allow no strong conclusions about the homology of this region of sc-mtTFB to conserved region 3 of sigma factors to be drawn. Why these two mutations have no effect in vivo is unclear. An sc-mtTFB-independent transcription mechanism, able to replace the normal sc-mtTFB-dependent system when sc-mtTFB is defective, might explain this discrepancy. However, such a substitution would be predicted to compensate for the $\Delta 115-119$ mutation. For this reason, the existence of an additional sc-mtTFB-independent transcription mechanism seems an unlikely possibility. However, it is plausible that other protein factors or template conditions are able to compensate directly for the low activity of these mutated proteins in vivo.

The results of this mutagenesis study confirm that sc-mtTFB is an essential protein component of the sc-mtRNA polymerase holoenzyme and that it is required for specific initiation of transcription. Two regions of sc-mtTFB defined in this study overlap amino acids that show similarity to portions of region 2.0 of bacterial sigma factors, which has been proposed to be involved in essential steps of transcription initiation. The common portions of these proteins probably represent conserved functional domains involved in open complex formation. In contrast, the sigma 2.4-like region of sc-mtTFB can be deleted without eliminating the ability of the protein to promote specific transcription initiation (a loss of function that would be predicted for a similar deletion in sigma factors). This observation, coupled with the fact that regions outside of those that have similarity to sigma factors appear to affect sc-mtTFB function, suggests that sc-mtTFB has structural and functional characteristics that are distinct from sigma factors. Additional study of the simple two-component sc-mtRNA polymerase system should provide insight into the many ways that proteins can interact to promote transcription initiation.

ACKNOWLEDGMENTS

We thank K. Chao for the gift of purified yeast mtRNA polymerase, native recombinant sc-mtTFB, and the plasmid pE1-1. We also thank A. F. Davis and J. D. Garman for critical reading of the manuscript and many helpful discussions.

This work was supported by grant GM33088-24 from the National Institute of General Medical Sciences. G.S.S. is supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation, Postdoctoral Fellowship DRG-1208.

REFERENCES

1. Aiyer, S. E., Y.-L. Juang, J. D. Helmann, and P. L. deHaseth. 1994. Mutations in sigma factor that affect the temperature dependence of transcription from a promoter, but not from a mismatch bubble in double-stranded DNA. *Biochemistry* **33**:11501–11506.
2. Biswas, T. K. 1992. Transcriptional commitment of mitochondrial RNA polymerase from *Saccharomyces cerevisiae*. *J. Mol. Biol.* **226**:335–347.
- 2a. Chao, K., and D. A. Clayton. Unpublished observations.
3. Christianson, T., and M. Rabinowitz. 1983. Identification of multiple transcription initiation sites on the yeast mitochondrial genome by in vitro capping with guanylyltransferase. *J. Biol. Chem.* **258**:14025–14033.
4. Clayton, D. A. 1991. Replication and transcription of vertebrate mitochondrial DNA. *Annu. Rev. Cell Biol.* **7**:453–478.
5. Dairaghi, D. J., G. S. Shadel, and D. A. Clayton. *J. Mol. Biol.*, in press.
6. Daniels, D., P. Zuber, and R. Losick. 1990. Two amino acids in an RNA polymerase σ factor involved in the recognition of adjacent base pairs in the –10 region of a cognate promoter. *Proc. Natl. Acad. Sci. USA* **87**:8075–8079.
7. Diffley, J. F. X., and B. Stillman. 1991. A close relative of the nuclear, chromosomal high-mobility group protein HMG1 in yeast mitochondria. *Proc. Natl. Acad. Sci. USA* **88**:7864–7868.
8. Dombroski, A. J., W. A. Walter, T. M. Record, Jr., D. A. Siegle, and C. A. Gross. 1992. Polypeptides containing highly conserved regions of transcription initiation factor σ^{70} exhibit specificity of binding to promoter DNA. *Cell* **70**:501–512.
9. Fisher, R. P., T. Lisowsky, M. A. Parisi, and D. A. Clayton. 1992. DNA wrapping and bending by a high mobility group-like transcriptional activator protein. *J. Biol. Chem.* **267**:3358–3367.
10. Gardella, T., H. Moyle, and M. M. Susskind. 1989. A mutant *Escherichia coli* σ subunit of RNA polymerase with altered promoter specificity. *J. Mol. Biol.* **206**:579–590.
11. Guthrie, C., and G. R. Fink. 1991. A guide to yeast genetics and molecular biology. *Methods Enzymol.* **194**:3–37.
12. Hager, D. A., and R. R. Burgess. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results from the sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. *Anal. Biochem.* **109**:76–86.
13. Jang, S.-H., and J. A. Jaehning. 1991. The yeast mitochondrial RNA polymerase specificity factor, MTF1, is similar to bacterial σ factors. *J. Biol. Chem.* **266**:22671–22677.
14. Jones, C. H., M. Tatti, and C. P. Moran, Jr. 1992. Effects of amino acid substitutions in the –10 binding region of σ^E of *Bacillus subtilis*. *J. Bacteriol.* **174**:6815–6821.
15. Juang, Y.-L., and J. D. Helmann. 1994. A promoter melting region in the primary σ factor of *Bacillus subtilis*: identification of functionally important aromatic amino acids. *J. Mol. Biol.* **235**:1470–1488.
16. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-directed mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:1367–1382.
17. Lesley, S. A., and R. R. Burgess. 1989. Characterization of the *Escherichia coli* transcription factor σ^{70} : localization of a region involved in the interaction with core RNA polymerase. *Biochemistry* **28**:7728–7734.
18. Lisowsky, T., and G. Michaelis. 1988. A nuclear gene essential for mitochondrial replication suppresses a defect of mitochondrial transcription in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **214**:218–223.
19. Lonetto, M., M. Gribskov, and C. A. Gross. 1992. The σ^{70} family: sequence conservation and evolutionary relationships. *J. Bacteriol.* **174**:3843–3849.
20. Mangus, D. A., S.-H. Jang, and J. A. Jaehning. 1994. Release of the yeast mitochondrial RNA polymerase specificity factor from transcription complexes. *J. Biol. Chem.* **269**:26568–26574.
21. Masters, B. S., L. L. Stohl, and D. A. Clayton. 1987. Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. *Cell* **51**:89–99.
22. Osinga, K. A., E. De Vries, G. T. V. Van der Horst, and H. F. Tabak. 1984. Initiation of transcription in yeast mitochondria: analysis of origins of replication and of genes coding for a messenger RNA and a transfer RNA. *Nucleic Acids Res.* **12**:1889–1900.
23. Parisi, M. A., and D. A. Clayton. 1991. Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. *Science* **252**:965–969.
24. Parisi, M. A., B. Xu, and D. A. Clayton. 1993. A human mitochondrial transcriptional activator can functionally replace a yeast mitochondrial HMG-box protein both in vivo and in vitro. *Mol. Cell. Biol.* **13**:1951–1961.
25. Rieman, G., and G. Michaelis. 1993. A point mutation in the core subunit gene of yeast mitochondrial RNA polymerase is suppressed by a high level of specificity factor MTF1. *Mol. Gen. Genet.* **237**:49–57.
26. Rong, J. C., and J. D. Helmann. 1994. Genetic and physiologic studies of *Bacillus subtilis* σ^A mutants defective in promoter melting. *J. Bacteriol.* **176**:5218–5224.
27. Schinkel, A. H., M. J. A. Groot Koerkamp, and H. F. Tabak. 1988. Mitochondrial RNA polymerase of *Saccharomyces cerevisiae*: composition and mechanism of promoter recognition. *EMBO J.* **7**:3255–3262.
28. Schinkel, A. H., M. J. A. Groot Koerkamp, E. P. W. Touw, and H. F. Tabak. 1987. Specificity factor of yeast mitochondrial RNA polymerase: purification and interaction with core RNA polymerase. *J. Biol. Chem.* **262**:12785–12791.
29. Shadel, G. S., and D. A. Clayton. 1993. Mitochondrial transcription initiation: variation and conservation. *J. Biol. Chem.* **268**:16083–16086.
30. Siegle, D. A., J. C. Hu, W. A. Walter, and C. A. Gross. 1989. Altered promoter recognition by mutant forms of the σ^{70} subunit of *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **206**:591–603.
31. Sikorski, R. S., and J. D. Boeke. 1991. In vitro mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. *Methods Enzymol.* **194**:302–318.
32. Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**:19–27.
33. Tabor, S. 1990. Expression using the T7 RNA polymerase/promoter system, p. 16.2.1–16.2.11. *In* F. A. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Seidman, and K. Struhl (ed.), *Current protocols in molecular biology*. Greene Publishing and Wiley-Interscience, New York.
34. Tatti, K. M., C. H. Jones, and C. P. Moran, Jr. 1991. Genetic evidence for the interaction of σ^E with the *spoIIID* promoter in *Bacillus subtilis*. *J. Bacteriol.* **173**:7828–7833.
35. Waldburger, C., T. Gardella, R. Wong, and M. M. Susskind. 1990. Changes in conserved region 2 of *Escherichia coli* σ^{70} affecting promoter recognition. *J. Mol. Biol.* **215**:267–276.
36. Waldburger, C., and M. M. Susskind. 1994. Probing the information content of *Escherichia coli* σ^{70} region 2.3 by combinatorial cassette mutagenesis. *J. Mol. Biol.* **235**:1489–1500.
37. Xu, B., and D. A. Clayton. 1992. Assignment of a yeast protein necessary for mitochondrial transcription initiation. *Nucleic Acids Res.* **20**:1053–1059.
38. Zhou, Y. N., W. A. Walter, and C. A. Gross. 1992. A mutant σ^{70} with a small deletion in conserved region 3 of σ has reduced affinity for core RNA polymerase. *J. Bacteriol.* **174**:5005–5012.
39. Zuber, P., J. Healy, H. L. Carter III, S. Cutting, C. P. Moran, Jr., and R. Losick. 1989. Mutation changing the specificity of an RNA polymerase sigma factor. *J. Mol. Biol.* **206**:605–614.