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Functional Conservation of Yeast mtTFB Despite Extensive Sequence Divergence

JOSÉ A. CARRODEGUAS,* SUYONG YUN,* GERALD S. SHADEL,† DAVID A. CLAYTON,†[1] AND DANIEL F. BOGENHAGEN*[2]

*Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, NY 11794-8651
†Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305

Transcription of mtDNA in the yeast *S. cerevisiae* depends on recognition of a consensus nonanucleotide promoter sequence by mtRNA polymerase acting with a 40-kDa dissociable factor known as mtTFB or Mtf1p. mtTFB has been cloned and characterized in *S. cerevisiae*, but has not been studied in similar detail in any other organism. Although it is known that mitochondrial transcription in the dairy yeast, *Kluyveromyces lactis*, initiates within the same consensus promoter sequence used in *S. cerevisiae*, no previous studies have focused on the proteins involved in transcription initiation in *K. lactis*. In this article, we report the cloning of mtTFB from *K. lactis* and from a yeast more closely related to *S. cerevisiae*, *S. kluyveri*. Both novel mtTFB genes were able to substitute for the *MTF1* gene in *S. cerevisiae*. Both proteins purified following expression in *E. coli* were able to support specific transcription initiation in vitro with the *S. cerevisiae* mtRNA polymerase. The *S. kluyveri* and *K. lactis* mtTFB proteins share only 56% and 40% identity with *S. cerevisiae* mtTFB, respectively. Alignments of the three mtTFB sequences did not reveal any regions larger than 30 amino acids with greater than 60% amino acid identity. In particular, regions proposed to show sequence similarity to bacterial σ factors were not more highly conserved than other regions of the mtTFB proteins. All three yeast mtTFB genes lack conventional amino-terminal mitochondrial targeting sequences, suggesting that all three proteins may be imported into mitochondria by the same unusual mechanism reported for *S. cerevisiae* mtTFB.

Mitochondria mtTFB Sigma factors Yeast transcription

MITOCHONDRIA play a central role in energy metabolism and in several other biosynthetic pathways in eukaryotic organisms. Mitochondrial DNA (mtDNA) codes for a limited number of enzyme subunits implicated in mitochondrial energy metabolism, as well as for the ribosomal RNAs and tRNAs sufficient for mitochondrial gene expression. The remaining protein subunits necessary for assembly of the oxidative phosphorylation and electron transport enzyme complexes and all other proteins required for mitochondrial gene expression are imported as nuclear gene products (2). Therefore, control of mitochondrial activity is highly dependent on accurate coordination between nuclear and mitochondrial gene expression.

Mitochondrial transcription initiation has been studied most thoroughly in the yeast, *S. cerevisiae*. In this organism, only two proteins are required for specific transcription initiation in vitro: a core RNA polymerase encoded by the *RPO41* gene (18,19,23) and a transcription specificity factor referred to as sc-mtTFB or Mtf1p, the product of the *MTF1* gene (17,20). Both of these genes are required for expression and maintenance of *S. cerevisiae* mtDNA. Recombinant mtRNA polymerase and mtTFB are sufficient to promote transcription initiation within core promoters consisting of matches to the sequence ATA TAAGTA (3,32). sc-mtTFB is a 40-kDa protein that binds the core mtRNA polymerase (22) and

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[1]Present address: Howard Hughes Medical Institute, Chevy Chase, MD.
[2]Address correspondence to Daniel F. Bogenhagen. Tel: (516) 444-3068; Fax: (516) 444-3218; E-mail: dan@pharm.sunysb.edu

shows potential sequence relationship with prokaryotic σ factors (17). Although mtTFB has been referred to as a specificity factor (28), the relative contributions of domains in mtRNA polymerase and mtTFB in promoter binding have not been determined and the importance of σ factor homology has been questioned (29,30). An additional mtDNA binding protein, the HMG-box protein known as sc-mtTFA or abf2 (10,11), does not appear to play a major role in transcription initiation in *S. cerevisiae* (32) and is dispensable under certain conditions (24,34).

Transcription of mtDNA has also been studied extensively in human and *Xenopus* systems. A human HMG-box factor similar to the *ABF2* gene product, h-mtTFA, plays a dominant role in transcription initiation by binding to promoter elements upstream of transcription start sites (14,26,29). Addition of the C-terminal tail of human mtTFA to yeast mtTFA produces a chimeric protein that is able to stimulate specific transcription by human mtRNA polymerase (9). Although human mtRNA polymerase has not been extensively purified, it appears to be encoded by a cDNA with extensive primary sequence homology to sc-mtRNA polymerase (D. Garman, personal communication). It has not yet been determined whether human mitochondria contain a factor analogous to sc-mtTFB. However, a protein that appears to be at least functionally similar to sc-mtTFB has been purified recently from *Xenopus* oocyte mitochondria. As in the case of sc-mtTFB, the *Xenopus* factor is a 40-kDa protein that is required along with a 140-kDa mtRNA polymerase for transcription initiation at a core consensus sequence (1,4–6).

Because mtTFB has been cloned only from *S. cerevisiae*, no information is available on the sequence or structure of this protein in other eukaryotes. It has been known for some time that transcription of mtDNA in the dairy yeast, *Kluyveromyces lactis*, requires the same core nonanucleotide promoter sequence as *S. cerevisiae* (25), although no characterization of the transcriptional machinery in *K. lactis* mitochondria has been reported. We considered it likely that *K. lactis* should contain a homologue of *S. cerevisiae* mtTFB. Initial efforts to clone *K. lactis* mtTFB using cross-hybridization with a probe derived from sc-mtTFB were unsuccessful, indicating a low degree of primary sequence conservation between these factors. Therefore, we used another yeast more closely related to *S. cerevisiae, S. kluyveri*, to clone an mtTFB homologue by cross-hybridization with sc-mtTFB. We then used conserved sequence blocks between both factors to design degenerate primers that enabled us to clone *K. lactis* mtTFB using a PCR-based approach. mtTFB genes from *S. kluyveri* and *K. lactis* can functionally replace sc-mtTFB in vivo and in vitro. The sequences of these three mtTFB proteins reveal extensive evolutionary divergence. However, these proteins have retained the ability to function with sc-mtRNA polymerase to initiate transcription from the same core consensus sequence as well as the ability to enter mitochondria without the use of a conventional cleaved mitochondrial targeting signal.

MATERIALS AND METHODS

Enzymes and Reagents

Restriction enzymes were purchased from Boehringer Mannheim and New England Biolabs. Taq DNA Polymerase was from Perkin Elmer, Sequenase from USB, T4 DNA ligase, shrimp alkaline phosphatase, and DNA polymerase I (Klenow fragment) were from New England Biolabs.

Nonradioactive nucleotides were ultrapure reagents from Pharmacia. Radioactive nucleotides were obtained from ICN except for [α-^{35}S]dATP, which was obtained from New England Nuclear.

PCR

PCR with degenerate primers was carried out in a Perkin Elmer Cetus thermal cycler 480 using touchdown PCR (12). Following a 2-min incubation at 94°C, cycles were carried out at 94°C, 0.5 min; 54°C, 0.5 min; 72°C, 1 min, decreasing the annealing temperature 2°C each cycle until reaching 40°C, at which 23 cycles were carried out, ending with a 10-min incubation at 72°C. PCR reactions were carried out in 50-μl volumes containing 300 pmol of each primer, 0.25 mM dNTPs, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, and 2.5 units of Taq DNA polymerase. Genomic DNA (100 ng) was used for the first PCR. Products from a first round of PCR were diluted 1/100 with water and amplified in a second round of PCR with internal primers using the same conditions.

Degenerate primers were synthesized by Operon Technologies, comprising the following sequences: FBF1, YGFKYL (TAYGGITTYAAR TAYYT); FBF2, CIGNKNW (TGYATIGG IAAYAARAAYTGG); FBR1, WPFKPDI (antisense strand ATRTCIGGYTTRAAIGGCCA); and FBR2, DNWDYVT (antisense strand GTI

ACRTARTCCCARTTRTC). Inosine was used in positions with three- or fourfold degeneracy. PCR products were cloned by TA cloning in the pCRII vector from Invitrogen.

Genomic DNA and DNA Blot Hybridization

Yeast genomic DNA was prepared by SDS treatment and organic extraction from *S. kluyveri* (ATCC strain 22512) or from *K. lactis* (obtained from N. Dean, SUNY Stony Brook). Samples (10 μg) of genomic DNA were digested with the indicated restriction enzymes, run in a 0.7% agarose gel and blotted onto Nytran membranes (Schleicher and Schuell). Probes were synthesized by random priming with [α-^{32}P]dATP (13). Prehybridization and hybridization were carried out at 45°C for cross-hybridization and at 65°C when homologous probes were used in a solution containing 6 × SSC, 5 × Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured calf thymus DNA. Membranes were washed with 2 × SSC, 0.5% SDS followed by 0.2 × SSC, 0.1% SDS at the temperature used for hybridization, and exposed to Kodak XAR film with intensifying screens at −80°C.

DNA Sequencing

Chain terminator sequencing was performed using the Sequenase 2.0 kit from USB and [α-^{35}S]dATP. Products were resolved in 8% polyacrylamide/8 M urea sequencing gels. Dried gels were exposed to Kodak XAR film for autoradiography.

Construction of Expression Vectors for sk- and kl-mtTFBs

Both sk-mtTFB and kl-mtTFB cloned genes were engineered by PCR to introduce an *Nde*I site with the ATG matching the translation start codon. An *Afl*III site located 25 bp downstream of the sk-mtTFB stop codon was made blunt using the Klenow fragment of DNA polymerase I and ligated to a *Hinc*II-*Hin*dIII adaptor by cloning into pUC 19. The whole gene was transferred into pET22b(+) (Novagen) using the *Nde*I and *Hin*dIII sites of the vector. A similar approach was used with kl-mtTFB, using an *Rsa*I site located 11 bp beyond the stop codon. *S. cerevisiae* mtRNA polymerase and sc-mtTFB were prepared from *E. coli* BL21(DE3) cells containing clone pGS317 and pGS344, respectively (30).

Expression and Purification of Recombinant Proteins

E. coli BL21(DE3) was transformed with the expression constructs and 100-ml cultures (2X YT with 50 μg/ml ampicillin) were grown to an OD_{600} of 0.5 before expression was induced by addition of IPTG to a final concentration of 1 mM. The culture was then incubated for 3 h. Cells were concentrated by centrifugation and resuspended in buffer containing 0.2 M sucrose, 50 mM HEPES, pH 7.5, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1 μM pepstatin, treated for 5 min at 37°C with 1 mg/ml lysozyme and centrifuged. Cells were resuspended in 10 ml of H buffer (50 mM HEPES, pH 7.5, 0.1 mM EDTA, 2 mM DTT, 0.5 mM benzamidine-HCl, 1 μM pepstatin, 5 μg/ml leupeptin, and 0.2 mM PMSF) containing 200 mM KCl and 20% glycerol and incubated on ice during treatment with two pulses of 15 s separated by 20 s with the micro-tip of a Fisher Scientific model 550 sonicator. Debris and inclusion bodies were sedimented by centrifugation for 10 min at 14 krpm in a Sorvall SS34 rotor. When mtRNA polymerase was expressed, protein was prepared from the supernatant following this step. When mtTFB was expressed, the pellet was resuspended in 6 ml of H buffer containing 200 mM KCl and 8 M urea. This suspension was sonicated and centrifuged as described above to provide a supernatant containing solubilized inclusion body proteins.

For mtTFB purification, proteins solubilized in urea were subjected to DEAE Sephacel chromatography at 200 mM KCl in a buffer containing 5% glycerol, 20 mM Tris, pH 8.0, 1 mM EDTA, 2 mM DTT, 2 mM benzamidine-HCl, 1.25 μg/ml leupeptin, and 1.25 μg/ml aprotinin. The DEAE flow-through fraction was diluted to 50 mM KCl using the same buffer and applied to a 1-ml Poros HS column. Proteins were eluted with a linear KCl gradient from 50 mM to 1 M. Elution of the proteins was monitored by UV absorbance and the peak fractions were analyzed by SDS-PAGE. The same chromatography steps were used for purification of sc-mtRNA polymerase, using a nonspecific RNA polymerase assay to monitor column fractions (5). Proteins were stored at −80°C after addition of one-half volume of storage buffer (20 mM Tris, pH 8.0, 5 mM DTT, 75% glycerol, 5 μg/ml leupeptin, 5 μg/ml aprotinin).

In vitro Transcription Reactions

In vitro transcription reactions used a DNA template prepared as an *Afl*III/*Nde*I fragment of

DNA plasmid pSCP56 containing the oligonucleotide 5′-GATCCGTATATAAGTAATAG-3′ cloned between the *Bam*HI and *Eco*RI sites of plasmid pUC9. Transcription initiated at the promoter consensus sequence (underlined) in this fragment provides a 222-nucleotide run-off transcript.

Transcriptions were carried out in 40-μl reactions containing 8 μl of 5× transcription buffer (50 mM Tris, pH 8.0, 50 mM $MgCl_2$, 25 mM DTT, 0.4 mM PMSF, 25% glycerol), 2 μl 20× nucleoside triphosphate mix (20 mM ATP, 2 mM UTP, 2 mM CTP, 0.2 mM GTP), 1 μl [α-^{32}P]GTP (10 μCi), 25 μg/ml BSA, 0.5 U/μl RNAsin (Promega), 30 ng template DNA, 2 μl sc-mtRNA polymerase, 1–4 μl mtTFB. After incubation at 30°C for 20 min, 170 μl of stop mix (10 mM Tris, pH 8.0, 1 mM EDTA, 0.3 M sodium acetate, 0.5% SDS, 10 μg/ml tRNA, 10 μg/ml glycogen) was added, followed by extraction with 200 μl of phenol:chloroform (1:1) and precipitation with 500 μl of ethanol at −80°C. Samples were centrifuged for 15 min in an Eppendorf microcentrifuge and pellets were washed with 200 μl of 70% ethanol, dried under vacuum, and resuspended in 10 μl of formamide loading buffer. After heating at 65°C for 5 min, samples were run in a 6% sequencing gel. The dried gel was exposed to Kodak XAR film for autoradiography.

Functional Analysis of Putative sc-mtTFB Homologues by In Vivo Plasmid Shuffle

The *S. cerevisiae* strain GS113 (*a,his3-D200 leu2-3,-112 ura3-52 trp1-D1 ade2 Dmtf1::HIS3* pE1-1[l-YESR::*MTF1 URA3 CEN4/ARS1*]) used in the plasmid shuffle experiments was created as follows. The strain GS100 (30), which contains the *S. cerevisiae MTF1* gene on the plasmid pE1-1, was transformed with a linear restriction fragment containing a disrupted version of the *MTF1* gene into which the *HIS3* gene was inserted (Fig. 4). To select for a chromosomal disruption of the *MTF1* gene, His$^+$ transformants were screened for dependence on the plasmid-borne copy of the *MTF1* gene (pE1-1) for mitochondrial function. First, the transformants were grown on FOA-containing medium to force the loss of the pE1-1 plasmid and then checked for the inability to grow on medium containing glycerol as the sole carbon source (YPG), indicating the loss of mitochondrial function (the expected phenotype for a *MTF1* disruption). One transformant met these criteria and was designated GS113. Disruption of the chromosomal copy of the *MTF1* gene in GS113 was confirmed by Southern analysis of its genomic DNA (data not shown).

All of the plasmids used to complement *MTF1* in the plasmid shuffle assay were derivatives of pRS314 (31). The plasmid pGS345 contains the *S. cerevisiae MTF1* gene (encoding sc-mtTFB) and has been described previously (30). The plasmid pRS314-SK consists of a 5.0-kb *Hin*dIII fragment, harboring the gene for the putative *S. kluyveri* mtTFB protein (sk-mtTFB), inserted into pRS314. The plasmid pRS314-SK was digested with *Pst*I and ligated to create a ~500-bp deletion of the *S. kluyveri* DNA insert. This deletion removed ~110 bp of the coding region of interest and ~400 bp of upstream *S. kluyveri* DNA, which would presumably contain the requisite promoter for the gene. The putative *K. lactis* mtTFB (kl-mtTFB) gene, contained on a ~2.5-kb *Xba*I fragment, was isolated on a ~2.5-kb *Kpn*I-*Sac*I restriction fragment (these restriction sites flank *Xba*I in the polylinker) and inserted into pRS314.

The plasmid shuffle assay for *MTF1* complementation was performed as described previously (30), except GS113 was used as the parent strain. The plasmids pRS314 (no *MTF1* insert), pGS345 (*S. cerevisiae MTF1* insert), pRS314-SK (putative sk-mtTFB gene insert), pRS314-SKΔP (deletion of promoter and 5′ end of predicted sk-mtTFB gene), and pRS314-KL (putative kl-mtTFB gene insert) were used to transform GS113. Trp$^+$ transformants were then grown on FOA-containing medium to force the loss of pE1-1 (wild-type *MTF1* gene). Complementation of *MTF1* in these strains by the *S. kluyveri* and *K. lactis* genes was scored as the ability to grow on medium containing glycerol as the sole carbon source (YPG), indicating maintenance of mitochondrial function.

RESULTS

Cloning of mtTFB From S. kluyveri *and* K. lactis

We attempted to use cross-hybridization with a probe derived from sc-mtTFB to clone candidate mtTFB genes from *S. kluyveri* and *K. lactis*. This approach identified a candidate fragment in digests of *S. kluyveri* DNA, but not of *K. lactis* DNA, even when hybridization was performed at very low stringency. A 2.6-kb *Eco*RI fragment of *S. kluyveri* genomic DNA was ligated into a pBluescript KS vector. Partial sequencing of this clone identified an open reading frame with homology to sc-mtTFB. However, the open reading

frame began at one end of the insert and the putative sc-mtTFB homologue appeared to lack residues that would correspond to the first 24 amino acids of sc-mtTFB. Using a fragment of this clone as probe, we cloned a 5-kb *Hin*dIII fragment from *S. kluyveri* genomic DNA that contains the *Eco*RI clone and extends farther upstream, including the missing amino-terminal sequence. The complete ORF codes for a protein of 338 amino acids with 56% identity and 15% similarity to sc-mtTFB. This protein was tentatively designated as sk-mtTFB based on this sequence homology.

Regions conserved between sc-mtTFB and sk-mtTFB were used to design degenerate primers to clone a related gene from *K. lactis*. Products obtained by touchdown PCR with different combinations of primers were separated on a 1% agarose gel, blotted, and hybridized with a probe derived from sk-mtTFB at low stringency. A 0.5-kb product obtained with primers FBF2/FBR1 reacted weakly with this probe. Cloning and sequencing of this product revealed an open reading frame with homology to sc-mtTFB. The 0.5-kb PCR product hybridized to unique *Xba*I, *Eco*RI, and *Hin*dIII fragments in *K. lactis* DNA. The 2.5-kb *Xba*I fragment was cloned and found to contain a reading frame for a 335-aa protein with 40% identity and 13.4% similarity to sc-mtTFB and with 41.8% identity and 14.6% similarity to the putative sk-mtTFB. As noted for sk-mtTFB, this gene was tentatively designated as kl-mtTFB based on sequence homology. DNA blot hybridization confirmed that the putative sk-mtTFB and kl-mtTFB genes are single-copy genes (Fig. 1).

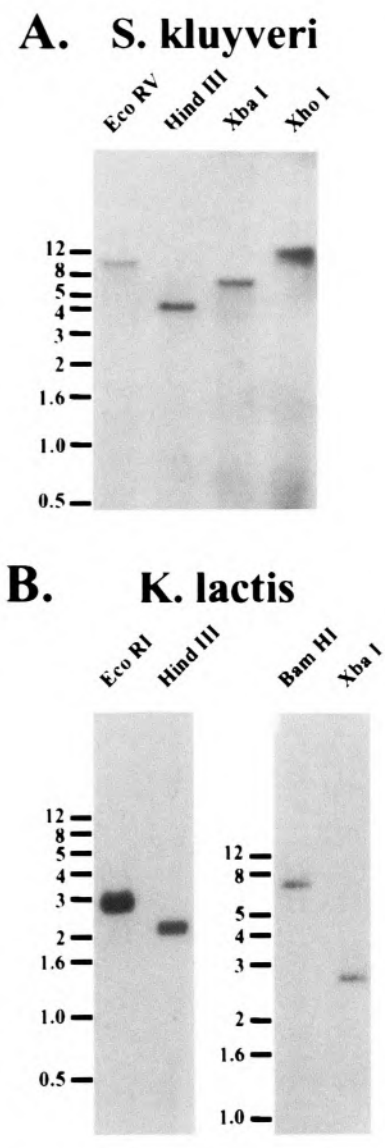

FIG. 1. Putative sk-mtTFB and kl-mtTFB genes occur as single-copy genes. Autoradiograms are shown of DNA blots in which 10 μg of genomic yeast DNA was hybridized with homologous labeled probes from putative mtTFB genes as described in Materials and Methods. The positions of mobility markers in a commercial 1-kb DNA ladder (Life Technologies) are noted on each gel.

An alignment of the two putative novel mtTFB protein sequences with that of sc-mtTFB is shown in Fig. 2. Thirty percent of the amino acid residues are identical in these three sequences. It is apparent that there is no single extensive domain that is highly conserved among all three proteins. Jang and Jaehning (17) presented an alignment of sc-mtTFB sequences with blocks 2.1/2.2, 2.3/2.4, and 3.0 of bacterial σ factors. Together these motifs comprise 125 residues of sc-mtTFB, more than one-third of the protein. Only 24% of these residues are conserved among all three mtTFB proteins. Thus, the putative σ factor homology motifs are not particularly highly conserved in yeast mtTFB sequences. A summary of results obtained in site-specific mutagenesis of sc-mtTFB in a previous study by Shadel and Clayton (30) is presented in Fig. 2. Only a small fraction of the amino acid changes introduced by Shadel and Clayton (30) caused either a defective in vivo phenotype or a significant loss of in vitro transcription activity. It is interesting to note that the critical residues Y42, D52, and Y108 are conserved in all three sequences, with an aromatic amino acid (Y $\rightarrow$ F) substitution at position 108 of kl-mtTFB. Many of the other mutations generated by Shadel and Clayton (30) that failed to show a significant phenotype affect residues that are also poorly conserved.

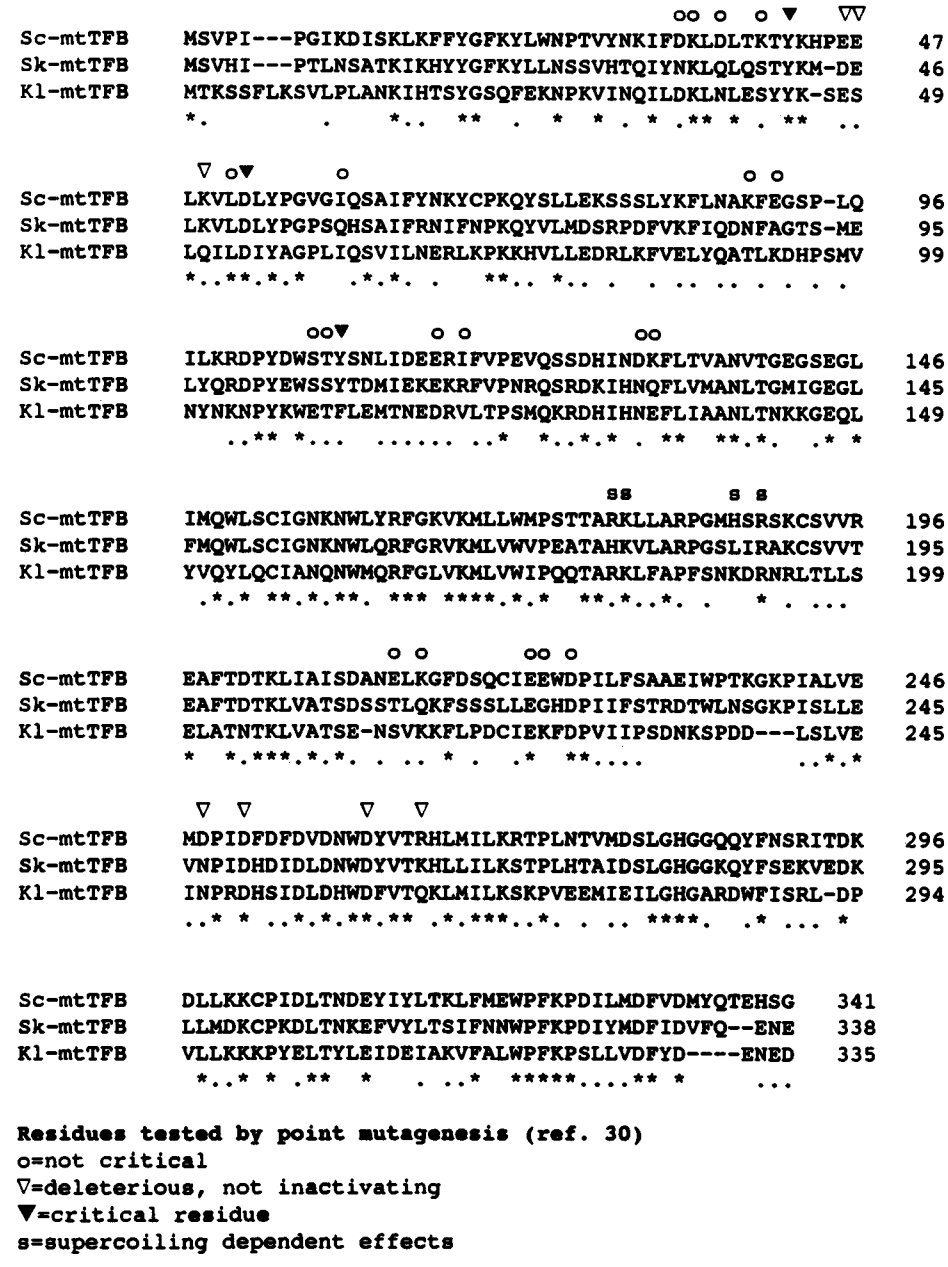

FIG. 2. Alignment of three yeast mtTFB protein sequences. Sequences were aligned using the program Clustal (PCGene). Residues marked by asterisks (*) are identical in all three sequences. Residues marked by periods (.) either match at two of three positions or have chemically similar residues at the indicated position in all three proteins, as defined in Fig. 5. The symbols above the sequence for sc-mtTFB show the effects of altering the indicated amino acids in the sc-mtTFB sequence (30), as noted in the figure and discussed in the text. Genbank accession numbers for the sequences are "U81619" and "U81620" for *S. kluyveri* and *K. lactis* mtTFB, respectively.

sk-mtTFB and kl-mtTFB Can Substitute for sc-mtTFB in Transcription In Vitro and for Mitochondrial Function In Vivo

Recombinant sk-mtTFB and kl-mtTFB were tested for functional replacement of sc-mtTFB in in vitro transcription using recombinant sc-mtRNA polymerase and a DNA template containing an *S. cerevisiae* mitochondrial promoter. The relative amount of all three recombinant mtTFBs was estimated by SDS-PAGE and Coomassie blue staining, followed by densitometry of the dried

gel. Samples of the three mtTFB proteins were adjusted to contain the same concentrations of the 40-kDa polypeptides and used in parallel run-off transcription assays in reactions as described in Materials and Methods. All three proteins were able to direct specific transcription initiation by sc-mtRNA polymerase to produce a 222-nucleotide RNA (band a, Fig. 3). A minor RNA species with lower gel mobility (band b) was produced in the presence of sk-mtTFB or kl-mtTFB. sc-mtRNA polymerase alone produced nonspecific products labeled c and d in Fig. 3. The results shown in Fig. 3 are representative of several sets of transcription reactions performed with different preparations of sk- and kl-mtTFB. In all cases, kl-mtTFB produced a lower yield of specific transcripts than sk-mtTFB, and both heterologous factors were reproducibly weaker than sc-mtTFB.

To test if sk-mtTFB and kl-mtTFB can substitute for sc-mtTFB in vivo, we carried out plasmid shuffle experiments as described in Materials and Methods. An *S. cerevisiae* strain carrying the wild-type *MTF1* gene on the single-copy plasmid pE1-1 was transformed with a single-copy plasmid containing either the sk-mtTFB or the kl-mtTFB gene. Cells were then grown on FOA-containing media to force the loss of the wild-type *MTF1* gene and to test whether the heterologous gene product would be sufficient for maintenance of mitochondrial function as assessed by survival on a nonfermentable carbon source (YPG). To test if complementation was due to the mtTFB gene present in the cloned genomic DNA fragment, a deletion of the sk-mtTFB gene (ΔP) was used in the same kind of experiment, indicating that complementation was dependent on an intact mtTFB gene. Both heterologous mtTFB genes permitted the recipient yeast to survive on glycerol (Fig. 4), although the growth rate was reduced for both strains under these conditions. This is consistent with the reduced activity of kl-mtTFB in in vitro transcription (Fig. 3).

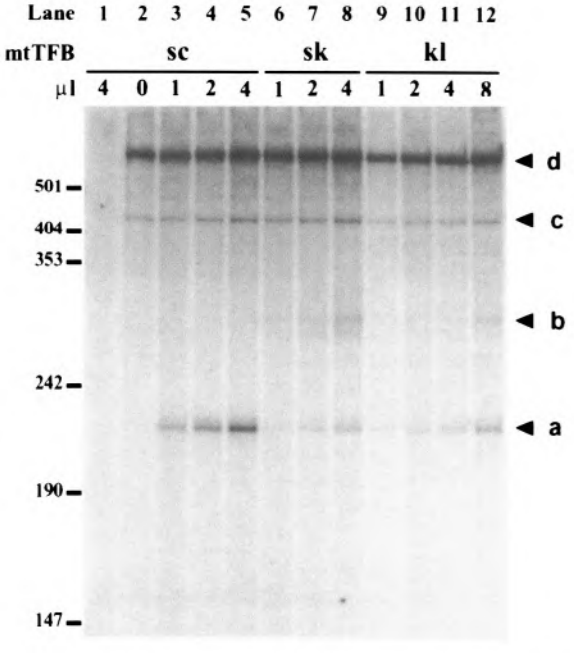

FIG. 3. Recombinant mtTFB proteins from heterologous yeasts can replace sc-mtTFB in in vitro transcription by sc-mtRNA polymerase from a canonical nonanucleotide promoter. In vitro transcription experiments were performed as described in Materials and Methods using sc-mtRNA polymerase alone (lane 2) or mtRNA polymerase plus the indicated amount of recombinant mtTFB proteins expressed from genes obtained from *S. cerevisiae* (sc), *S. kluyveri* (sk), or *K. lactis* (kl). Lane 1 shows a control reaction from which mtRNA polymerase was omitted. In vitro transcripts were fractionated by electrophoresis on a polyacrylamide-urea gel and detected by Phosphorimager analysis. The positions of labeled *Msp*I fragments of pUC18 DNA as gel mobility markers are indicated at the left. The labels on the right are discussed in the text.

DISCUSSION

We used the sequence of *S. cerevisiae* mtTFB to clone homologous genes from two other yeasts, *S. kluyveri* and *K. lactis*. We have referred to these novel gene products as sk-mtTFB and kl-mtTFB, respectively, following the proposed nomenclature of Xu and Clayton (32). Although the three proteins are very similar in size, ranging from 335 to 341 amino acids, alignment of the three protein sequences shows that they have diverged considerably. The sk-mtTFB and kl-mtTFB proteins are 56% and 40% identical to sc-mtTFB, respectively (Fig. 2). Nevertheless, both novel mtTFB genes can substitute for sc-mtTFB in vivo (Fig. 4) and can function with sc-mtRNA polymerase in run-off transcription in vitro (Fig. 3).

The divergence in sequence between kl-mtTFB and sc-mtTFB is surprising considering the fact that these yeasts are generally considered to be closely related. For comparison, we note that the homologous TFIIIB genes from these two organisms are 68% identical. It may be argued that nuclear gene products involved in mitochondrial replication or transcription may not be similarly constrained. However, in a recent project to clone mtDNA polymerase γ genes, Ye et al. (33) found that a fragment of the *K. lactis* DNA pol γ was so closely related to that of *S. cerevisiae* as to provide little genetic variability. Ye et al. (33) found that the DNA pol γ genes of *S. cerevisiae, P. pastoris,* and the distantly related fission yeast, *S. pombe*,

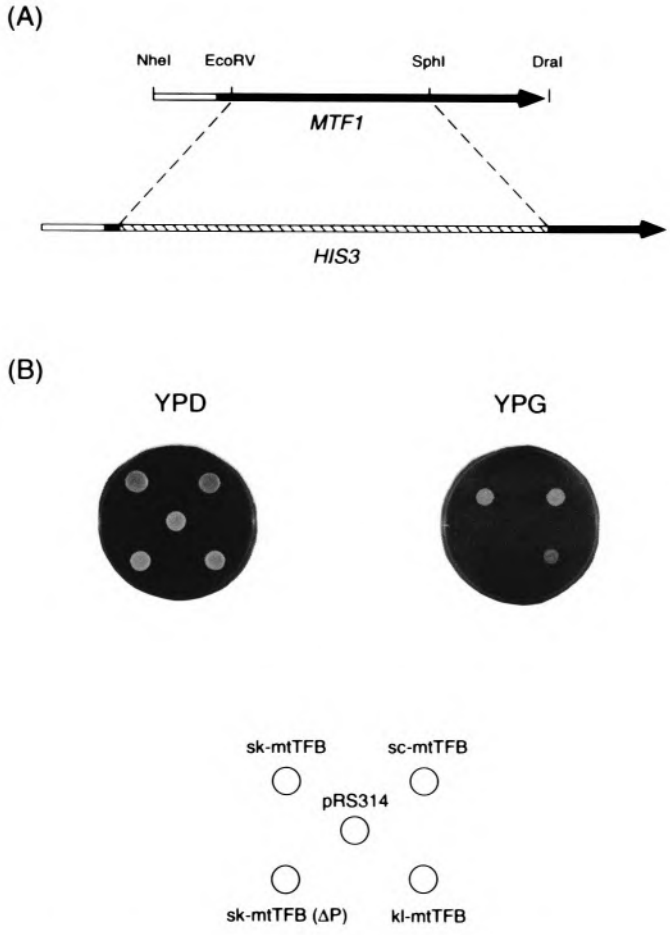

FIG. 4. Disruption of the *S. cerevisiae MTF1* gene and complementation of sc-mtTFB function by *S. kluyveri* and *K. lactis* sc-mtTFB homologues in vivo. (A) The *S. cerevisiae MTF1* gene (black arrow) was disrupted by insertion of the *HIS3* gene (dashed rectangle) into the coding region (between the existing *Eco*RV and *Sph*I sites in the *MTF1* gene). A His$^+$ transformant that contained the chromosomal *HIS3* disruption and a copy of the *MTF1* gene on a plasmid was designated GS113 and used in the plasmid shuffle experiments (see Materials and Methods for details). (B) Rescue of the *MTF1* deletion by mtTFB homologues from *S. kluveri* and *K. lactis* sc-mtTFB. GS113 was transformed with plasmids containing the *TRP1* gene as a marker and either the *S. cerevisiae MTF1* gene (sc-mtTFB), the *S. kluveri* mtTFB gene (sk-mtTFB), the *S. kluveri* mtTFB gene with a deletion of the promoter and the beginning of the coding region (sk-mtTFBΔP), the *K. lactis* mtTFB gene (kl-mtTFB), or no mtTFB insert (pRS314). These strains were replica plated onto YPD (dextrose) and YPG (glycerol) medium after growth on 5-FOA-containing medium to force the loss of the *S. cerevisiae MTF1* gene. Growth on YPG indicated rescue of wild-type sc-mtTFB function by the introduced mtTFB homologues (sk-mtTFB and kl-mtTFB) as manifested by maintenance of mitochondrial function. The order in which the strains appear on the plates is given in the key at the bottom of the figure.

contain domains as large as 113 amino acids with 73% identity. Similarly, the mtRNA polymerase genes of *S. cerevisiae* and *N. crassa*, a distantly related filamentous fungus, contain a central domain of 222 amino acids with 60% identity (8). In contrast, the alignment of mtTFB sequences in Fig. 2 shows no region as large as 30 amino acids with more than 60% sequence identity.

The comparison between sc-mtTFB and kl-mtTFB is of particular interest because Osinga et al. (25) reported that both organisms employ a common consensus sequence as a mitochondrial

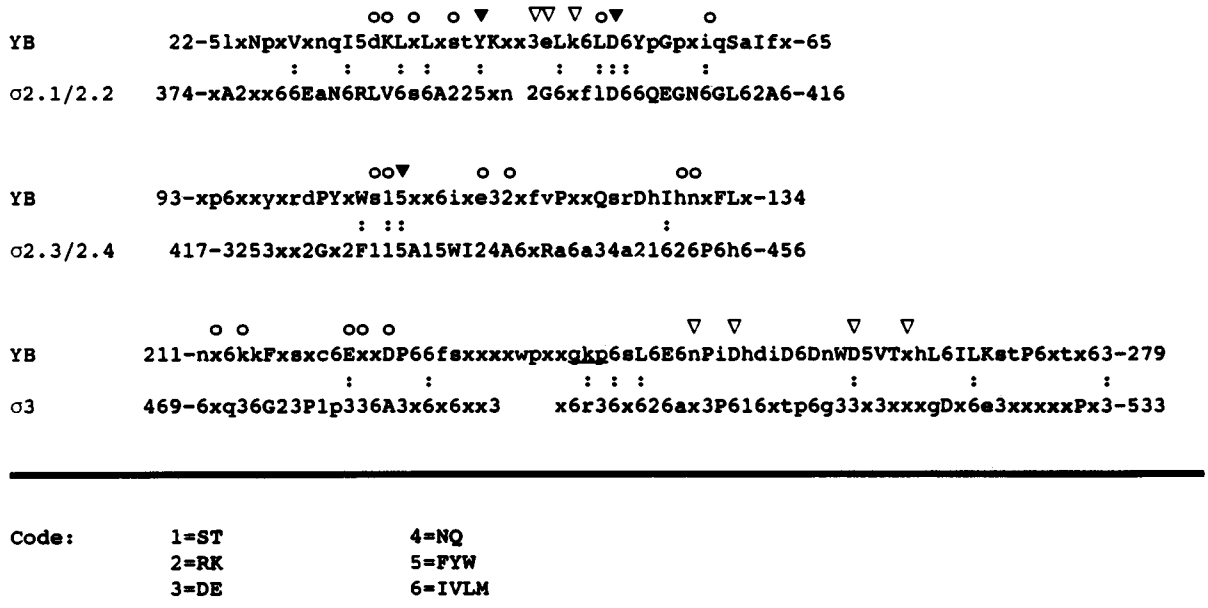

FIG. 5. The σ factor homologies in mtTFB sequences. A consensus sequence was derived for the three yeast mtTFB sequences (YB) and domains were aligned to σ2.1/2.2, σ2.3/2.4, and σ3 sequence motifs as identified by Lonetto et al. (21). The alignments are positioned to reflect the sequence alignments reported by Jang and Jaehning (17). Amino acids shown in upper case are highly conserved, whereas those shown in lower case are well conserved (two of three for the yeast mtTFB sequences). Note that the underlined gkp in the sequence aligned with σ3 represents an insertion in the sc-mtTFB and sk-mtTFB sequences with respect to the kl-mtTFB sequence. The numerical code used to denote similar amino acids follows that used by Lonetto et al. (21); x denotes any amino acid. The residues are numbered as in the sc-mtTFB and *E. coli* σ70 sequences. The symbols above the YB sequence refer to the importance of individual residues as determined by point mutagenesis (30), as defined in Fig. 2.

promoter. The observation that kl-mtTFB provided on a single-copy plasmid can substitute for sc-mtTFB is remarkable given the extensive divergence of primary sequence. We are not aware of any studies of mtRNA polymerase in *K. lactis*, although efforts to characterize mtRNA polymerase sequences are progressing for several other organisms. Cermakian et al. (7) identified potential mtRNA polymerase sequences related to T7 RNA polymerase in a number of organisms, but did not examine *K. lactis*. Chen et al. (8) recently reported a complete sequence of a *Neurospora crassa* mtRNA polymerase, which is related to that of *S. cerevisiae* and which shows weak homology to T7 RNA polymerase. Thus, we may anticipate that *K. lactis* mtRNA polymerase will exhibit homology to sc-mtRNA polymerase, and that mtTFB and mtRNA polymerases will be shown to coevolve to retain the ability to recognize a core consensus promoter sequence surrounding the transcription start site.

mtTFB appears to function as a dissociable transcription initiation factor, in much the same sense as the classical *E. coli* σ70 factor (22). Eubacterial σ factors represent a diverse set of proteins [reviewed in (15,16)]. Although many primary σ factors are closely related to σ70, the σ54 family shows little, if any, primary relationship to the σ70 family. Jang and Jaehning (17) suggested that regions of sc-mtTFB show homology with the eubacterial σ70 family of transcription factors. The results reported in this article can be combined with the site-specific mutagenesis of Shadel and Clayton (30) to reassess the significance of σ factor homologies at the primary sequence level. These two approaches provide complementary types of information. The previous site-specific mutagenesis study has the advantage that the effects of targeted mutations can be assessed within the same general protein background. In contrast, the evolutionary comparison of mtTFB sequences provides a more comprehensive collection of mutations that can be tolerated without loss of mtTFB function than can be achieved with site-specific mutagenesis. It is important to recognize that the interpretation of sequence differences among mtTFB proteins is complicated by the fact that sk-mtTFB and kl-mtTFB proteins exhibit reduced ability to support in vitro transcription by sc-mtRNA polymerase. In each case, it may be that the lower transcription efficiency results from only a few of the large number of amino acid changes with respect to the sc-mtTFB sequence.

An alignment of consensus mtTFB sequences

(designated YB for yeast B) with consensus sequences of various domains of the σ70 family of factors (21) is presented in Fig. 5. These analyses employ a number code to denote conservation of chemically similar amino acid residues, as described in the figure legend and in Lonetto et al. (21). In general, the homology between mtTFB sequences and σ factors is quite limited. The sequence relationships noted by Jang and Jaehning (17) between sc-mtTFB and σ factor regions 2 and 3 are not strengthened by the inclusion of additional yeast mtTFB sequences. For example, Jang and Jaehning (17) noted the conservation of residues D_{34}--D_{37}--K_{40} of sc-mtTFB with corresponding residues of the σ domain 2.1 in individual members of the σ70 family. As shown in Fig. 2, several of these residues are not conserved among the other yeast mtTFB sequences and were not found by Shadel and Clayton (30) to be important for activity in sc-mtTFB. However, a small number of residues that were scored as essential for mtTFB activity following point mutagenesis are conserved in both yeast mtTFB sequences and σ factors. These three residues are marked by filled triangles in Fig. 5 and probably represent the best evidence for a relationship between the primary sequences of mtTFB and σ factors.

The region of mtTFB that is most highly conserved, from Q_{149} to R_{189} of sc-mtTFB, overlaps the locations of two pairs of mutations in sc-mtTFB, R178A-K179A, and H187A-R189A, which have been shown to produce a protein defective in in vitro transcription on linear templates (30). Another relatively large block of conserved amino acids in mtTFB proteins, W321-D333 in sc-mtTFB, is located within the 50 C-terminal amino acids shown to be dispensable for normal activity in sc-mtTFB (30). These residues may play a functional role, because Shadel and Clayton (30) reported that C-terminal deletion to residue 292 produced a temperature-sensitive phenotype. Further deletion to residue 268 caused a loss of activity, possibly due to deletion of two additional blocks of conserved amino acids in this region, ILK and LGHG.

The results presented here demonstrate that yeast mtTFB can retain function despite extensive sequence divergence. At this juncture, we do not fully understand the constraints that have controlled the evolution of yeast mtTFB sequences. The factor must retain the ability to interact with mtRNA polymerase and to facilitate promoter recognition and early steps in transcription initiation. The protein must also retain sequences that target it to mitochondria because all three mtTFB sequences lack a conventional cleaved N-terminal mitochondrial targeting signal (Fig. 2). Sanyal and Getz (27) have shown that sc-mtTFB (Mtf1p) is imported into mitochondria by an unusual mechanism that does not require a cleavable signal sequence, a specific receptor, a transmembrane electrochemical potential gradient, or ATP hydrolysis. It appears that this mechanism may hold for sk-mtTFB and kl-mtTFB as well. The variety of yeast mtTFB sequences reported here will be valuable for future efforts to swap domains between different mtTFB proteins to identify sequences that impair the function of kl-mtTFB with sc-mtRNA polymerase. Such experiments will be more readily interpretable if they are guided by experimental data on the three-dimensional structure of mtTFB proteins and are beyond the scope of the present analysis. In the course of our experiments, we have noted that the proportion of mtTFB protein that is soluble in *E. coli* is greater for *K. lactis* mtTFB than for the other two proteins. Therefore, it may be profitable to pursue structural analysis of the *K. lactis* mtTFB expressed with the T7 system.

One of the reasons we initiated the current line of study was to determine whether the sequences of yeast mtTFB proteins would be sufficiently conserved to provide the information necessary to clone the protein in higher eukaryotes. We would be particularly interested in attempting to clone the protein in *X. laevis*, which is the only higher eukaryote from which a factor has been purified that is required for basal transcription from core mitochondrial promoters (1,6). Preliminary experiments have shown that none of the three yeast mtTFB proteins is capable of complementing *X. laevis* mtRNA polymerase for specific transcription (D. Bogenhagen, unpublished experiments). The rapid evolution of mtTFB sequences within yeasts suggests that it will be difficult to use these sequences to obtain a clone of mtTFB from a higher eukaryote.

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